A DEOXYRIBONUCLEASE FROM CALF SPLEEN*

I. PURIFICATION AND PROPERTIES

By JAMES F. KOERNER† and ROBERT L. SINSHEIMER‡

(From the Department of Physics, Iowa State College, Ames, Iowa)

(Received for publication, February 18, 1957)

The deoxyribonucleases[1] of animal tissues are of two types which are readily distinguishable by the conditions necessary for activity on their substrate, DNA. The pancreatic DNase, which has been crystallized by Kunitz (1), is active in neutral solution in the presence of magnesium or certain other divalent cations (2). The DNase predominant in most other tissues is active at a lower pH, in the presence of adequate ionic strength, but does not specifically require divalent ions. The difference of pH requirement for the activity of the DNase of pancreas and that of other tissues upon the DNA in histological preparations was observed by Catcheside and Holmes (3). The acid-active DNase was partially purified from spleen by Maver and Greco (4) and its action upon DNA solutions was studied. The presence of this activity was soon confirmed and extended to other tissues (5–9). Methods of subcellular fractionation have indicated that the enzyme is located in the mitochondria (10–13).

Partial purifications of the acid-active DNase of thymus have been described by Webb (14) and by Laskowski *et al.* (15), and partial purifications of the DNase of spleen have been described by McDonald (16) and by Maver and Greco (17).

The actions of thymic DNase and pancreatic DNase have been compared by Privat de Garilhe and Laskowski (18, 19) by means of ion exchange chromatography of their digests of calf thymus DNA. Under their conditions, the digests obtained with thymic DNase contained more

* The research described in this paper was supported by a grant from the National Cancer Institute of the United States Public Health Service.

Portions of this manuscript are taken from a dissertation submitted by J. F. Koerner in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Iowa State College.

† Present address, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts.

‡ Present address, Division of Biology, California Institute of Technology, Pasadena, California.

[1] The following abbreviations are used: DNase, deoxyribonuclease; DNA, deoxyribonucleic acid; T-A buffer, a buffer composed of tris(hydroxymethyl)aminomethane and acetate; the molarity refers to the total concentration of tris(hydroxymethyl)aminomethane.

mononucleotides than did those obtained with the pancreatic DNase, although the remaining fractions were of greater average size in the digest with thymic DNase.

This paper describes a purification procedure for splenic DNase and some properties of the purified product. The procedure is specifically intended to remove other enzymes with possible action upon polydeoxyribonucleotides. Another paper describes the nature of the polynucleotides formed by this purified enzyme preparation.

Materials and Methods

Assay for Splenic DNase—This procedure was based upon the increase of the ultraviolet absorption of DNA during degradation, first described by Kunitz (1), and adapted for the optimal conditions of activity of the splenic DNase.

The substrate solution contained thymus DNA to give an absorbance at 260 mμ of 1.0, and was 0.33 M in sodium formate buffer, pH 4.5. 2.9 ml. of substrate solution were warmed in a test tube on a 37° water bath. The sample to be assayed (with a volume no greater than 0.1 ml.) was added, and the solution quickly stirred and transferred to a 1 cm. path quartz cell in a cuvette holder previously warmed to 37° in a temperature-regulated cuvette housing attachment to the Beckman spectrophotometer. The A_{260} was read each minute for 10 to 15 minutes against a reference cell of substrate solution. The linear portion of the increase in absorption, after the initial induction period of lesser slope, was graphically extrapolated to give the rate of increase of absorption per 10 minutes time, and this rate multiplied by 1000 was defined as the total number of DNase units in the sample volume assayed. This assay was linear and reproducible within about 10 per cent over the range of 100 to 500 DNase units per sample. Because of the induction period of the degradation, assays on less than 100 units were less reliable, although semiquantitative results could be obtained on samples with as few as 30 units.

When the turbid suspensions encountered during the initial stages of the DNase preparation were assayed, 1 volume of the suspension was added to 1 volume of a solution which was 1 M in KCl and 0.3 M in ammonium acetate buffer, pH 4.5, the mixture was centrifuged, and the clear supernatant solution was assayed. This procedure recovered all the DNase activity that was active at pH 4.5 and that could be removed with salt solutions.

Assay for Splenic Phosphodiesterase—A mixture of 0.1 ml. of 2 M ammonium acetate buffer, pH 4.5, 1 ml. of 0.001 M calcium [bis(*p*-nitrophenyl)-phosphate]$_2$ (20), water, and a sample to give 1.5 ml. total volume was incubated for 12 hours at 37°. 1.5 ml. of 1 M ammonium hydroxide

were then added and the A_{440} was read against a blank of buffer and substrate which received identical treatment. The A_{440} obtained was defined as the total phosphodiesterase unit in the sample.

Since a number of enzymes active against this substrate are present in spleen, the assay as described was arbitrary and did not attempt to attain optimal conditions for any of them. It was generally quite reproducible and linear on a given sample up to 0.1 to 0.2 phosphodiesterase unit. Since phosphodiesterases active against the polynucleotides obtained with spleen DNase might not have been detected with this substrate, and conversely enzymes inactive on polydeoxyribonucleotides seemed to degrade this substrate, this assay could be used only as an empirical criterion for the evaluation of fractionation procedures.

Assay for Splenic Acid Phosphatase—A mixture of 1 ml. of 0.002 M disodium *p*-nitrophenylphosphate (20), 0.1 ml. of 2 M ammonium acetate buffer, pH 5.5, enzyme, and water to give a total solution volume of 1.5 ml. was incubated for 30 minutes at 37°. 1.5 ml. of 1 M ammonium hydroxide were then added. The A_{440} read against a blank of reagent and buffer was defined as the total phosphatase unit in the sample.

This assay determined a definite phosphatase fraction in spleen. If this phosphatase was present in a DNase preparation, it liberated inorganic phosphate during a DNA digestion, apparently by the removal of the monoesterified phosphate of the polynucleotides.

Purification of Splenic DNase—All operations were carried out in the cold room at 1°. 1800 gm. of spleen from cattle were cut into small pieces and ground in aliquots in a Waring blendor with a total of 9 liters of 0.05 M ammonium acetate buffer, pH 4.5. Each aliquot was ground for about 2 minutes at the full speed of the blendor. This homogenate was strained through cheesecloth and was then stored for 3 to 4 days. 550 gm. of Celite 545 were added and the mixture was collected by suction onto an 18.5 × 22.5 inch rectangular filter.[2]

The precipitate was washed successively with 2 liters of 0.05 M ammonium acetate buffer, pH 4.5, 8 liters of 0.1 M ammonium acetate buffer, pH 4.5, and 1 liter of a solution 0.2 M in ammonium acetate buffer, pH 4.5, and 0.005 M in ammonium sulfate. The DNase fraction was then extracted with 2 liters of a solution 0.2 M in ammonium acetate buffer, pH 4.5, and 0.05 M in ammonium sulfate. The extract was stored in the cold for 1 week. After this time, as much of the clear supernatant liquid as

[2] The filter used, accommodating a sheet of chromatographic paper, consisted of a rectangular metal tray with an outlet tube leading to a suction flask. In the bottom of the tray was a heavy mesh metal grille upon which rests a fine mesh brass screen. These two parts were sealed to the edge of the tray with wax. This type of filter gives a greater flow rate per unit area than a Büchner funnel and can be made in very large sizes.

possible was decanted from the precipitate, and the remainder collected by centrifugation. The precipitate was discarded.

This extract was heated with stirring on a 62° water bath until its temperature reached 58°. It was held at 58–60° for 15 minutes and was then cooled to 1°. 3 gm. of Celite were added and the suspension was filtered through an 11 cm. diameter Büchner funnel.

To 1 volume of the heat-treated filtrate was added 0.11 volume of 1.5 M formic acid to bring the pH to 3.5. 0.43 volume of absolute ethanol was added to 1 volume of this solution at pH 3.5 and the mixture was cooled to 1° with a dry ice bath. 3 gm. of Celite were added and the suspension was filtered onto a very thin Celite pad on a Büchner funnel 11 cm. in diameter. To 1 volume of this filtrate, 0.4 volume of absolute ethanol was added. The suspension was allowed to stand for 30 minutes, 2 gm. of Hyflo Super-Cel were added, and it was then filtered onto a thin Hyflo Super-Cel pad 11 cm. in diameter. To 1 volume of this filtrate, 0.5 volume of absolute ethanol was added. The suspension was allowed to stand for 30 minutes, 4 gm. of Hyflo Super-Cel were added, and it was then filtered onto a thin Hyflo Super-Cel pad 11 cm. in diameter. The precipitate was washed with 200 ml. of 66 per cent ethanol. The filter pad was suspended in 66 per cent ethanol and transferred to a filter 3 cm. in diameter. The DNase fraction was washed from this filter pad by allowing 50 ml. of water to percolate through the filter. The turbid aqueous filtrate was centrifuged and the precipitate was discarded.

All these filtrations had to be started with very gentle suction or the precipitate would run through the filter. As filtration proceeded, the suction could be increased.

Chromatography on Celite—To prepare the column, Celite 545 was suspended in distilled water, allowed to settle about 20 minutes, and the fine particles were decanted and discarded. This washing was repeated several times. A thick suspension of washed Celite was then poured into a column 4.5 cm. in diameter equipped with a sintered glass-bottomed plate. Enough Celite was added to give a column height of about 25 cm. The column was washed with 1 liter of a solution 0.02 M in T-A buffer at pH 8.5 and 4 M in NaCl, then with 250 ml. of water, and was then rapped sharply against a desk top, after which it could be compacted by the application of slight air pressure. The final length of the column was adjusted to 20 cm.

Before the chromatography, a series of solutions was prepared, each of 20 ml. volume, 0.05 M in T-A buffer at pH 8.5, and 0.001 M in ethylenediaminetetraacetate, and with the following sequence of sodium chloride concentrations in the series: 0.005, 0.010, 0.017, 0.025, 0.033, 0.043, 0.055, 0.069, 0.083, 0.10, 0.12, 0.14, 0.17, 0.20, 0.23, 0.26, 0.30, 0.34, 0.39, 0.45, 0.52, 0.60, 0.69, 0.79, 0.91, 1.05 M.

The amount of solution of ethanol precipitate which contained 3,000,000 units of DNase was added to the Celite column. The above eluting solutions were added in order, each one being forced onto the column with sufficient air pressure to add the 20 ml. in about 40 seconds. The effluent was collected in 20 ml. fractions and the active fractions were pooled. The activity appeared in the effluent during the addition of the solutions ranging from 0.26 to 1.05 M in sodium chloride content.

Fig. 1 illustrates the position of the DNase, the two phosphodiesterase peaks, and the phosphatase in a typical chromatogram. The larger phosphodiesterase peak was just beginning to appear when this particular experiment was terminated. This peak has been observed in its entirety in other experiments.

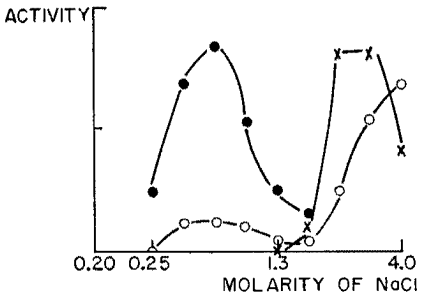

FIG. 1. Chromatography of splenic deoxyribonuclease on Celite. ●, DNase activity; ○, phosphodiesterase activity; ×, phosphatase activity.

To concentrate the eluate, the active fractions were adjusted to pH 5.5 with 1 M acetic acid, and the solution was added to a Celite column 1 cm. in diameter and 10 cm. in length. The column was then washed with 10 ml. of a solution 0.01 M in ammonium acetate buffer, pH 5.5, and 0.001 M in ethylenediaminetetraacetate. The DNase was eluted with a solution 1 M in sodium chloride, 0.1 M in T-A buffer, pH 8.5, and 0.001 M in ethylenediaminetetraacetate. The first 4 ml. of effluent collected after addition of the eluting solution were discarded; then 0.5 ml. fractions were collected. The active fractions were pooled. The salt could be removed by dialysis.

Results

Purification of DNase—Table I lists the concentrations, yields, and purifications attained at each step of the DNase preparation. The ratio of DNase to phosphodiesterase activity (Table I) is computed for equal volumes of sample in the two assays. The ratio of DNase activity to A_{280} is defined as the number of DNase units contained in a solution

1 ml. in volume and having an absorbance of 1.0 at 280 mμ with a cell length of 1 cm.

Thermal Stability of DNase—The splenic DNase is more stable to heating in acidic than in alkaline solution (14). It withstands heating in 0.1 M formic acid at 60° for 30 minutes with little loss of activity. At pH 4.5 it has a half life of approximately 100 minutes at 65°, when in 0.2 M acetate buffer. At pH 8.5, its stability has decreased to such an extent that it has a half life of only 10 minutes at 45°.

TABLE I

Purification of Splenic DNase

Material	Approximate volume	Total DNase units	Yield for single step	DNase / Phosphodiesterase	DNase / A_{280}
	ml.		*per cent*		
Homogenate	10,000	34,000,000		500	
DNase extract	1,700	15,000,000	45	8,200	4,400
Supernatant of heat fractionation	1,700	15,000,000	100	12,000	6,700
Solution of ethanol ppt.*	40	12,000,000	80	12,000	150,000–200,000
	Carried out on 3,000,000 units of DNase				
Celite chromatogram effluent†	200	3,000,000	100	100,000–400,000	200,000–400,000
DNase concentrate	3.5	2,000,000	67	50,000–200,000	

* DNase to phosphatase ratio of solution of ethanol precipitate, 800.

† Phosphatase undetectable in effluent of Celite chromatogram.

Activation, pH Optimum, and Inhibition—The requirement of salt for splenic DNase activity which was noted by other workers has been verified. Fig. 2 shows the activation at pH 4.5 by potassium chloride and by magnesium chloride. Salts of various monovalent anions and cations seem to be equivalent in their action. The optimal salt concentration is about 0.15 to 0.25 M. At higher concentrations, the activity again decreases. The optimal concentration for the divalent magnesium ion is about 0.04 M. 0.1 M solutions have little activity. The maximal activity is about half as great as is attained with monovalent ions.

The variation of activity with pH, with 0.15 M sodium chloride as the activator, is shown in Fig. 3. The variation of activity with pH with 0.05 M magnesium chloride as activator is similar to that illustrated for 0.15 M sodium chloride.

Fig. 3 also illustrates the activation by a series of dilute acetate buffers

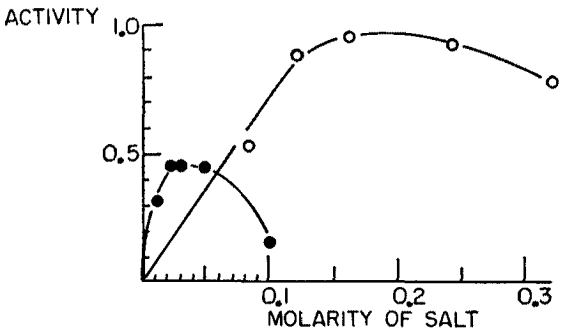

FIG. 2. Activation of splenic deoxyribonuclease by salts. ○, KCl; ●, $MgCl_2$

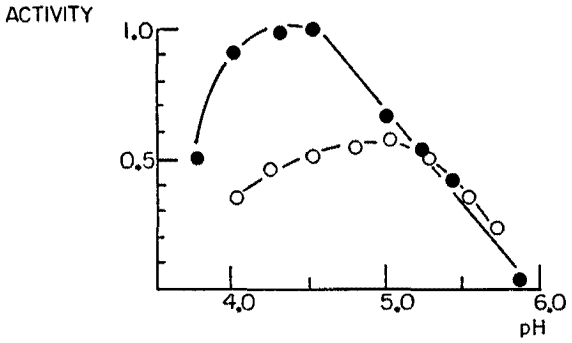

FIG. 3. Variation of activity of splenic deoxyribonuclease with pH. ●, activity in the presence of 0.15 M NaCl plus dilute acetate buffer; ○, activity in the presence of 0.15 M sodium acetate buffer.

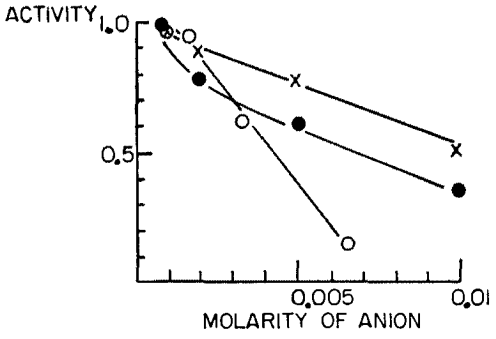

FIG. 4. Inhibition of splenic deoxyribonuclease activity by polyvalent anions. ○, sulfate; ●, phosphate; ×, arsenate.

of varying pH values and constant total acetate concentration. As is shown, a pH maximum other than 4.5 may be observed under these conditions because such buffers are varying both in pH and in ionic strength. Such activity curves can be duplicated by activity measurements obtained

in the presence of a negligibly small buffer concentration and the appropriate concentration of sodium chloride necessary at each pH to give an ionic strength equal to that of the acetate buffer previously used. It should be noted that an "optimum" at a pH other than 4.5 obtained in this manner is never as high as could be obtained at pH 4.5 under ideal conditions of ionic strength. This observation may account for some of the discrepancies of pH optima reported in the literature (9–11).

Many polyvalent anions are inhibitory to the DNase activity. Fig. 4 shows the inhibition of activity by the addition of sulfate, phosphate, and arsenate ions to solutions with 0.33 M sodium formate buffer, pH 4.5, as the activator.

DISCUSSION

After homogenization of the spleen for 2 minutes at pH 4.5, the DNase activity was bound to the insoluble material. A similar step carried out at pH 3.8 or pH 7 would have yielded most of the activity in the supernatant solution. The homogenate at pH 4.5 had then to be stored in the cold for several days before filtration and extraction because, if filtered immediately, different preparations yielded extracts which required different ethanol concentrations for the step in ethanol precipitation. There was no appreciable loss of DNase activity during storage.

After extraction of the DNase from the insoluble residue, it was stored for 1 week before the procedure was continued. The activity was usually observed to increase during this storage. Whatever the initial activity of the solution, the activity after storage amounted to about 45 per cent of that which could be removed from the homogenate by adding an equal volume of 1 M KCl and assaying the supernatant fluid. The missing activity could not be recovered from any of the wash solutions or by extraction of the residue with 1 M KCl. That most of the loss was not due to adsorption onto the Celite filter aid was shown by the fact that nearly as much loss occurred when the extraction procedure was carried out by collecting the precipitates in a centrifuge without the use of Celite. An activity increase upon storage of even greater magnitude than that observed in this preparation was noted by Webb in his preparation of thymic DNase (14).

The heat fractionation used in this procedure was similar to that described by McDonald (16).

During ethanol precipitation, the inactive material was removed in two steps, the second fraction being taken at an ethanol concentration which would have caused an appreciable precipitation of DNase if approached in one step. The active DNase fraction obtained by ethanol precipitation still contained two phosphodiesterases and a very powerful phosphatase

activity. As will be shown in another paper, this phosphodiesterase activity, assayed on bis(*p*-nitrophenyl)phosphate, does not seem to be involved in the degradation of DNA by DNase preparations at this stage of purification, but such evidence could be gained only by further fractionation of the activities. The phosphatase present at this stage does attack the polynucleotides in the DNase digest, as was shown by the liberation of inorganic phosphate in digests made with these preparations.

The Celite chromatographic procedure described separated the DNase completely from the phosphatase and from much of the phosphodiesterase. The phosphodiesterase appeared as two peaks and is hence considered as two different enzymes, although it could be a single protein with an anomalous elution pattern.

The DNase eluate from the Celite column was extremely dilute. Although reasonably stable in the presence of ethylenediaminetetraacetate, it was not possible to dialyze this solution or carry out conventional concentration procedures without heavy losses. The column concentration procedure yielded a solution which was stable and could be dialyzed against water or pH 4.5 or pH 8.0 buffer without loss.

SUMMARY

A procedure for the purification of splenic deoxyribonuclease is described. This procedure separates the deoxyribonuclease activity from acid phosphatase and from much of the phosphodiesterase detected with bis(*p*-nitrophenyl)phosphate.

The pH optimum of the purified enzyme is near pH 4.5 under conditions of optimal ionic strength.

BIBLIOGRAPHY

1. Kunitz, M., *J. Gen. Physiol.*, **33**, 349, 363 (1950).
2. Miyaji, T., and Greenstein, J. P., *Arch. Biochem. and Biophys.*, **32**, 414 (1951).
3. Catcheside, D. G., and Holmes, B., *Symposia Soc. Exp. Biol.*, **1**, 225 (1947).
4. Maver, M. E., and Greco, A. E., *J. Biol. Chem.*, **181**, 853, 861 (1949).
5. Webb, M., *Nature*, **169**, 417 (1952).
6. Brown, K. D., Jacobs, G., and Laskowski, M., *J. Biol. Chem.*, **194**, 445 (1952).
7. Siebert, G., Lang, K., Lucius-Lang, S., Herkert, L., Stark, G., Rossmüller, G., and Jöckel, H., *Z. physiol. Chem.*, **295**, 229 (1953).
8. Cunningham, L., and Laskowski, M., *Biochim. et biophys. acta*, **11**, 590 (1953).
9. Fellas, V. M., Meschan, I., Day, P. L., and Douglass, C. D., *Proc. Soc. Exp. Biol. and Med.*, **87**, 231 (1954).
10. Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, **198**, 155 (1952).
11. Allfrey, V., and Mirsky, A. E., *J. Gen. Physiol.*, **36**, 227 (1952).
12. Webb, M., *Exp. Cell Res.*, **5**, 16 (1953).
13. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F., *Biochem. J.*, **60**, 604 (1955).

14. Webb, M., *Exp. Cell Res.*, **5,** 27 (1953).
15. Laskowski, M., Steberl, E. A., Akka, R., and Watson, P., *Biochim. et biophys. acta*, **13,** 595 (1954).
16. McDonald, M. R., in Colowick, S. P., and Kaplan, N. O., Methods in enzymology, New York, **2,** 444 (1955).
17. Maver, M. E., and Greco, A. E., *Federation Proc.*, **13,** 261 (1954).
18. Privat de Garilhe, M., and Laskowski, M., *J. Biol. Chem.*, **215,** 269 (1955).
19. Privat de Garilhe, M., and Laskowski, M., *Biochim. et biophys. acta*, **14,** 147 (1954).
20. Sinsheimer, R. L., and Koerner, J. F., *J. Biol. Chem.*, **198,** 293 (1952).
21. Bessey, O. A., Lowry, O. H., and Brock, M. J., *J. Biol. Chem.*, **164,** 321 (1946).