STUDIES ON THE NONSPECIFIC PHOSPHODIESTERASE ACTIVITY OF SPLENIC ACID DEOXYRIBONUCLEASE

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Various methods of purification of acid deoxyribonuclease (DNase II) have been described (reviewed by Laskowski, 1967 and by Bernardi, 1968). Recently, Bernardi and Griffe (1964) prepared a very highly purified DNase II from hog spleen. However, this pure enzyme was able to cleave bis-nitrophenyl phosphate, and thus possessed a nonspecific phosphodiesterase activity. Both the DNase and phosphodiesterase activities can be inactivated by heat and both are inhibited competitively by s-RNA. The ratio of DNase to nonspecific phosphodiesterase after heating, or when inhibited by the s-RNA, remained constant. Bernardi and Griffe concluded that the nonspecific phosphodiesterase activity is an intrinsic activity of the DNase II molecule.

We prepared DNase II from sheep spleen and other tissues using heating as one of the first steps. The specific activity of our pure DNase II was similar to that reported by Bernardi and Griffe (1964). However, using the assay procedure of Bernardi and Bernardi (1968), we found that our enzyme was free of any traces of nonspecific phosphodiesterase activity.
Mouse liver DNase II was also found to be free of nonspecific phosphodiesterase (Hodes et al, 1967). This suggested that the activity observed by Bernardi could have been a contaminant.

If both activities are due to a single active site, one should expect

that bis p-nitrophenyl phosphate, which is the substrate for the nonspecific phosphodiesterase, should inhibit competitively the activity of DNase II on DNA, and that DNA should inhibit competitively the activity of the enzyme on bis p-nitrophenyl phosphate. Alternatively, if the nonspecific phosphodiestrerase does not share a common site with DNase II, but rather has an active site of its own, possibly on a separate protein, one should expect that neither will DNA inhibit the nonspecific phosphodiesterase nor will bis p-nitrophenyl phosphate inhibit DNase II. It is the purpose of this paper to describe such an approach in attacking that problem.

Materials and Methods

Hog spleen DNase II was prepared as previously described (Bernardi, 1964). Sheep spleen DNase II was prepared largely according to Swenson and Hodes (1969), with an additional DEAE cellulose chromatography.

Calf Thymus DNA was prepared by the procedure of Kay, Simmons and Dounce (1951). ^{14}C Thymidine DNA was extracted from a culture of human kidney cells according to Thomas et al (1966) and had a specific activity of 8000 cpm/μg DNA.

Sodium bis p-nitrophenyl phosphate was purchased from B.D.H. Chemicals, Ltd., England. DNase II was assayed on Calf Thymus DNA by measurement of acid soluble nucleotides (Hodes et al 1967).

A Unit of DNase II is defined as the amount of enzyme required to cause Δ A 260 of 0.001 per minute under the above conditions.

Specific activity is defined as DNase units per mg protein.

When ^{14}C DNA was used as substrate, the assay method of Kates and McAuslan (1967) was used.

Phosphodiesterase was assayed as previously described (Bernardi and Bernardi, 1968) with substitution of the sodium salt of bis p-nitrophenyl phosphate for

the calcium salt. Substrate concentration could thus be increased to 5 or 10 mM.

Protein was estimated from its absorbancy at 280 m μ.

Results

DNase II enzymes that were prepared from sheep and hog spleen according to Swenson and Hodes (1969) (method I) and also from hog spleen according to Bernardi and Griffe (1964) (method II), were assayed for their nonspecific phosphodiesterase activities. The three preparations had similar specific activities on DNA but as can be seen in Table 1 the DNase II from hog spleen

Table 1 The activities of DNase II preparations from hog and sheep spleens on DNA and on bis p.nitrophenyl phosphate (nonspecific phosphodiesterase). DNase units and specific activity are defined in "methods". The nonspecific phosphodiesterase activity was determined by measuring p-nitrophenol formation from the bis p-nitrophenyl phosphate, assuming ε_{400} of 12000 (Razzel and Khorana, 1961). Activity is expressed as Δ A 400 per unit time.

TABLE I

enzyme source	method of preparation	DNase II specific activity	non specific phosphodiesterase as Δ A 400 per 750 DNase units after 1 hour	after 18 hrs
Hog spleen	method II	110000	0.083	0.81
Hog spleen	method I	92000	0.005	0.004
Sheep spleen	method I	103000	0	0.002

that was purified by method II was active against bis p-nitrophenyl phosphate, whereas the sheep and the hog spleen DNase that were prepared by method I showed no such activity even when incubated with large amounts of enzyme for 18 hours at 37°.

A preliminary control experiment was performed to find out if DNA has any influence on the activity of spleen phosphodiesterase (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) that was obtained from Worthington Co., DNA did not inhibit the enzyme at any concentrations up to 250 μg/ml.

A similar experiment on the effects of DNA on the activity of purified DNase II from hog spleen (prepared by method II) on bis p-nitrophenyl phosphate was performed using concentrations of DNA many times greater than the concentration of bis p-nitrophenyl phosphate. As can be seen in Fig. 1, the nonspecific phosphodiesterase activity was not inhibited at all by DNA, even when the incubation was continued for 18 hours.

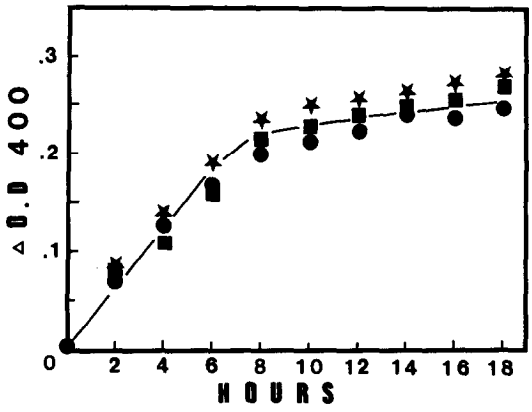

Fig. 1 Influence of CT-DNA on the nonspecific phosphodiesterase activity of hog spleen DNase II. Three sets of tubes in triplicate were incubated for 18 hours at 37 . At the times indicated, samples were drawn from each tube and assayed for the nonspecific phosphodiesterase activity on bis p.nitrophenyl phosphate. Set I ●——● contained no DNA, Set II ■——■ contained 0.1m g/ml DNA, and Set III ✶——✶ contained 0.5 mg/ml DNA.

The experiment concerning the influence of bis p-nitrophenyl phosphate on the activity of purified hog spleen DNase II on DNA posed a different problem. If a standard DNase assay were to be used, the concentration of bis p-nitrophenyl phosphate would be too low because of its solubility

limits. In order to overcome this problem, we used ^{14}C DNA as substrate for DNase II. We were thus able to use extremely low DNA concentrations and the ratio of bis p-nitrophenyl phosphate to DNA was large. The experiment was performed with three DNase II concentrations chosen so that complete DNA degradation (no acid insoluble ^{14}C in the reaction mixture) occurred between 15 and 45 minutes after the incubation was initiated at 37° (Fig. 2). There was no influence of bis p-nitrophenyl phosphate on the degradation of DNA by DNase II.

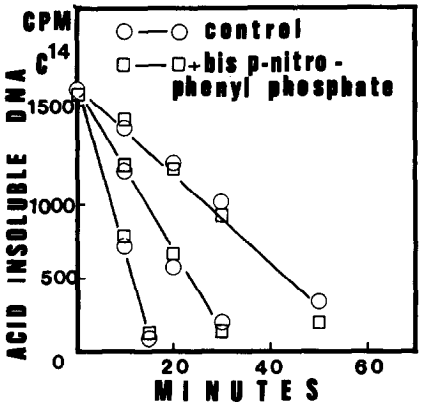

Fig. 2 Effects of bis p-nitrophenyl phosphate on the degradation of C^{14} DNA (specific activity, 8000 cpm/μg DNA) by hog spleen DNase II. The experiment was performed at 3 different enzyme concentrations. The concentration of the bis p-nitrophenyl phosphate was 0.02 M. DNase II was measured by counting the acid precipitable C^{14} DNA, using the method of Kates and McAuslan (1967).

Discussion

The data presented in this paper suggest that the nonspecific phosphodiesterase activity observed with the highly purified DNase II of Bernardi and Griffe is a contaminant, or at least does not occupy the same active site as the DNase activity. Swenson and Hodes have recently shown that although on chromatography of spleen homogenate on carboxy methyl cellulose (CMC), there was a nonspecific phosphodiesterase peak overlapping the DNase peak, the two activities could be separated from each other if the homogenate was heated prior to chromatography

on CMC, (Swenson and Hodes, 1969). Our DNase II preparation is heated at 50° for 1 hour prior to the CMC step.

Swenson and Hodes repeated the observation of Bernardi and Griffe (1964) that both DNase II and the nonspecific phosphodiesterase activities were equally sensitive to heating at 60° . However, when the enzyme was heated at 50° , the nonspecific phosphodiesterase activity was inactivated more rapidly than the DNase II activity, thus invalidating the finding at 60° as a support for Bernardi's assumption.

It should be mentioned that while in Bernardi's laboratory the author tried to separate the two activities of his highly purified hog spleen DNase II (prep. HS 21) from each other. Heating at pH 3 and 4.5 did not selectively inactivate one of the two activities. Rechromatography on CMC of the pure enzyme also failed to separate the two activities. It is possible that heating is only effective when crude extract is heated prior to CMC chromatography. The reason could be either that heating changes the chromatographic behavior of both DNase II and nonspecific phosphodiesterase, causing them not to chromatograph coincidently any longer and enabling their separation from each other, as suggested by Swenson and Hodes (1969), or as suggested by Bernardi (personal communication) there could be a partial digestion of the DNase II nolecules by proteolytic enzymes, when the enzyme is heated. The partial digestion of the DNase II causes the molecule to lose its activity on bis p-nitrophenyl phosphate.

Our data suggests that the activities of DNase II and of nonspecific phosphodiesterase do not occupy the same site on the DNase II molecule. It is probable that the two activities belong to two different proteins.

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