Activation of Deoxyribonucleases by Divalent Cations

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ABSTRACT The activation of DNase I by Mg, Mn, Co, Ni, Fe, Cd, Zn, Ba, Sr, Ca, and Cu ions has been studied by several methods, at different pH and salt concentrations. Mg, Mn, and Co are the best activators for initial stages of degradation. A synergistic effect is shown only by the pair Mg-Ca. Optimal pH of action is always situated at 6.5. DNase II is activated to about the same degree by alkaline earths and Mn ions. Cd and Cu are strong inhibitors. Optimal pH is always 4.6. By titration of liberated secondary phosphate groups, two stages in the hydrolysis of DNA by DNase I are evidenced: a rapid phase activated most by Mg and a slow phase activated by Ca. Some possible mechanisms of action of both enzymes are outlined and the general influence of metal ions is discussed.

Two main categories of deoxyribonucleases specifically hydrolyze the phosphoester bonds in deoxyribonucleic acid (DNA): the first one (DNase I), extracted and crystallized from pancreas by Kunitz (1950), requires divalent cations (in particular Mg); the second one, DNase II, extracted from thymus or spleen and purified by chromatography (Koerner and Sinsheimer, 1957; Shimomura and Laskowski, 1957; Fredericq and Oth, 1958), is already active in the absence of metallic cations but is activated by several divalent cations.

Wiberg (1958) showed that Mg and Mn are the most powerful activators of DNase I. He also reported a very interesting synergistic effect of Ca ions when added to Mg ions, although they had themselves very little activity. His method of determination was, however, restricted to the earlier stages of hydrolysis and under conditions in which DNA was partially denatured. The synergistic effect of Ca and Mg was confirmed by Feinstein (1960) using a viscometric assay and by one of us (Fredericq, 1959), using a titration of secondary phosphate; differences in the activation of hydrolysis of DNA and oligonucleotides were also observed.

In the present paper we describe a study of the activation of DNase I by a variety of divalent cations and its extension to subsequent stages of DNA

degradation. The effect of ions on the activity of DNase II was also determined under various conditions. A comparison of the activity requirements of both DNases was made and some conclusions are presented regarding the possible interpretation of metal specificities. Definite differences between the mechanisms of action of the enzymes are deduced from these and other studies.

EXPERIMENTAL

Calf thymus DNA was prepared after Kay *et al.* (1952). Its molecular weight deduced from intrinsic viscosity was 7,000,000. It was dialyzed against distilled water at pH 6. The concentration was determined spectroscopically, admitting an absorbancy of 200, in 0.1 M NaCl, in 1 per cent solutions, at 260 mμ.

DNase I was a crystallized enzyme obtained from Worthington Biochemical Corporation, Freehold, New Jersey, and DNase II was a calf spleen enzyme, prepared and purified after Fredericq and Oth (1958).

The enzymic activities were determined by viscosity, spectroscopy, and acid precipitation by methods previously described (Fredericq and Oth, 1958; Kunitz, 1950). They are expressed in activity units: viscometric (Fredericq and Oth, 1958), spectroscopic (Kunitz, 1950); for acid precipitation, activity units give the variation of optical density at 260 mμ of the acid supernatant, per minute.

For routine measurements, 0.02 per cent DNA in acetate buffer (pH 6 for DNase I, pH 4.6 for DNase II) was used at 25°C. The total ionic strength was kept at 0.075, except with high concentrations of divalent cations. The metal salts were all chlorides.

The hydrolysis of DNA by DNase I in subsequent stages of degradation was followed by a titration of the secondary phosphate groups using a titration method under specified conditions (Fredericq, 1960).

Oligonucleotides were prepared by hydrolysis of DNA by DNase I and alcohol fractionation of the hydrolysates. The degree of polymerization (or number of nucleotide units per molecule) is estimated by titrating the secondary phosphate groups and dividing the total number of phosphorus atoms by the number of secondary phosphate groups. This gave degrees of polymerization ranging from 6 to 12.

RESULTS

DNase I

The influence of cation concentration upon activity of DNase I was studied at constant ionic strength (0.075), in acetate pH 6, and measured by the viscosity method. Curves are given in Fig. 1. The best activator, besides Mg and Mn is Co; somewhat lower lies Fe^{++}. All the curves are similar in shape to those already found for Mg and Mn (Wiberg, 1958), reaching a plateau at concentrations near 0.01 M. Co^{++} seems to be the best activator at low concentration (0.0005 M). Above 0.01 M, activation effects decrease for most ions and for Mg ions in particular. This was interpreted by Kunitz (1950) as

mainly an effect of ionic strength. But this interpretation is contradicted by the synergistic effect of Ca which is maximum at ionic strength around 0.15 (see below). Co and Mn could not be investigated above 0.01 M because they form gels with DNA. A much less effective class of activators includes Ba, Sr, Cd, and Ni. Their activation effect decreases at concentrations above 0.001 M. Ca activates slightly and Zn not at all.

Similar results are obtained by the spectroscopic method of Kunitz (1950) and by determination of acid-soluble products at low ionic concentrations. However, activation of the enzyme by Mg at concentrations above 0.01 M is weaker when measured by the increase of absorbancy at 260 mμ.

The influence of pH upon activity is shown on Fig. 2. Essentially the same

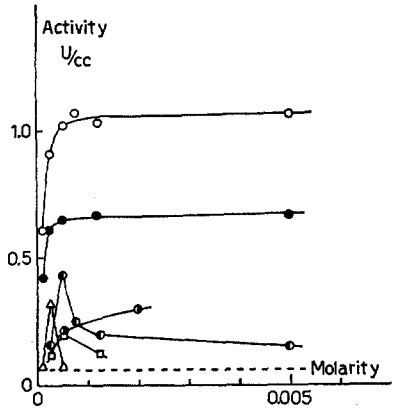

FIGURE 1. Activity of DNase I as a function of metal ion concentration, at pH 6. ○ Co; ● Fe^{++}; ◐ Ni; △ Ba; □ Sr; ◑ Cd; - - - - pure NaAc.

influence is apparent for the three activators, Mg, Co, and Ca. The optimum is always situated around pH 6.5.

A few cations form gels with DNA at pH above 6 and above some critical concentration. The maximum tendency is shown by Cd, Co, and Mn.

The synergistic effect reported by Wiberg (1958) for the mixture Mg-Ca in proportions 10 to 1, was confirmed here using different methods. In Table I the results are given. It can be seen that the synergistic ratio, defined as the ratio of activation by Mg and Ca to the activation by Mg alone is 2.7 by viscosity, 4.8 by spectrophotometry, and 5.2 by acid precipitation. The first method gives only the very first stages of hydrolysis; the other ones are sensitive to what will be called later the rapid phase. They correspond more closely to the conditions of titration of Wiberg who found a ratio around 3.8.

DNase II

This enzyme has a noticeable activity in acetate without addition of divalent cations. Many cations, however, influence the enzymic activity in some way

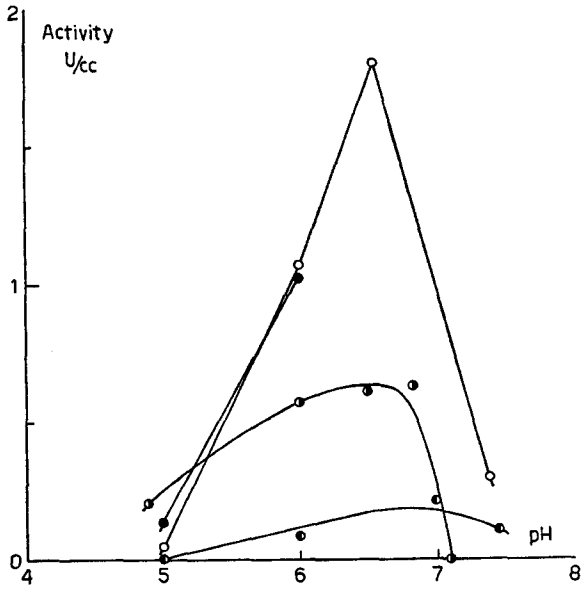

FIGURE 2. Activity of DNase I as a function of pH, in the presence of several metal ions. ○ 0.005 M Mg; ◑ 0.045 M Mg; ● 0.005 M Co; ◐ 0.005 M Ca.

(Oth *et al.*, 1958). The dependence of their effects on concentration is shown in Figs. 3 and 4. We may distinguish two classes:

1. Activators (Fig. 3) among which are Mn, Zn, and the alkaline earths. The maximum activation effect is around two times the activity in sodium acetate for all of them. All the curves (except for Ba) present a maximum at a characteristic concentration varying from 0.002 to 0.01 M; this concentration is proportional to the DNA concentration, showing a stoichiometric relation.

2. Inhibitors (Fig. 4) in increasing order include Fe, Ni, Cd, Cu. With Cd and Cu, inhibition is total at concentrations above 0.003 M. Co exhibits slight activation at low concentrations.

TABLE I

ACTIVITY UNITS OF DNASE I DETERMINED BY SEVERAL METHODS AT pH 6, IONIC STRENGTH 0.15

Activators	Method		
	Viscosity	Spectroscopy	Acid precipitation
0.005 M Mg	1.07	0.82	0.33
0.045 M Mg	0.57	0.24	0.26
0.005 M Ca	0.084	0.41	0.057
0.045 M Mg+ 0.005 M Ca	1.53	1.14	1.34

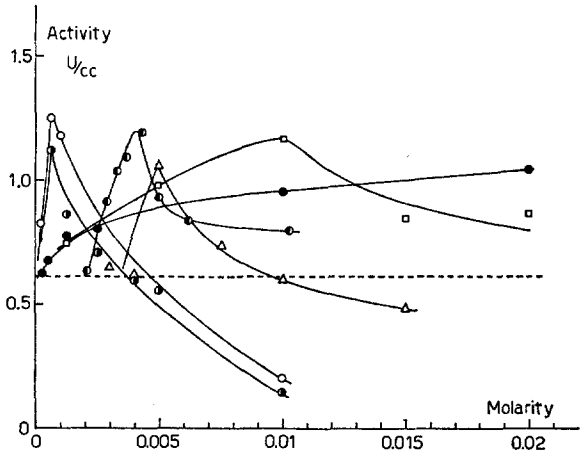

FIGURE 3. Activity of DNase II as a function of metal ion concentration at pH 4.6. ● Ba; □ Sr; ◐ Ca; △ Zn; ○ Mg; ◑ Mn; - - - - NaAc.

The spectroscopic method gives very similar results.

At pH 6, the concentrations of Mg, Mn, or Ca giving the maximum activation are the same as at pH 4.6. The influence of pH is also the same in the presence of several ions. The pH optimum for Mn, Mg, Ca, and Zn ions at their optimal concentration is always at pH 4.6. Above 6, the activating effects become negligible.

No synergistic effect of ions was found in the case of DNase II.

It is known that citrate and ethylenediaminetetraacetate (EDTA) have an activating effect. This is rather unexpected since these complexing agents should hinder activating divalent cations. We investigated the activation by EDTA. The influence of concentration is given in Fig. 5. It can be seen that

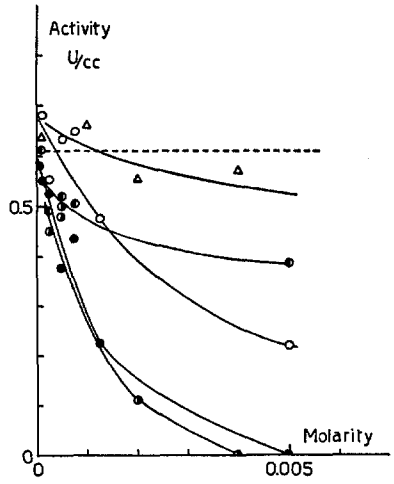

FIGURE 4. Activity of DNase II as a function of metal ion concentration, at pH 4.6. ○ Co; ◐ Ni; ◑ Cd; ● Cu; △ Fe; - - - - NaAc.

the activation starts at extremely low concentrations and stops above 0.001 M. At higher concentrations, EDTA is inhibitory. Small quantities of heavy metal ions are always present in preparations of DNA. For instance, in one of our preparations, we found by emission spectroscopy Ca, Mg, Al, Pb, and traces of Fe. The heavy metals are complexed by EDTA at very low concentration and consequently the activity of DNase is increased. When the concentration of EDTA becomes too high, it will then complex divalent cations such as Mg and Ca and reduce the activity of DNase. This hypothesis is confirmed by the fact that the activating effect of EDTA is higher on

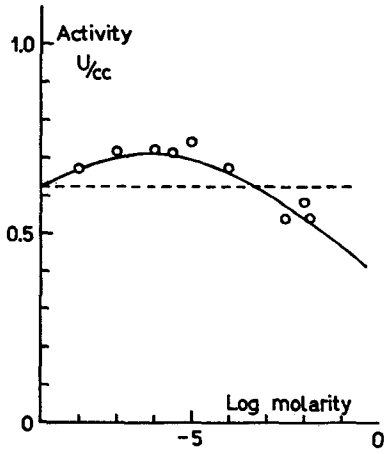

FIGURE 5. Activity of DNase II as a function of the logarithm of ethylenediaminetetraacetate concentration, at pH 4.6. - - - - pure NaAc.

preparations of DNase which have not been submitted to purification by chromatography.

DNase II loses a great deal of its activity by dialysis against distilled water. The presence of 0.001 M $MgSO_4$ protects it. 0.01 per cent sodium dodecyl sulfate completely inhibits the enzyme activity.

Subsequent Stages of Hydrolysis

The process of more complete hydrolysis of DNA by DNase I was followed at pH 6.2, at 37° C in the presence of high concentrations of DNase (1.25 μg/ml). A method of titration gave directly the number of secondary phosphate groups liberated as a function of time (Fig. 6). Total ionic strength was 0.15 and was adjusted when necessary by NaCl. In the presence of 0.005 M Mg^{++}, there is a very rapid phase which brings about the hydrolysis of 0.15 phosphate bond per phosphorus atom, followed by a very slow phase which continues several days. In the presence of Ca, the rapid phase is slower. In accordance with observations of Wiberg (1958) our measurements by methods sensitive to the earlier stages of degradation show that Ca sup-

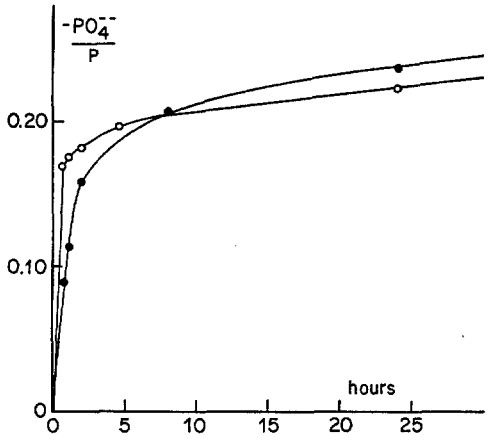

FIGURE 6. Hydrolysis of DNA at pH 6, by DNase I, in the presence of 0.005 M Mg (○) or 0.005 M Ca (●).

presses the early rapid action. Mg is a much better activator at these stages, yet the slow phase is stimulated by Ca more than by Mg, so that after some time the hydrolysis in the presence of Ca goes further than in the presence of Mg. High concentrations of Mg (0.05 M) have a lower activating effect particularly on the rapid phase.

We tried to separate the second stage of degradation by studying the hydrolysis of oligonucleotides. They were prepared by partial hydrolysis of DNA and fractionated by ethanol. In this way we separated them from high

TABLE II

ACTIVITY OF DNASES DETERMINED BY TITRATION OF SECONDARY PHOSPHATE GROUPS

At 37°C, 10 μg of DNase per ml, 0.5 mg of DNA per ml.

Enzyme	Substrate	Activator	No. of secondary phosphate per phosphorus atom		Percentage of bonds split
			Time = 0	Time = 24 hrs.	
DNase I	DNA	0.005 M Mg	0	0.20	20
	DNA	0.005 M Ca	0	0.22	22
	DNA	Mg + Ca	0	0.24	24
	Oligonucleotides	0.005 M Mg	0.15	0.18	18
	Oligonucleotides	0.005 M Ca	0.15	0.21	28
	Oligonucleotides	Mg + Ca	0.15	0.24	37
DNase II	DNA	0.001 M Mg	0	0.18	
	Oligonucleotides	0.001 M Mg	0.15	0.16	

molecular weight molecules which could survive from the original substrate. Their average degree of polymerization is in the neighborhood of 9 so they correspond to about 11 per cent of hydrolysis.

The oligonucleotides were fractionated and the fraction with the lowest degree of polymerization (6.5) was selected. It was submitted to the action of DNases I and II. The results of hydrolysis after 24 hours of action of enzymes at 37°C in the presence of various ions are given in Table II, in comparison with the hydrolysis of native DNA under the same conditions. In the last column are given the percentages of bonds split, that is, the total number of phosphoester bonds split in 24 hours related to the number of bonds present in the substrate at time zero. It may be seen that in the case of the oligonucleotides, the activating effect of Ca is noticeably higher than that of Mg. The effect of Ca and Mg together seems to reflect an addition of the separate effect of each ion. Both ions seem necessary to reach maximum degradation. This confirms the data of Fig. 6: Ca ion would be a better activator of the slow phase, that is the phase in which the substrate is already reduced to the state of a mixture of oligonucleotides. On the other hand, DNase II is almost unable to hydrolyze further the oligonucleotides resulting from the action of DNase I. This confirms results of Privat de Garilhe and Laskowski (1955).

DISCUSSION

The behavior of DNase II is relatively simple as regards activation. It appears that the good activators are all among the group of metals which are at the bottom of the scale as regards complex formation: alkaline earths and Mn; Zn also is very near Mn in its capacity to form complexes with many ligands (Martell and Calvin, 1953). The ions with high complex-forming abilities are inhibitors. This fits with the general theory of metal activation: a good activator must have some capacity to form complexes but too high a power will give rise to inhibitory properties (Klotz, 1954). The correspondence of the optimum values of activator and DNA concentrations indicates the formation of stoichiometric complexes. Important discrepancies exist between our results and those of Kurnick and Sandeen (1959) and Shack (1959) as regards optimal concentrations, degrees of activation, and optimal pH of action of salts. Since these authors used crude mouse spleen extracts, a comparison is impossible.

The behavior of DNase I is quite different and much more involved. Strong specificities are evident and they could not be related to complex-forming abilities or simple factors such as size, charge, etc. Moreover the synergistic effect of Mg and Ca introduces some puzzling problems.

It appears that the hydrolysis of DNA and its catalysis by DNase I occur in several stages, among which two distinct phases can be recognized: a rapid

one involving about 15 per cent of the total number of phosphoester bonds and a slow one which involves about 20 per cent more. Mg (as well as Mn and Co) is a specific activator of the first phase, Ca being a better activator for the second one. A complete explanation of these facts would require a much more thorough knowledge of the mechanisms of DNA splitting. We must content ourselves with some hypothetical considerations. We have already postulated (Fredericq, 1960) that the rapid phase is mostly due to the splitting of all the big molecules; from ultracentrifugal analysis, it appears that the degradation is not an "all or none" process (Fredericq, 1960), and that no high molecular material is left after some time. We have also supposed that DNase I is able to attack double chains as well as single chains resulting from the separation of the helical strands with low molecular weight. If this general scheme is correct, we could then suppose that Mg is a specific activator for the hydrolysis of double stranded high molecules; this would be related to its particular effect of maintaining together the DNA chains (Zubay, 1959). When the hydrolysis has split DNA into small pieces, they will spontaneously separate into single stranded oligonucleotides which will be attacked by DNase I much more slowly and this would constitute the slow phase. Ca is a good activator for this kind of substrate.

With regard to DNase II, it appears that it is probably unable to attack single stranded substrates (Fredericq, 1960). It is difficult at the present time to establish any correlation between ion binding and activation. Some attempts were made in that direction (Wiberg, 1958) but since that time, conflicting results have been published on the binding power of DNA for divalent cations. It appears that the first measurements were made on denatured material (Wiberg and Neumann, 1957); more recent results on native DNA indicates a weak binding of Mg ions (Zubay and Doty, 1958).

The only common point in the activation of DNases I and II is the privileged position of Mg and Mn ions. It appears that these two ions are general activators of many enzymes hydrolyzing peptide or phosphoester bonds.

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