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Synergistic Effects in the Activation of a Streptococcal Deoxyribonuclease by Bivalent Metal Ions

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This paper is a full account of our finding (Stone & Burton, 1961) that suitable pairs of bivalent metal ions show large synergistic effects on both the initial rate and the final extent of hydrolysis of deoxyribonucleic acid by a streptococcal deoxyribonuclease. The enzyme preparation was the same as that used by Potter & Laskowski (1959), whose results indicate that the enzyme is a deoxyribonuclease oligonucleotidohydrolase (EC 3.1.4.5). Streptodornase has often been used as a trivial name for the streptococcal deoxyribonucleases, of which Wannamaker (1958) has distinguished three types, produced in different proportions by different strains.

MATERIALS AND METHODS

Streptococcal deoxyribonuclease. This was Streptodornase (Lederle Laboratories, Pearl River, N.Y., U.S.A.), prepared from the culture medium of a group C strain (H46A) of *Streptococcus haemolyticus*. The preparation differed from Varidase, which contains both streptokinase and streptodornase. The material contained 4% of protein as estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine plasma albumin (Armour Laboratories, Eastbourne) as standard. Phosphate salts form the bulk of the enzyme preparation, contributing 40 μ M-phosphate to the incubation mixtures. Solutions were prepared in water and stored at 4° for a maximum period of 2 days.

Bovine pancreatic deoxyribonuclease. Crystalline enzyme was purchased from Sigma Chemical Co. (St Louis 18, Mo., U.S.A.).

Snake-venom diesterase and 5'-nucleotidase. A preparation that contained both these enzymes was kindly supplied by Dr P. Esnouf. It had been obtained from Russell's viper venom and was inactive on deoxyribonucleoside 3'-phosphates.

Deoxyribonucleic acid. Unless otherwise indicated, DNA (P content, 7.9%) had been prepared from herring testes essentially according to Emanuel & Chaikoff (1953). Solutions (2 mg./ml.) were prepared in 0.01 M-NaCl. Heat-denatured DNA refers to such a solution heated in a boiling-water bath for 30 min. and then cooled rapidly to room temperature. The extinction coefficient of this solution at 260 m μ was 136% of that before heating. Heated DNA was stored at 4° for up to 1 month. During this period the extinction was not altered and the DNA did not become susceptible to streptococcal deoxyribonuclease in the presence of Mg²⁺ as the sole activating metal ion. DNA from *Alcaligenes faecalis* and *Pseudomonas aeruginosa* was prepared as described by Burton (1960). Calf-thymus DNA was prepared according to Chargaff (1955).

Metal salts. These were analytical-reagent grade: Mg, Mn, Ca, Sr, and Ba were added as the chlorides (standardized by titration against AgNO₃); Fe²⁺ and Zn were added as the sulphates.

Buffers. A M solution of tris was adjusted to pH 7.2 at room temperature with N-HCl and diluted to the required molarity of tris. Similarly, m-glycine was adjusted to pH 8.6 with N-NaOH and diluted to the required molarity of glycine.

Glass-distilled water was used for the preparation of all solutions used.

Measurement of deoxyribonuclease activity. For all experiments except those in Table 1, the activity was measured by the release of acid-soluble products. Streptodornase (10 μ g. of the preparation), tris-HCl buffer, pH 7.2 (10 μ moles of tris), DNA (0.6 mg.) and metal salts as indicated were incubated at 37° in a volume of 1.3 ml. After a suitable time the reaction was stopped by the addition of 0.15 ml. of 2.5 N-perchloric acid. The mixture was stored at 4° for 30 min., centrifuged and the precipitate discarded. The extinction coefficient of the supernatant was measured at 260 m μ .

Estimation of metals as described below indicated that about 5 μ M-Ca and less than 0.2 μ M-Mg were contributed to the incubation mixture by the DNA [in Stone & Burton

(1961) the amount of Ca contributed by the DNA was [incorrectly given]. Streptodornase contributed less than $2\mu\text{M}$ -Ca and less than $0.2\mu\text{M}$ -Mg.

Under several ionic conditions with either native or heated DNA as substrate, the increase of the extinction coefficient of the supernatant at $260\text{ m}\mu$ (ΔE) was proportional to the period of incubation at 37° and to the concentration of enzyme. The same concentration of enzyme has been used in all the experiments presented and activity is expressed as $\Delta E/\text{min.}$ of incubation at 37° . All activities reported are therefore comparable even though different periods of incubation were used in order to obtain suitable degrees of hydrolysis.

For the data of Table 1, the hydrolysis was followed by measuring the production of acid. An automatic titrating apparatus (Radiometer, Copenhagen, Denmark) was used with 7 mM -NaOH in the syringe-burette. The titration vessel, fitted with an efficient stirrer, was kept at 37° . Solutions were prepared with CO_2 -free water and a stream of CO_2 -free air was passed over the surface of the liquid. The pH was kept at 7.5, at which the secondary phosphate is incompletely ionized. The extent of ionization was assessed by titration to pH 8.0 at the end of the period of enzyme action. The amount of alkali used was corrected for the titration of a control mixture, which was identical except that the enzyme had been inactivated by heating at 100° . The difference between the two titrations was between 10 and 25% of the total amount of alkali consumed during the enzyme action.

Chemical estimations

Metals. DNA (7 mg.) or streptodornase (1.4 mg.) was heated for 20 min. with boiling conc. HNO_3 and evaporated to dryness under an infrared lamp. The residue was taken up in 2.5 ml. of 3N - HNO_3 . Phosphate was estimated as described below and Na was estimated in a Beckman flame photometer by comparison with standard solutions of NaCl in 3N - HNO_3 . Calcium and Mg were then estimated with the use of standard solutions of CaCl_2 and MgCl_2 in 3N - HNO_3 , which also contained $\text{NH}_4\text{H}_2\text{PO}_4$ and NaCl in amounts equivalent to the phosphate and sodium in the ashed materials. Emissions were read at $422.7\text{ m}\mu$ for Ca, $285.2\text{ m}\mu$ for Mg and $589.3\text{ m}\mu$ for Na.

Inorganic phosphate. The method of Chen, Toribara & Warner (1956) was scaled down to 0.4 ml. final volume. EDTA (10 mM) was added to the colour reagent to prevent the spurious development of blue colour.

Total phosphate. Samples were ashed and phosphate was estimated as described by Burton & Petersen (1960).

Extinction measurements. A Beckman model DU quartz spectrophotometer with 1 cm. cells was used.

RESULTS

Metal requirements with native DNA as substrate. The effects of MgCl_2 , MnCl_2 and FeSO_4 on deoxyribonuclease activity are shown in Fig. 1. Magnesium chloride was the best activator, and gave the greatest effect at concentrations between 0.4 and 0.6 mM. Magnesium sulphate and MgCl_2 were equally effective. The optimum concentrations of MnCl_2 and FeSO_4 were about 0.15 and 0.05 mM respectively and they both gave about 40% of the

activity with optimum MgCl_2 . Higher concentrations of these activators decreased the rate of hydrolysis. We did not find any activation by the chlorides of Ca, Sr, Ba, Zn, Cd or Na or by Na_2SO_4 .

Fig. 2 shows that the activity was increased fivefold when 0.15 mM- CaCl_2 was added to a reaction mixture that already contained 0.6 mM- MgCl_2 . Strontium chloride also increased the activity to a lesser degree. Barium chloride and MnCl_2 were slightly inhibitory, whereas CdSO_4 , FeSO_4 and ZnCl_2 were potent inhibitors. Calcium chloride also increased the rate in the presence of the optimum concentration of MnCl_2 (Fig. 3). Strontium chloride increased the rate of hydrolysis slightly; BaCl_2 was inhibitory.

The $\text{Fe}^{2+}/\text{Ca}^{2+}$ pair was also synergistic. With 0.05 mM- FeSO_4 alone, the activity ($\Delta E/\text{min.}$) was 0.08. It was increased by adding CaCl_2 and the maximum value was 0.26 at 0.4 mM- CaCl_2 . The activity fell to 0.16 at 0.75 mM- CaCl_2 . The $\text{Ca}^{2+}/\text{Sr}^{2+}$ pair was not synergistic.

The effects of MgCl_2 and CaCl_2 are shown more fully in Fig. 4. In Fig. 4 (a) CaCl_2 was maintained at a constant concentration and the effects of different concentrations of MgCl_2 were studied; Fig. 4 (b) shows the reverse procedure. The inhibitory effects of a high concentration of one metal were overcome when sufficient of the other metal was added. However, when the enzyme was activated by a mixture of 4 mM- MgCl_2 and 4 mM- CaCl_2 , the inhibition by ZnCl_2 was as pronounced as when MgCl_2 was the sole activator; for example, 0.05 mM- ZnCl_2 decreased the activity by 70%.

Fig. 1. Activation by single metal ions (incubation times in parentheses): $\bullet$, MgCl_2 (24 min.); $\blacktriangle$, MnCl_2 (60 min.); $\circ$, FeSO_4 (60 min.). The reaction was linear with time and activities ($\Delta E/\text{min.}$) are given as the increase in extinction of the acid supernatant/min. of incubation at 37° . For other details see the Materials and Methods section.

Concentrations of NaCl up to 70 mM did not affect the activity in the presence of 4 mM-MgCl₂, so increased ionic strength was not responsible for

Fig. 2. Effects of several metal ions on enzyme activity in the presence of 0.6 mM-MgCl₂: $\bullet$, CaCl₂; $\circ$, SrCl₂; $\blacktriangle$, BaCl₂; $\triangle$, MnCl₂; $\blacksquare$, FeSO₄; $\square$, ZnSO₄. Incubation times: $\bullet$, 6 min.; $\circ$, 12 min.; $\blacktriangle$, $\triangle$, $\blacksquare$, $\square$, 20 min. The effect of CdSO₄ is not shown; this salt was slightly less inhibitory than FeSO₄.

Fig. 3. Effects of several metal ions on enzyme activity in the presence of 0.15 mM-MnCl₂: $\bullet$, CaCl₂; $\blacktriangle$, SrCl₂; $\blacksquare$, BaCl₂. Incubation times: $\bullet$ and $\blacktriangle$, 10 min.; $\blacksquare$, 30 min.

the stimulating action of Ca. Neither did the Ca act by inhibiting precipitation of the DNA fragments. The rate at which the enzyme was inactivated in the presence or absence of DNA, and in the absence of CaCl₂, was too low (about 13% loss of activity in 80 min. at 37°) for the effects to be explicable by stabilization of the enzyme. When the substrate or the enzyme was omitted and the mixtures were incubated for 1 hr. at 37° with 0.6 mM-MgCl₂ or a mixture of 0.6 mM-MgCl₂ and 0.6 mM-CaCl₂ before addition of the missing component, the complete systems gave the same activities as controls preincubated in the absence of the activating cations.

Effect of DNA concentration. Alterations of DNA concentration caused complex effects which have not been fully studied. For example, with MgCl₂ alone as activator, doubling the DNA concentration approximately doubled the optimum concentration of MgCl₂ and reduced the maximum rate.

Heat-denatured DNA as substrate. Heat-denatured DNA was not attacked in the absence of

Fig. 4. Synergism between activating ions with native DNA as substrate. (a) Different concentrations of MgCl₂ with: $\blacktriangle$, 4 mM-CaCl₂; $\circ$, 0.1 mM-CaCl₂; $\triangle$, no CaCl₂. (b) Different concentrations of CaCl₂ with: $\blacksquare$, 4 mM-MgCl₂; $\bullet$, 0.6 mM-MgCl₂; $\square$, 0.08 mM-MgCl₂; ∇ , without MgCl₂. Note that curves $\blacksquare$, $\bullet$ and $\square$ do not pass through the origin. Incubation times: $\blacktriangle$ and ∇ , 15 min.; $\circ$ and $\bullet$, 6 min.; $\triangle$ and ∇ , 24 min.; $\blacksquare$, 10 min. Also see legend to Fig. 1.

added metal salts or when various amounts of Mg^{2+} , Mn^{2+} , Fe^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+} or Zn^{2+} ions were present. Although each cation by itself was ineffective by itself a mixture of Mg^{2+} and Ca^{2+} ions allowed the enzyme to degrade heat-denatured DNA. The Mg^{2+}/Sr^{2+} pair was about one-tenth as effective. Ba^{2+} , Zn^{2+} or Na^+ ions did not act synergistically with Mg^{2+} ions.

Fig. 5 shows the results obtained for different concentrations of $MgCl_2$ and $CaCl_2$. In the presence of 4 mM- $MgCl_2$ the optimum rate required 8 mM- $CaCl_2$, as against 2 mM- $CaCl_2$ when native DNA was used at the same concentration of $MgCl_2$. The highest activity was about 40% of that obtained with native DNA as substrate.

Re-naturation of DNA. Bacterial DNA preparations are known to revert to a form that is similar to native DNA when they are cooled slowly after denaturation by heat (Marmur & Lane, 1960; Doty, Marmur, Eigner & Schildkraut, 1960; Marmur & Doty, 1961; Rownd, Lanyi & Doty, 1961). Solutions of DNA from *P. aeruginosa* and *A. faecalis* (2 mg./ml. in 0.15M-NaCl) were heated in a boiling-water bath for 10 min. and then cooled

to room temperature over a period of 2 hr. As expected, such re-natured DNA preparations were degraded by the streptococcal nuclease in the presence of 0.6 mM- $MgCl_2$ and no added $CaCl_2$. The rates were 60–75% of those obtained with the native DNA samples under the same conditions.

By contrast, the DNA preparations of herring testes and calf thymus heated and cooled in this manner were not degraded by the enzyme unless both $MgCl_2$ and $CaCl_2$ were added; these DNA preparations are not expected to become re-natured (Doty *et al.* 1960). The bacterial DNA preparations were not attacked in the absence of Ca^{2+} ions if, during the cooling period, the concentration of NaCl had been low (10 mM) or if the solutions had been cooled rapidly.

Denaturation of DNA at various temperatures. The 'melting-out' of crystalline structure when DNA is heated, as judged by the increase of extinction coefficient and other methods, occurs within a relatively small range of temperature (Doty, Boedtker, Fresco, Haselkorn & Litt, 1959; Marmur & Doty, 1959). Fig. 6 shows that the hyperchromic effect and the fall in susceptibility to the streptococcal nuclease both occur in virtually the same range of temperature. The extinction of the heat-denatured DNA was decreased by about 13% by the subsequent addition of 0.6 mM- $MgCl_2$ or a mixture of 4 mM- $MgCl_2$ and 8 mM- $CaCl_2$. The similarity of the extinctions under the two conditions contrasts with the different enzymic activities.

Proportion of internucleotide bonds hydrolysed. Since the measurement of activity in the above-described experiments might not be simply related to the number of bonds hydrolysed, the hydrolysis of the DNA was followed directly by measuring the production of acid during the enzyme action. Except for the omission of the tris buffer and the slightly higher pH, the compositions of the reaction mixtures were the same as those used for the previous experiments. The results (Table 1) confirm the synergistic effects of Mg^{2+} and Ca^{2+} ions upon the rates of hydrolysis and also show that there are pronounced effects upon the final extent of hydrolysis. The differences in the rates of hydrolysis of native and heat-denatured DNA are also evident when the activity is followed by this method. In all cases, the products were susceptible to further attack by pancreatic deoxyribonuclease, as has been noted earlier by Potter & Laskowski (1959).

For comparison, some similar experiments were performed with pancreatic deoxyribonuclease, which shows smaller synergistic activation effects (Wiberg, 1958; Fredericq, 1959). In the presence of 6 mM- $MgCl_2$, 31% of the phosphodiester linkages of native DNA were hydrolysed. With a mixture of 6 mM- $MgCl_2$ and 6 mM- $CaCl_2$, about 55% of the

Fig. 5. Synergism between activating ions with heated DNA as substrate. (a) Different concentrations of $MgCl_2$ with: O, 4 mM- $CaCl_2$; Δ , no $CaCl_2$. (b) Different concentrations of $CaCl_2$ with: ●, 4 mM- $MgCl_2$; ■, 0.6 mM- $MgCl_2$; ▲, no $MgCl_2$. All curves pass through the origin. Incubation times: O and ●, 15 min.; Δ and ▲, 20 min.; ■, 10 min.

Fig. 6. Activity of enzyme on DNA heated at different temperatures. DNA was denatured by heating solutions (2 mg./ml. in 0.01M-NaCl) for 30 min. at the indicated temperatures. (a) Hyperchromicity produced on denaturation: the DNA was diluted 100-fold into 0.01M-NaCl (■), 0.6 mM-MgCl₂ (○) or a mixture of 4 mM-MgCl₂ and 8 mM-CaCl₂ (▲), and extinctions were read at 260 m μ and room temperature. (b) Deoxyribonuclease activity: in the presence of 0.6 mM-MgCl₂ (●) (15 min. incubation) or in a mixture of 4 mM-MgCl₂ and 8 mM-CaCl₂ (△) (6 min. incubation).

internucleotide bonds were hydrolysed. In contrast with the streptococcal nuclease, the pancreatic enzyme caused appreciable hydrolysis (27%) in the presence of 6 mM-CaCl₂ alone, but, in agreement with Wiberg (1958), the rate was less than one-tenth of that with MgCl₂ alone.

Nature of hydrolysis products. Potter & Laskowski (1959) found that the streptococcal nuclease hydrolysed the phosphoester bonds at the 3'-position of the nucleotides and we have confirmed that this also occurs under our more favourable conditions of 0.6 mM-MgCl₂, or 6 mM-MgCl₂ plus 6 mM-CaCl₂. This was shown by the action of venom phosphodiesterase plus 5'-nucleotidase upon final reaction mixtures that had been heated at 90° for 5 min. to destroy the nuclease. The venom enzymes liberated 98% of the phosphorus of the nuclease digests as inorganic orthophosphate.

Purity of streptococcal deoxyribonuclease. Wannamaker (1958) has separated three deoxyribonu-

Table 1. Percentage of internucleotide bonds hydrolysed by streptococcal deoxyribonuclease

The reaction mixture initially contained 3.8 mg. of DNA, 0.1 mg. of deoxyribonuclease preparation and the activating salts in a total volume of 7.7 ml. at pH 7.5 and 37°. The reactions were complete in 10–30 min. The number of bonds hydrolysed is expressed as the percentage of the total internucleotide bonds in the DNA.

DNA	MgCl ₂ (mM)	CaCl ₂ (mM)	Bonds hydrolysed	
			Initial rate (%/min.)	Final amount (%)
Native	0.6	—	0.47	7.0
	—	0.6	0	0
	6.0	—	0.23	—*
	0.6	0.6	2.7	13.7
	6.0	6.0	3.8	16.7
Heat-denatured	6.0	—	0	0
	—	6.0	0	0
	6.0	6.0	1.7	21.4

* Not determined.

cleases (A, B and C) from the culture media of streptococci. The preparation that we have used appears to be deoxyribonuclease A, since its pH-activity curve had a maximum at pH 8.3–8.6, resembling Wannamaker's (1958) curve for deoxyribonuclease A, and also since Wannamaker found that nuclease A was the predominant deoxyribonuclease in crude extracts of strain H46A. On starch-block electrophoresis, by Wannamaker's procedure, the streptodornase preparation gave a single peak of deoxyribonuclease activity. Fractions from the maximum and from each side of it were examined for activity with Mg²⁺ ions alone and native DNA as substrate and also with both Mg²⁺ and Ca²⁺ ions and either native or heat-denatured DNA. In all fractions, the ratios of the three activities were closely similar. There is therefore no evidence that any of the different types of activity observed in this work are due to the presence of more than one deoxyribonuclease.

DISCUSSION

The bivalent cations studied fall into three groups: 1, Mg²⁺, Mn²⁺ and Fe²⁺ ions; 2, Ca²⁺ and Sr²⁺ ions; 3, Cd²⁺ and Zn²⁺ ions. A metal from group 1 acts synergistically with one from group 2 whereas group 3 metals are potent inhibitors. The results are consistent with the idea that the synergism depends upon the binding of both activating metals at specific sites in the enzyme-substrate complex. The observation that the inhibition by excess of Mg²⁺ ions can be reversed by increasing the concentration of Ca²⁺ ions and vice versa suggests that the two ions compete with each other for their respective sites.

Smaller synergistic effects have been reported for certain other deoxyribonucleases (Kay, 1954; Wiberg, 1958; Fredericq, 1959). The non-enzymic reaction between ATP and inorganic phosphate to give ADP and inorganic pyrophosphate is also synergistically catalysed by suitable pairs of bivalent metal ions (Lowenstein, 1960). Further study of this interesting reaction might indicate the modes of action of the two metals in each pair.

Although native DNA was hydrolysed in the presence of Mg^{2+} ions with no other added bivalent cation, it is conceivable that a second metal is always required for the action of the enzyme. The DNA contributed about $5 \mu M$ -Ca to the reaction mixtures and the curves of Figs. 2 and 3 for calcium suggest that this amount might be sufficient to account for the low activity in the absence of added Ca^{2+} ions.

With heat-denatured DNA as substrate there was a clear requirement for both Mg^{2+} and Ca^{2+} ions. In addition, at each concentration of Mg^{2+} ions the optimum concentration of Ca^{2+} ions was higher for heated DNA than for native DNA. In this connexion, Shack & Bynum (1959) have reported that the affinity of DNA for Mg^{2+} ions decreases on denaturation.

Bernheimer & Ruffier (1951) and Bernheimer (1953) have described differences in the abilities of ribonucleic acids from various sources to inhibit deoxyribonuclease from group A streptococci. These workers also found that the deoxyribonucleases of groups B and C streptococci were not inhibited by ribonucleic acid. However, there are considerable quantities of calcium in highly purified preparations of ribonucleic acid (Wacker & Vallee, 1959), and so the effects must have been complicated by differences in the calcium contents of the RNA preparations and in the sensitivities of the various deoxyribonucleases to Ca^{2+} ions.

SUMMARY

1. A commercially prepared extracellular deoxyribonuclease from a group C strain (H46A) of *Streptococcus haemolyticus* was activated by Mg^{2+} , Mn^{2+} or Fe^{2+} ions, with optimum concentrations of about 0.6, 0.15 and 0.05 mM respectively. Higher concentrations of the metal ions were inhibitory.

2. Although Ca^{2+} or Sr^{2+} ions did not by themselves activate the enzyme, they increased the reaction rate in the presence of Mg^{2+} , Mn^{2+} or Fe^{2+} ions. Ca^{2+} ions were shown to prevent the inhibition by excess of Mg^{2+} or Mn^{2+} ions.

3. With Mg^{2+} ions at the optimum concentration of 0.6 mM, estimation of the acid produced indicated that the reaction stopped when about 7% of the phosphodiester bonds of the DNA had been hydrolysed. With a mixture of 6 mM- Mg^{2+} and

6 mM- Ca^{2+} ions, the reaction was approximately eight times as fast, and about 17% of the bonds were split.

4. Under both types of activation, hydrolysis occurred at the phosphoester bonds attached to the 3'-position of the nucleotides.

5. If the deoxyribonucleic acid had been heated at 100° for 30 min., it was not attacked when any single metal was added, but the addition of Mg^{2+} ions together with either Ca^{2+} or Sr^{2+} ions allowed hydrolysis to proceed. The rate was less than that obtained with unheated deoxyribonucleic acid under the same conditions.

6. These results suggest that the depolymerization of DNA by the nuclease involves two metal-binding sites, or groups of sites, one having a specific requirement for Mg^{2+} , Mn^{2+} or Fe^{2+} ions and the other for Ca^{2+} or Sr^{2+} ions.

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