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THE EXTRACELLULAR NUCLEASE ACTIVITY OF *MICROCOCCUS SODONENSIS*

II. CHARACTERIZATION AND MODE OF ACTION

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SUMMARY

1. The purified extracellular nuclease of *Micrococcus sodonensis* has been shown to contain non-specific phosphodiesterase (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) activity against native and denatured DNA, RNA, polyadenylic acid and adenylyl-3′,5′-adenosine. It is exonucleolytic in its action, requiring a free 3′-hydroxyl group.

2. An associated phosphomonoesterase activity specific for 5′-ribo- and deoxyribomononucleotides was demonstrated.

3. Mn^{2+} alone is required for monoesterase activity while the diesterase requires Mg^{2+} in addition to Mn^{2+}, with Ca^{2+} having a stimulatory effect.

4. Monoesterase activity is more heat resistant than the diesterase but both are destroyed by heating for 5 min at 90°. The heat sensitivity of both activities is increased in the presence of Mg^{2+} and Mn^{2+} and decreased in the presence of Ca^{2+}.

5. pH optimum for both activities is 8.8.

6. The possibility of the existence of a single protein exhibiting both diesterase and monoesterase activity is discussed.

INTRODUCTION

In an attempt to classify nucleases from different sources, LASKOWSKI[1] suggested 4 criteria; type of substrate, type of attack, products formed and preferential

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Abbreviations: ApA, adenylyl-3′,5′-adenosine; pTp, thymidine 3′,5′-diphosphate; poly (A) polyadenylic acid. Esterified phosphoric acid is indicated by p placed at the left of the symbol (*e.g.* pA) if linked to the 5′-hydroxyl of the nucleoside, and at the right (*e.g.* Ap) if linked to the 3′-hydroxyl. Deoxyribonucleosides or nucleotides are indicated by d, placed at the left of the symbol (*e.g.* dA, deoxyadenosine; dpA, deoxyadenosine 5′-phosphate). M or N, unspecified ribo- or deoxyribonucleosides.

linkages. Some enzymes are specific for the sugar moiety, *e.g.* pancreatic ribonuclease and deoxyribonuclease (deoxyribonucleate oligonucleotidohydrolase, EC 3.1.4.5), while others, such as Micrococcal nuclease (ribonucleate (deoxyribonucleate) 3′-nucleotidohydrolase, EC 3.1.4.7) from *Staphylococcus aureus*, are non-specific phosphodiesterases attacking DNA and RNA. As well as a specificity for the sugar moiety there may also be a specificity for the configuration of the molecule. *Escherichia coli* exonuclease I (ref. 2) and *S. aureus* nuclease[3,4] preferentially attack denatured DNA, while *E. coli* exonuclease II and III show a distinct preference for native DNA[5].

Nucleases from different sources vary in their mode of attack on polynucleotide substrates. The attack may be exonucleolytic with the production of either 5′- or 3′-mononucleotides (*e.g.* snake venom[6] or spleen[7] phosphodiesterases, respectively), or endonucleolytic with the production of oligonucleotides terminated with 3′- or 5′-phosphates (*S. aureus* nuclease[8] or pancreatic deoxyribonuclease[9] respectively).

The present report deals with the characterization of the activity of purified *M. sodonensis* nuclease and an attempt is made to draw some conclusions concerning its mode of action. It has been suggested[10], that as well as being a criterion of pathogenicity, nuclease production may be of taxonomic value. In view of the difficulty encountered in separating the two genera, Staphylococcus and Micrococcus, it becomes necessary to investigate the characteristics of the nucleases produced by members of these two genera if differentiation on these grounds is to be possible.

MATERIALS AND METHODS

Enzymes

Purified *M. sodonensis* nuclease was obtained as described in the preceding paper. Pancreatic deoxyribonuclease (Worthington Biochemical), purified Russell's viper venom (Dr. B. G. LANE) and spleen phosphodiesterase (Mann Biochemicals) were also used in these studies.

Substrates

DNA (A grade), yeast RNA (C grade; purified by the method of EAVES AND JEFFRIES[11]) and the purine and pyrimidine nucleotides and nucleosides used were obtained from Calbiochem. Poly(A) was obtained from Mann Biochemicals while ApA was the kind gift of Dr. B. G. LANE. Denatured DNA was obtained by heating a stock solution (2 mg/ml) for 10 min at 100° and cooling rapidly.

Assay of enzyme activity

Diesterase activity was assayed as described in the preceding paper employing the following final concentrations of the various substrates: native and denatured DNA, 1 mg/ml; RNA, 4 mg/ml; poly (A), 1 mg/ml; ApA, 2 μmoles/ml.

Monoesterase activity was assayed using the following reaction mixture: mononucleotide substrate, 2 μmoles; Tris, 33.0 μmoles; $MgCl_2$, 13.0 μmoles; $CaCl_2$, 1.7 μmoles; $MnCl_2$, 1.7 μmoles; purified enzyme (Sephadex fraction) 0.1 ml, in a total volume of 1 ml, final pH 8.8. The mixture was incubated at 37° for the designated time intervals and the release of P_i was followed by the technique of AMES AND DUBIN[12].

pH dependency studies

Standard reaction mixtures were prepared employing the following buffers to provide the indicated pH values; pH 5 and 6, 0.1 M monosodium phosphate; pH 7, 8.7 and 9, 0.1 M Tris; pH 10 and 11, 0.1 M boric acid + 0.1 M KCl.

In all cases, buffers were adjusted to the requisite final pH with HCl or NaOH.

Exonuclease and endonuclease activity

Two methods were employed, both using denatured DNA as substrate: (a) a comparison of the products soluble in trichloroacetic acid and in 0.25% uranyl acetate–10% trichloroacetic acid according to the method of HELLEINER[13], and (b) the decrease in viscosity (Ostwald viscometer) as compared with the appearance of ultraviolet-absorbing, acid-soluble products.

Chromatography

One-dimensional paper chromatography was employed using the method described by SINGH AND LANE[14]. Separation of nucleosides from DNA and RNA digests was achieved after 18 h using 75% ethanol as the developing solvent. AMP and ApA digests were chromatographed using saturated $(NH_4)_2SO_4$–isopropanol (80:2, v/v) as the developing solvent. Good separation was obtained after 3–4 h. Spots were located with a hand ultraviolet lamp, eluted with distilled H_2O and quantitated by measuring absorption of the eluate at 260 mμ.

RESULTS

Heat stability

Fig. 1a shows the results of an experiment in which purified enzyme (400 units/ml) was heated for 5 min at various temperatures. Heating at 45° had little or no effect upon either the diesterase or monoesterase activity, however, diesterase activity was destroyed after 5 min at 55°. Monoesterase activity was destroyed only after heating at 90° for 5 min.

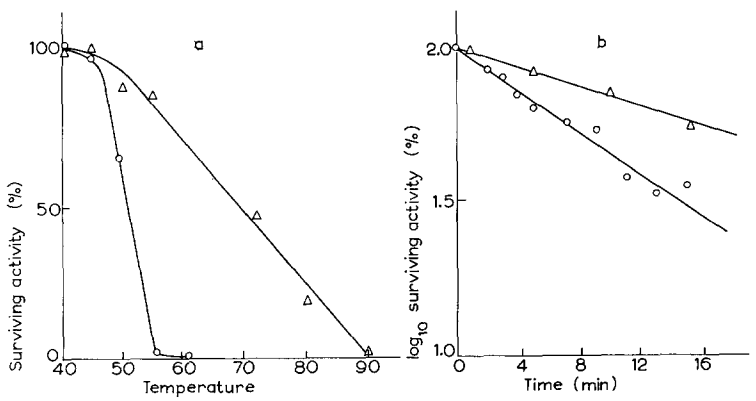


Fig. 1. Heat inactivation of *M. sodonensis* nuclease. a. Effect of heating for 5 min at varying temperatures. b. Effect of heating at 50° for varying time intervals. △—△, monoesterase activity; ○—○, diesterase activity.

TABLE I

EFFECT OF CATIONS ON INACTIVATION OF *M. sodonensis*

Nuclease by 5 min heating at 50°.

Addition	*Relative diesterase activity (%)*	*Relative monoesterase activity (%)*
None	66	88
Mg^{2+}	12	39
Mn^{2+}	7	30
Ca^{2+}	130	102
Ca^{2+}, Mg^{2+}, Mn^{2+}	103	120

The effect of heating the purified enzyme at 50° for various time intervals is shown in Fig. 1b. A linear inactivation curve was obtained for both activities.

Additional experiments to demonstrate the effect of cations on heat stability were carried out. It was found that the addition of either Mg^{2+} or Mn^{2+} to the enzyme prior to heating markedly increased the heat sensitivity of both activities (Table I). Ca^{2+} was protective or stimulatory in both cases, and the protective action was sufficient to counteract the adverse effect of Mg^{2+} and Mn^{2+} when a mixture of the 3 cations was used.

pH dependence

Optimal diesterase activity occurred from pH 8.2–9 with less than 11% remaining at pH 11 and a complete loss of activity at pH 6. The monoesterase activity followed essentially the same pH-dependence curve. pH 8.8 has been used routinely in the assay system.

Cation activation

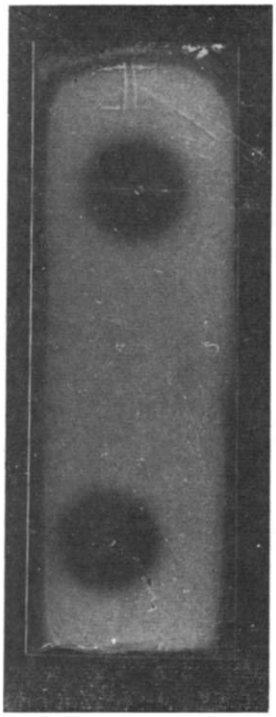
The effect of various combinations and concentrations of Mg^{2+}, Mn^{2+} and Ca^{2+} on diesterase activity was investigated.

Mg^{2+} and Mn^{2+} were both required for activation and the addition of Ca^{2+} had a synergistic effect. Optimum activity was obtained with $1.3 \cdot 10^{-2}$ M Mg^{2+}, $1.7 \cdot 10^{-3}$ M Mn^{2+} and $1.7 \cdot 10^{-3}$ M Ca^{2+}. An increase in the concentration of Mg^{2+} or Ca^{2+} had little or no effect but the concentration of Mn^{2+} was critical and any increase above $1.7 \cdot 10^{-3}$ M was inhibitory.

Mn^{2+} alone was required for monoesterase activity. As with the diesterase system a level of $1.7 \cdot 10^{-3}$ M Mn^{2+} was optimum and an increase over this level resulted in inhibition of activity.

Substrate specificity

Diesterase activity was tested against RNA and poly (A) as well as denatured and native DNA. Activity on poly (A) was tested by means of the diffusion plate assay (Fig. 2). Activity on RNA and denatured and native DNA is shown in Fig. 3. There was a lag of slightly more than 30 min with denatured DNA and of 2 h with native DNA, but after the lag period the rates were parallel. The longer lag period



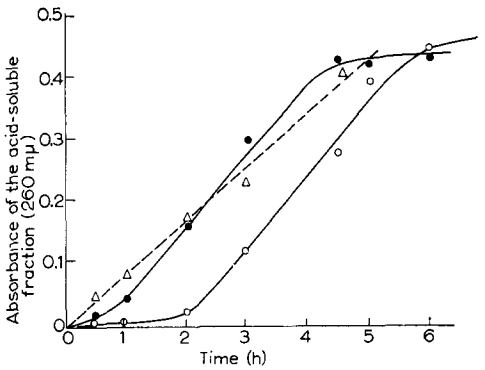


Fig. 2. Activity of purified *M. sodonensis* nuclease on polyadenylic acid. 0.06 ml of purified nuclease was impregnated into a filter-paper disc, and assayed on diffusion plate assay agar containing 0.5 mg poly (A) per ml, according to the method of BERRY AND CAMPBELL[18].

Fig. 3. Rate of diesterase activity of *M. sodonensis* nuclease on several polynucleotide substrates. ●—●, denatured DNA; ○—○, native DNA; △- - - -△, RNA.

with native DNA could be eliminated by preincubation of the assay mixture for 4 h at 37° before the addition of the enzyme. The effect did not appear to be that of denaturation since no hyperchromicity was observed.

Monoesterase activity was investigated using the various nucleotides. As shown in Table II all of the 5′-ribo- and deoxyribonucleotides were attacked yielding the corresponding nucleosides + P_i. The 3′-ribonucleotides, pTp, ADP and ATP were not dephosphorylated.

TABLE II

ACTIVITY OF *M. sodonensis* NUCLEASE ON RIBO- AND DEOXYRIBOMONONUCLEOTIDES

Substrate	*P_i per ml digest (mg)*	*Substrate*	*P_i per ml digest (mg)*
Cp	0	pC	0.014
Ap	0	pA	0.026
Gp	0	pG	0.030
Up	0	pU	0.017
pTp	0	dpT	0.020
ADP	0	dpG	0.024
ATP	0	dpA	0.031
		dpC	0.019

TABLE III

STUDY OF INITIAL REACTION RATES OF PURIFIED *M. sodonensis* NUCLEASE ON VARIOUS SUBSTRATES

Substrate	*Method of analysis*	*Initial rate (μmoles/h)*
RNA	Spectrophotometric	0.64
ApA	Chromatographic	0.50
AMP	Chromatographic	0.66
AMP	Release of P_i	0.50

Comparison of initial reaction rates

The rates of reaction on AMP, ApA and RNA were compared. Each reaction mixture contained 25 volumes % of the purified Sephadex fraction of the enzyme. RNA digestion was followed spectrophotometrically by measuring the appearance of acid-soluble products at 260 mμ. Digestion of ApA was estimated by chromatography and elution while both chromatography and phosphate assays were used in measuring activity on AMP. The results are tabulated in Table III.

As can be seen from these data the initial rates of activity on the various substrates are virtually identical.

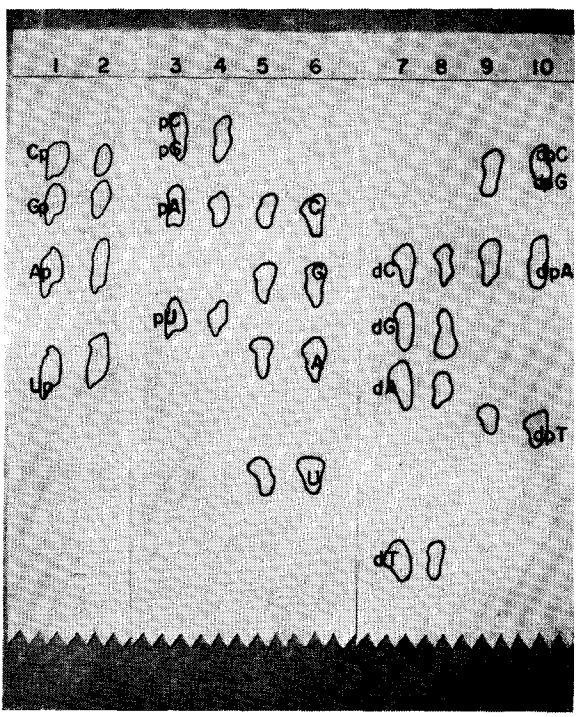


Fig. 4. Paper-chromatographic analysis of reaction products of several nucleases on DNA and RNA. 1. known 3′-ribonucleotides. 2. Spleen phosphodiesterase on RNA. 3. known 5′-ribonucleotides. 4. Snake-venom phosphodiesterase on RNA. 5. *M. sodenensis* nuclease on RNA. 6. known ribonucleosides. 7. known deoxyribonucleosides. 8. *M. sodonensis* nuclease on DNA. 9. snake-venom phosphodiesterase on DNA. 10. known 5′-deoxyribonucleotides.

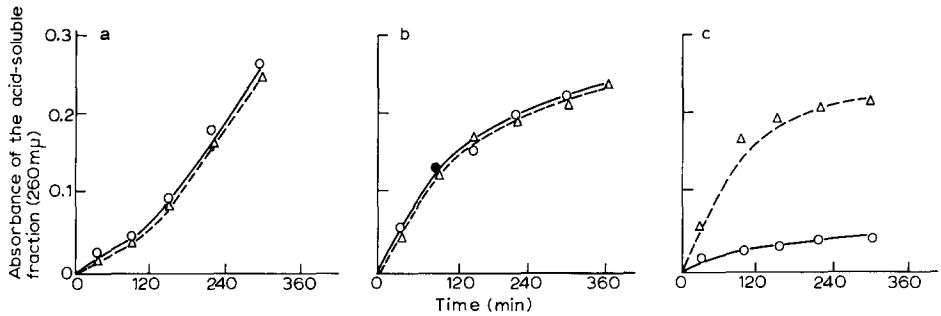


Fig. 5. Comparison of mode of action of various nucleases on denatured DNA. a. *M. sodonensis* nuclease. b. Snake-venom phosphodiesterase. c. Pancreatic deoxyribonuclease. △- - - -△, trichloroacetic acid soluble; ○—○, soluble in 0.25% uranyl acetate–10% trichloroacetic acid.

Products of digestion of DNA and RNA

The reaction products of *M. sodonensis* nuclease activity on DNA and RNA were analyzed by paper chromatography and compared with those of snake-venom phosphodiesterase and spleen phosphodiesterase (Fig. 4). Spleen phosphodiesterase released the 3′-mononucleotides and snake venom the 5′-mononucleotides while the products of the *M. sodonensis* enzyme were the corresponding nucleosides. At no time during the digestion were nucleotides detectable in the *M. sodonensis* digest. Analysis of the digests for P_i showed that activity had resulted in a release of P_i by the *M. sodonensis* enzyme, whereas neither spleen nor snake-venom phosphodiesterase exhibited this effect.

Evidence of exonucleolytic activity

The fact that nucleosides were the only reaction products detected at any time suggested an exonucleolytic type of attack by the *M. sodonensis* enzyme. Further support for this hypothesis was obtained by the following experiments.

The activity of the *M. sodonensis* nuclease was compared with that of a known exonuclease (snake-venom phosphodiesterase) and a known endonuclease (pancreatic deoxyribonuclease). The release of acid-soluble products is plotted in Fig. 5. In the case of pancreatic deoxyribonuclease, the internal bonds of the polymer were attacked

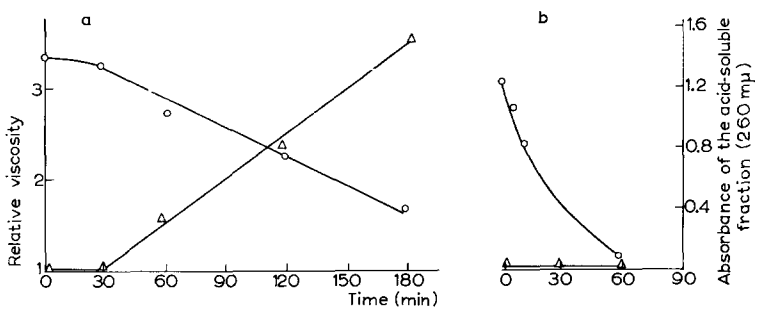


Fig. 6. Comparison of viscometric and spectrophotometric analyses of *M. sodonensis* and pancreatic nucleases. a. *M. sodonensis* nuclease. b. Pancreatic deoxyribonuclease. ○—○, viscosity; △—△, material soluble in 0.25% uranyl acetate–10% trichloroacetic acid.

releasing oligonucleotides, some of which were soluble in trichloroacetic acid but not in 0.25% uranyl acetate–10% trichloroacetic acid. Snake venom on the other hand releases 5'-mononucleotides one at a time from the 3'-hydroxyl end of the polymer. These products are soluble in both trichloroacetic acid reagents. The *M. sodonensis* nuclease is clearly exonucleolytic by these criteria.

Fig. 6 is the plot of the data from an experiment in which standard reaction mixtures, using pancreatic deoxyribonuclease and *M. sodonensis* nuclease, were analyzed for change in viscosity and for the appearance of soluble 260 mμ-absorbing products. In the case of pancreatic deoxyribonuclease there was a rapid decrease in viscosity preceding the appearance of products soluble in 0.25% uranyl acetate–10% trichloroacetic acid. The *M. sodonensis* nuclease showed a more gradual decrease in viscosity concurrent with the appearance of these soluble products. The latter occurrence is typical of an exonuclease and confirms the hypothesis that the *M. sodonensis* enzyme is exonucleolytic in its activity.

DISCUSSION

The nuclease of *M. sodonensis* is unique in several respects from other microbial nucleases that have been described. It differs markedly from the Micrococcal nuclease of *S. aureus*, for example, in that it is exonucleolytic, it readily degrades native as well as denatured DNA, is more heat labile (even in the presence of Ca^{2+}) and requires Mg^{2+} and Mn^{2+} for activation. In fact the only similarities between the two systems are their extracellular existence and pH optimum. The *M. sodonensis* system differs from other bacterial exonucleases such as *E. coli* exonucleases I, II and III, since it is extracellular, degrades native or denatured DNA, and RNA at essentially the same rate, thus exhibiting little or no preference for either the sugar moiety or the configuration of the polymer. Although kinetic data were not obtained, the *M. sodonensis* enzyme exhibits good activity against synthetic polymers (poly (A)) and replicative form (double stranded) MS_2 RNA prepared according to the technique of WEISSMANN *et al.*[15] (unpublished data). Regardless of the substrate used, the products of digestion by the *M. sodonensis* enzyme are nucleosides + P_i.

The observation that the products of polynucleotide digestion are nucleosides *plus* P_i, coupled with the fact that the monoesterase is active only against 5'-mononucleotides suggests that diesterase activity proceeds from the 3'-hydroxyl end. Further evidence for this lies in the fact that ApA digestion yields only adenosine + P_i, with no detectable pA or Ap produced, while pTp in untouched. The addition of another ionizable group to the 5'-phosphate of adenylic acid (*e.g.* ADP, ATP) inhibits the activity and indicates the specificity of the enzyme for the N·pN linkage.

The coincidence of mono- and diesterase activity in the purified preparation suggests either that there are two separate, but very similar, proteins present, or that there is a single protein responsible for both activities. Although definite proof is still lacking it is felt that there are compelling reasons why the latter should not be disregarded. The purification effected no separation of the two activities and the ratio of diesterase: monoesterase remained constant at each step in the sequence, resulting in a fraction which was homogeneous in both electrophoretic and ultracentrifugal analyses. The two activities have identical pH optima and require the same level of

Mn^{2+} for activity. They are both inhibited by excess Mn^{2+}, rendered more heat labile by Mg^{2+} or Mn^{2+}, and are heat stabilized by Ca^{2+}.

The initial reaction rates on AMP, ApA and RNA are essentially equal. No free mononucleotides were detectable at any stage of digestion of polynucleotide substrates. Since it is difficult to obtain concentrations of large polynucleotides which are saturating for an exonuclease, it is probable that the actual rate of activity on RNA is faster than the measured rate. This means that the rate of diesterase activity is at least as fast, and perhaps faster, than that of the monoesterase. Therefore the monoesterase, rather than the diesterase, must be rate limiting and if two separate enzymes were involved one would expect to be able to detect some free mononucleotides at some stage.

Diesterase activity requires Mg^{2+} in addition to Mn^{2+} and is more heat sensitive than is the monoesterase. This could suggest the existence of two enzymes but does not necessarily disprove the preceding hypothesis. It is conceivable that binding with a polymer involves an additional site on the protein which is Mg^{2+}-activated and is not involved in complexing with the mononucleotide. This more complex binding arrangement could be more sensitive to slight configuration changes in the protein brought about by heat than is the one involved in binding the mononucleotide.

If it is one enzyme it is necessary that it break the phosphodiester bond first on the 3′, then on the 5′ side of the phosphorus. It is suggested that the terminal nucleotide is complexed to the protein at the Mn^{2+}-dependent site and at a second Mg^{2+}-dependent site. The diester bond is broken on the 3′ side of the P releasing the enzyme-nucleotide complex and freeing the Mg^{2+}-dependent site. Then the monoester bond is broken releasing the enzyme, nucleoside and P_i. The Mn^{2+}-dependent site alone is involved when mononucleotides are the substrates.

The sharing of the Mn^{2+}-dependent site by both activities makes it unnecessary for the nucleotide to be released and recomplexed with another site either on the same or another protein. The kinetic data and the absence of detectable free nucleotides support this. The postulated sequence is as follows:

$$\text{----pNpNpM} + \text{enzyme} \xrightarrow[Mg^{2+}(Ca^{2+})]{Mn^{2+}} \text{---pNpNpM}\cdot\text{Enzyme}$$

$$\text{pM} + \text{Enzyme} \xrightarrow{Mn^{2+}} \text{pM}\cdot\text{Enzyme} + \text{----pNpN}$$

$$\text{pM}\cdot\text{Enzyme} \longrightarrow P_i + \text{M} + \text{Enzyme}$$

ACKNOWLEDGEMENTS

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REFERENCES

1 M. LASKOWSKI, *Ann. N.Y. Acad. Sci.*, 81 (1959) 776.
2 I. R. LEHMAN AND A. L. NUSSBAUM, *J. Biol. Chem.*, 239 (1964) 2628.

3 M. L. DIRKSEN AND C. A. DEKKER, *Biochem. Biophys. Res. Commun.*, 2 (1960) 147.
4 P. H. VON HIPPEL AND G. FELSENFELD, *Biochemistry*, 3 (1964) 27.
5 C. C. RICHARDSON, I. R. LEHMAN AND A. KORNBERG, *J. Biol. Chem.*, 239 (1964) 251.
6 W. E. RAZZELL AND H. G. KHORANA, *J. Biol. Chem.*, 234 (1959) 2114.
7 W. E. RAZZELL AND H. G. KHORANA, *J. Biol. Chem.*, 236 (1961) 1144.
8 M. ALEXANDER, L. A. HEPPEL AND J. HURWITZ, *J. Biol. Chem.*, 236 (1961) 3014.
9 J. L. POTTER, K. D. BROWN AND M. LASKOWSKI, *Biochim. Biophys. Acta*, 9 (1952) 150.
10 B. G. WECKMAN AND B. W. CATLIN, *J. Bacteriol.*, 73 (1957) 747.
11 G. N. EAVES AND C. D. JEFFRIES, *J. Bacteriol.*, 85 (1963) 273.
12 B. N. AMES AND D. T. DUBIN, *J. Biol. Chem.*, 235 (1960) 769.
13 C. W. HELLEINER, Ph. D. Thesis, 1955, Department of Biochemistry, University of Toronto.
14 H. SINGH AND B. G. LANE, *Can. J. Biochem.*, 42 (1964) 1011.
15 C. WEISSMANN, P. BORST, R. H. BURDEN, M. A. BILLETER AND S. OCHOA, *Proc. Natl. Acad. Sci. U.S.*, 51 (1964) 683.
16 S. A. BERRY AND J. N. CAMPBELL, *Biochim. Biophys. Acta*, 99 (1965) 566.