Studies on the Allosteric Properties of Nuclear Exoribonuclease from Ehrlich Ascites Tumor*

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ABSTRACT: The exoribonuclease isolated from the nucleus of Ehrlich ascites tumor cells has been found to have cooperative interactions with the inhibitor, polydextran sulfate (PDS), and with the positive effectors, phosphate and magnesium ions. When the enzyme is maximally activated by phosphate ion, PDS inhibition is diminished, and the inhibition is no longer cooperative. Cooperative inhibition by PDS is also diminished by repeated freezing and thawing of the enzyme. In contrast, magnesium ion, which stimulates enzyme activity, enhances inhibition by PDS. Denatured DNA is a partially competitive inhibitor of the enzyme, without cooperative interaction. NaCl decreases inhibition by both PDS and DNA, but does not abolish cooperative interaction with PDS.

We have previously suggested a possible role for nuclear exoribonucleases in regulating the metabolic fate of nuclear RNA (Lazarus and Sporn, 1967; Sporn *et al.*, 1969). Since the biological activity of many enzymes is now known to be controlled by their allosteric properties (Monod *et al.*, 1963, 1965; Stadtman, 1966; Koshland and Neet, 1968), a study of some of the allosteric properties of nuclear exoribonucleases was undertaken. In the present paper, we present data concerning the allosteric nature of the nuclear exoribonuclease isolated from Ehrlich ascites tumor cells. This enzyme has been found to be inhibited in a cooperative manner by polydextran sulfate (PDS).[1] In contrast, the enzyme is inhibited in a noncooperative manner both by certain fully competitive inhibitors, such as adenylate oligonucleotides with a terminal 2′,3′-cyclic phosphate group (Lazarus *et al.*, 1968) and by the partially competitive inhibitor, denatured DNA. The present paper also deals with the activation of Ehrlich tumor exoribonuclease by the positive effectors, phosphate and magnesium ions. This is the fourth paper in a series on nuclear exoribonucleases; for the previous paper, see Sporn *et al.* (1969).

Experimental Procedure

Materials. Exoribonuclease was purified from Ehrlich ascites tumor cell nuclei (Lazarus and Sporn, 1967); peak tubes from fraction 7 (second DEAE column chromatography) were used after being stored in a liquid nitrogen refrigerator. The enzyme has been purified over 200 times its original concentration in the whole cell. The final preparation has no detectable endonuclease activity, and 5′-mononucleotides are the sole product of exonucleolytic degradation of RNA (Lazarus and Sporn, 1967; Sporn *et al.*, 1969). Sources of other reagents were as follows: polydextran 500 (molecular wt, 5×10^5), polydextran DEAE 2000 (molecular wt, 2×10^6), polydextran sulfate 500 (molecular wt, 5×10^5), and polydextran sulfate 2000 (molecular wt, 2×10^6), Pharmacia, Piscataway, N. J.; calf thymus DNA, Worthington Biochemical, Freehold, N. J.; poly (A), Miles Laboratories, Elkhart, Ind.; and crystalline BSA, Pentex, Kankakee, Ill.

Enzyme Assay. The standard reaction mixture (1.0 ml) was as follows: 0.1 M Tris-Cl (pH 7.4)–4 mM $MgCl_2$–0.2 mM dithiothreitol–dialyzed bovine serum albumin 100–200 μg/ml–exoribonuclease, 0.3 unit/ml–poly (A) of varying concentrations (0.1 to 3.0 mM in adenine mononucleotide equivalent); and effectors of varying concentrations. The reaction was run at 37° for 30 min, and stopped by the addition of 2.0 ml of ice-cold 0.6 M perchloric acid. The precipitate was collected by centrifugation at 1900*g* for 30 min. The optical density of the supernatant was determined at 260 mμ in a Beckman DB or DU spectrophotometer. A unit of enzyme is defined as that amount which forms 1 μmole of adenosine 5′-phosphate per hr in the presence of 3 mM poly(A) and 25 mM potassium phosphate (pH 7.4); the assay is linear for 60 min (Lazarus and Sporn, 1967).

Other Methods. The DNA was denatured at 100° for 10 min and then quenched in ice. The concentration of poly(A) was determined using $\epsilon(P)$ (257 mμ) = 9.9×10^3 (Sarkar and Yang, 1965). A solution of native DNA in 0.01 M potassium phosphate (pH 6.8) was taken to have $\epsilon(P)$ (260 mμ) = 6300 (Kay *et al.*, 1952).

Results

Inhibition of Exoribonuclease by PDS. The cooperative nature of the inhibition of native exoribonuclease by PDS is readily seen in Figure 1, curve A. This curve of inhibition by a polyanionic sulfate is clearly sigmoidal in nature. This is in marked contrast to the competitive inhibition by the polyanions which are analogs of poly(A), namely oligoribonucleotides of adenine with a terminal 2′,3′-cyclic phosphate (Lazarus *et al.*, 1968). The initial stimulation of activity at low concentration of PDS was a consistent finding. The initial binding of PDS, at low concentration, to a few sites apparently causes the entire enzyme to be in a configuration which has increased activity for degradation of poly(A). As the PDS concentration increases, the configuration produced is a less active form. The inhibition caused by PDS is reversible and is not progressive with time of incubation.

The inhibition is caused by the multiple sulfate groups on

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[1] Abbreviation used is: PDS, polydextran sulfate 2000.

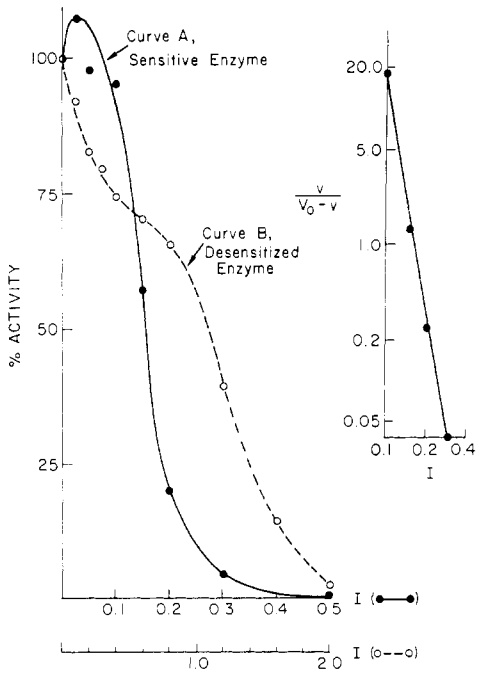

FIGURE 1: Inhibition of exoribonuclease by PDS 2000. The reaction mixture (1 ml) contained 0.1 M Tris-Cl, pH 7.4; 0.004 M $MgCl_2$; poly(A), 1 mg; exoribonuclease, 0.3 unit; bovine serum albumin, 100 μg–0.2 mM dithiothreitol; I (PDS 2000, μg/ml). The mixture was incubated at 37° for 30 min and the reaction was stopped by addition of 2 ml of 0.6 M perchloric acid at 0°. After centrifugation at 1900*g* for 30 min, the OD_{260} of the supernatant was measured. When [I] = 0 (100% activity of enzyme), the reaction velocity was 0.5 OD_{260} unit per 30 min. Curve A represents native enzyme and curve B represents enzyme partially desensitized by freezing and thawing several times. The insert is a Hill plot of curve A; V_0 is the reaction velocity in the absence of PDS; v is velocity in its presence.

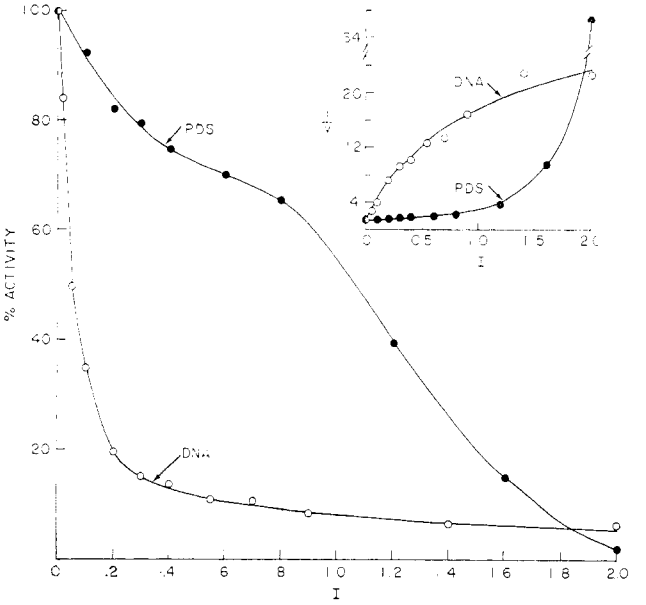

FIGURE 2: Inhibition of partially desensitized exoribonuclease by PDS 2000 and denatured DNA. The enzyme was frozen and thawed several times before the assay. Reaction conditions were the same as for Figure 1. The value of [I] is plotted in μg/ml for PDS and (μg/ml) (10^{-1}) for denatured DNA. The insert shows a plot of $1/v$ *vs.* [I], with v expressed as OD_{260} units per 30 min.

polydextran sulfate, not by the polydextran backbone. Equal weights of polydextran sulfate 500 and 2000 cause equal inhibition, while there was no enzyme inhibition by an equivalent weight of polydextran 500 or polydextran DEAE 2000. Other polyanionic sulfates, such as polyglucose sulfate (Wood and Mora, 1958) and heparin, are powerful inhibitors of exoribonuclease (H. M. Lazarus and M. B. Sporn, unpublished data), but their kinetics have not been explored. The mononucleoside 3′- and 5′-sulfates are poor inhibitors (Sporn *et al.*, 1969).

Adaptions of the Hill (1913) equation, $y = Kx^n/(1 + Kx^n)$, originally proposed to describe hemoglobin oxygenation, have been widely used in studies of enzyme kinetics to determine the molecular order of participation of inhibitors (Atkinson, 1966; Loftfield and Eigner, 1969). If one assumes that the velocity of the exoribonuclease reaction is inversely proportional to the fractional saturation of the inhibitor binding sites, the Hill equation for an inhibitor (Monod *et al.*, 1963) may be put in form $\log [v/(V_0 - v)] = \log K - n \log [\text{PDS}]$, where V_0 is reaction velocity in the absence of inhibitor, v is reaction velocity in the presence of inhibitor, n is the number of sites at which inhibitor may bind, and K is a constant. The inhibition of native exoribonuclease by PDS (Figure 1, curve A) gives a Hill plot (Figure 1, insert) with the value of n, the slope, equal to minus 5.5. As is the case with many other enzymes, the ability to undergo cooperative effects can be altered by various physicochemical treatments such as freezing and thawing, heat, etc. (Changeux, 1961; Monod *et al.*, 1965). This is the case for inhibition of exoribonuclease by PDS.

Effect of Freezing and Thawing of Exoribonuclease on PDS Inhibition Kinetics. Exoribonuclease becomes partially desensitized to inhibition by PDS if the enzyme is frozen and thawed two or more times, although catalytic activity is fully retained. With partially desensitized enzyme, the shape of the curve for inhibition by PDS is different from the curve obtained with sensitive enzyme (compare Figure 1, curves A and B). For an equal degree of inhibition, more PDS is needed for inhibition of the desensitized enzyme. There is no stimulation of activity at low concentrations of PDS. The inhibition of frozen and thawed enzyme by PDS at low concentrations is apparently noncooperative, while with higher concentrations of PDS, the inhibition again becomes cooperative. Figure 2 (insert) shows that with partially desensitized enzyme the lower concentrations of PDS give a linear region in a plot of $1/v$ *vs.* [I], while at higher concentrations the plot of $1/v$ *vs.* [I] is hyperbolic. This fact is further substantiated by the Hill equation which, for the lower PDS concentrations, gives n = minus 0.7, while for the higher PDS concentrations, n approaches minus 6. Figure 2 also shows that denatured DNA inhibits exoribonuclease; the nature of this inhibition is different from that caused by PDS and will be discussed in greater detail below.

Contrast of the Effects of Phosphate Ion and NaCl on PDS Inhibition. A plot of PDS inhibition of exoribonuclease in the presence of phosphate ion produces a nonsigmoid curve, as shown in Figure 3, which should be contrasted with the sigmoid curves shown in Figures 1 and 2. As seen by the linearity of the $1/v$ *vs.* [I] plot, the cooperative inhibition by PDS is lost in the presence of phosphate (see Figure 3, insert, which should be contrasted with Figure 2, insert). Moreover, in the presence of phosphate ion, PDS is a markedly less potent inhibitor of exoribonuclease, as shown in Figure 4.

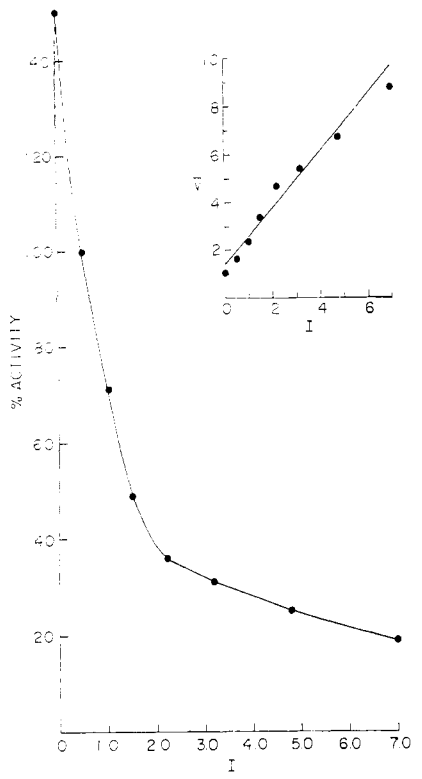

FIGURE 3: Inhibition of exoribonuclease by PDS 2000 in the presence of phosphate ion. Reaction conditions and enzyme preparation were the same as for Figure 2, with the addition of 40 mM potassium phosphate, pH 7.4. In this figure, 100% activity is the enzyme activity in the absence of phosphate ion and PDS. I is PDS 2000 (μg/ml). The insert shows a plot of $1/v$ *vs.* [I], with v expressed as OD_{260} units per 30 min.

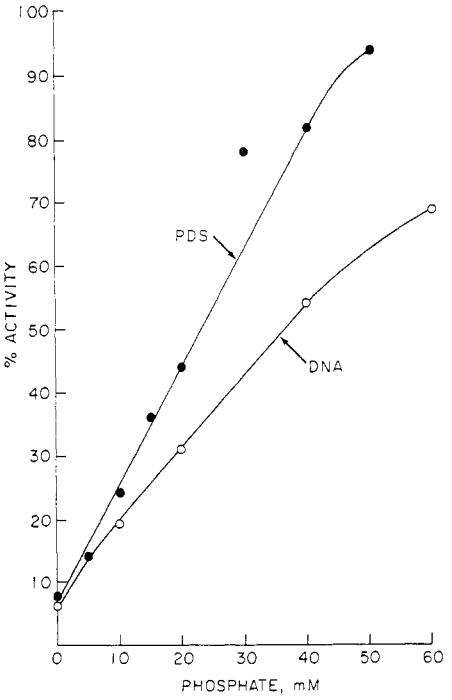

FIGURE 4: Stimulation of exoribonuclease by potassium phosphate in the presence of PDS 2000 and denatured DNA. Reaction conditions and the enzyme preparation were the same as in Figure 2. In this figure, 100% activity is enzyme activity with no effectors; *i.e.*, no phosphate, DNA or PDS. The concentration of PDS was 2 μg/ml and of DNA was 10 μg/ml.

However, NaCl, which also decreases the inhibitory action of PDS on exoribonuclease, does not affect the cooperative nature of this inhibition, as shown in Figure 5. Mora (1962) showed that NaCl decreased the inhibition of pancreatic ribonuclease by PDS and suggested that the binding between protein and polyanion is electrostatic. However, the effect of phosphate ion on the binding of PDS to exoribonuclease appears to be more than the effect of the ion interfering with electrostatic binding. Phosphate ion converts the PDS–enzyme interaction from a cooperative into a noncooperative form, as may be seen by comparing Figure 3 and Figure 2, while NaCl appears only to decrease the electrostatic binding between enzyme and inhibitor. Thus, the ability of phosphate to prevent PDS from binding cooperatively to exoribonuclease probably implies that phosphate affects conformational changes in the protein molecule over and above the salt effects.

Inhibition by Denatured DNA. Exoribonuclease is inhibited by another polyanion, denatured DNA (dnDNA), as shown in Figures 2, 4, 5, and 6. The interaction of this inhibitor with the enzyme is not altered by repeated freezing and thawing, as is PDS enzyme interaction. The degree of enzyme inhibition by dnDNA is lessened by 0.2 M NaCl, as shown in Figure 5. In the absence of NaCl, dnDNA caused greater than 95% inhibition of enzyme activity for all concentrations of dnDNA shown in Figure 5; whereas in the presence of 0.2 M NaCl maximal inhibition by dnDNA was only 70%. Thus, dnDNA binding to exoribonuclease involved electrostatic bonds weakened by NaCl. The inhibition of exoribonuclease by native DNA is 20% of that for dnDNA. The inhibition of exoribonuclease by dnDNA shows no evidence of cooperativity (Figures 2 and 5). The character of the inhibition by dnDNA appears to be that of a partially competitive inhibitor (Dixon and Webb, 1964). The inhibitor affects the affinity of the enzyme for the substrate, as shown by the Lineweaver–Burk (1934) plot in Figure 6. However, at fixed substrate concentration, the inhibition does not increase indefinitely with increasing inhibitor concentration (as in the fully competitive case), but increases to a definite limit at which all enzyme is combined with inhibitor, and can then increase no further (Figure 2, insert). The plot of $1/v$ *vs.* [I] for dnDNA (Figure 2, insert) clearly demonstrates this fact as it does not give a straight line (as it does in the fully competitive case for oligoribonucleotides of adenine with a terminal 2′,3′-cyclic phosphate (Lazarus *et al.*, 1968)) but gives a line with decreasing slope. Dixon and Webb (1964) have assumed that in the case of partially competitive inhibition, inhibitor and substrate do not bind at an identical site on the enzyme, but rather, the inhibitor causes a conformational change which decreases the affinity of the catalytic site of the enzyme for the substrate. However, catalysis of bound substrate is not influenced by the inhibitor, as shown by the V_{max} being unaltered in a Lineweaver–Burk plot (Figure 6). Similar examples of partially competitive inhibition have been noted with aspartate transcarbamylase (Gerhart and Pardee, 1963) and glutamine synthetase (Woolfolk and Stadtman, 1967). Whereas it is reasonable to consider that the partially competitive kinetics observed for dnDNA represent the effects of dnDNA on a single enzyme, it cannot be discounted that there are multiple molecular forms of exoribonuclease in the present enzyme preparation, some of which are not competitively inhibited by dnDNA. Examples of isozymes which differ in their sensitivity to competitive

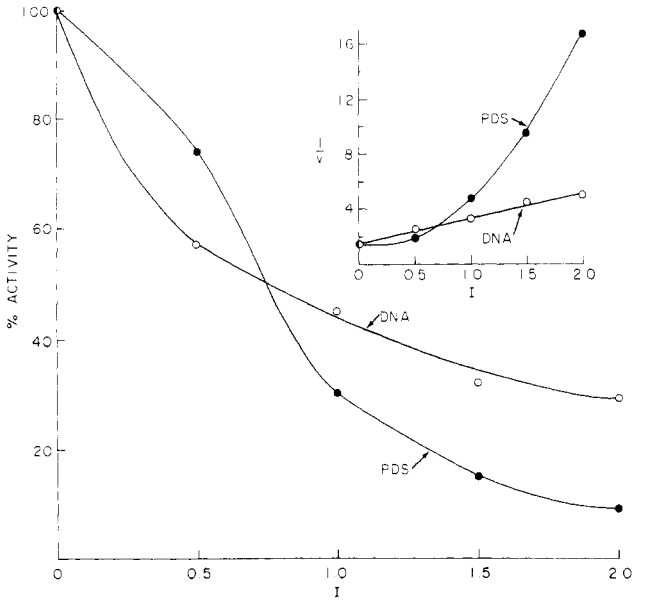

FIGURE 5: Effect of NaCl on inhibition of exoribonuclease (native enzyme) by PDS 2000 and denatured DNA. Reaction conditions were the same as for Figure 1, with the concentration of poly(A) = 0.5 mg/ml, and the addition of 0.2 M NaCl. The value of [I] is plotted in μg/ml for PDS and (μg/ml) (1/75) for DNA. In this figure, 100% activity is activity in the absence of inhibitor and in the presence of 0.2 M NaCl, which is 30% the velocity of the reaction without NaCl. The insert shows a plot of $1/v$ *vs.* [I], with v expressed as OD_{260} units per 30 min.

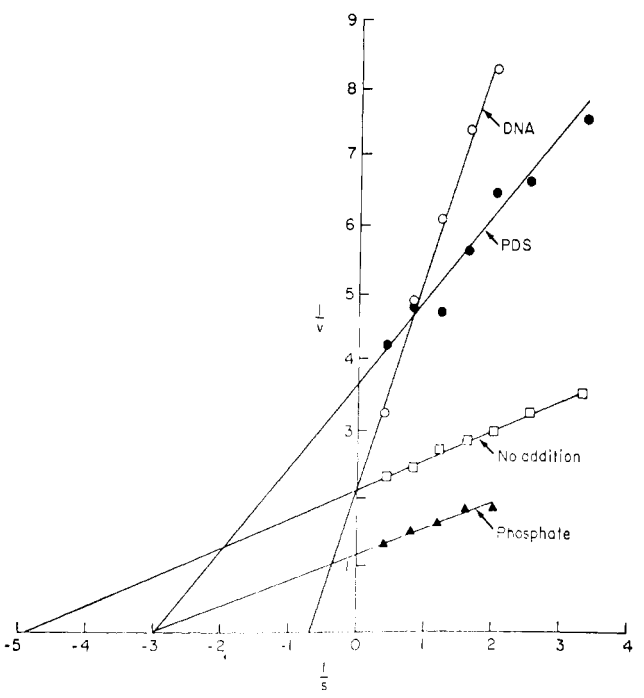

FIGURE 6: Lineweaver–Burk plots. Reaction conditions were as in Figure 1; v is expressed as OD_{260} units per 30 min. The concentration of poly(A) at 1/[S] = 1 was 1.2 mM (adenine equivalent). The phosphate ion concentration was 30 mM, the PDS 2000 concentration was 0.15 μg/ml, and the denatured DNA concentration was 0.5 μg/ml. For the line marked, "no addition," the standard assay without any effectors was used. Native enzyme was used in all experiments.

inhibition are well known (Stadtman *et al.*, 1961; Stadtman, 1968).

Phosphate as a Positive Effector. Stimulation of nuclear exoribonuclease (Lazarus and Sporn, 1967) and the leukemic cell phosphodiesterase (Anderson and Heppel, 1960) by phosphate ion has been previously noted. The stimulatory effect of phosphate on exoribonuclease activity is shown in Figure 7. The positive effect on enzyme activity is shown more strikingly in the presence of the inhibitors, PDS or dnDNA (Figure 4). A Hill plot indicates that the phosphate effect involves a cooperative interaction with the protein, as it gives an interaction coefficient of 1.3–1.9. The equation used for the Hill plot for the positive effector was log $[v/(V_{max} - v)] = n \log$ [phosphate] $- \log K$ with $v = V_{max}$ at [phosphate] = 40 mM. In the presence of negative effectors, the positive effect of phosphate continues up to 60 mM phosphate; in the absence of negative effectors, phosphate no longer increases activity at concentrations greater than 40 mM; actually, at 60 mM the stimulation falls from 60 to 20%.

Lineweaver–Burk Plots with Different Effectors. In order to obtain further data on the mode of action of both negative and positive effectors, a Lineweaver–Burk plot was made for exoribonuclease activity in the absence of effector and with the effectors PDS, dnDNA, and phosphate ion, as shown in Figure 6. The dnDNA appears to be a fully competitive inhibitor, but from Figure 2 the $1/v$ *vs.* [I] plot shows that it is not; instead, it acts as a partially competitive inhibitor (Dixon and Webb, 1964). The PDS does not behave as a purely competitive or noncompetitive inhibitor. The positive effector, phosphate ion, at a concentration which gives maximal stimulation of enzyme action, produces a line parallel to that obtained in the absence of effector. The shape of the $1/v$ *vs.* 1/[S] plot for the substrate, poly(A), appears to be linear and shows no cooperativity with and without the respective effectors. This was found in the absence of effectors down to a substrate level of 0.084 mM in adenine equivalents, where the velocity is 29% of maximum velocity for substrate saturation. However, at these low concentrations of poly(A), some preparations of exoribonuclease have shown cooperative effects (H. M. Lazarus and M. B. Sporn, unpublished).

Effect of Magnesium Ion. To learn the possible role of the divalent cation needed for enzyme action, the activity was studied at varying $MgCl_2$ concentrations up to 4 mM (Figure 8); further increases in concentration caused inhibition. There is no activity in the absence of Mg ion, and the curve at low concentrations of Mg ion is not linear. A similar

TABLE I: Effect of Magnesium Ion on Inhibition of Exoribonuclease by Polydextran Sulfate.[a]

Final $MgCl_2$ Concentration (mM)	Per Cent Inhibition of Enzyme Activity
0.1	5
0.4	8
1.5	32
4.0	42

[a] Reaction conditions were as described for Figure 1, with the final concentration of PDS 2000 = 0.15 μg/ml. Native enzyme was used.

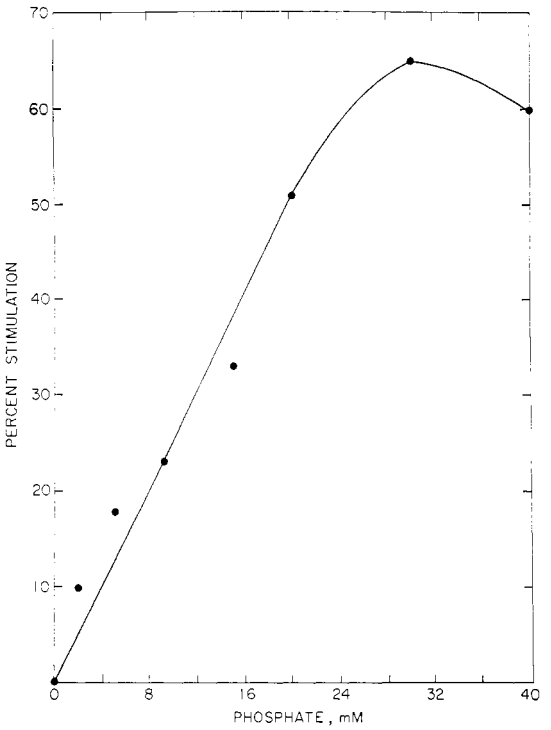

FIGURE 7: Stimulation of exoribonuclease by phosphate ion. Reaction conditions were as for Figure 1, with the addition of potassium phosphate, pH 7.4, to final concentrations as indicated. The point for 0% stimulation represents enzymatic activity without potassium phosphate.

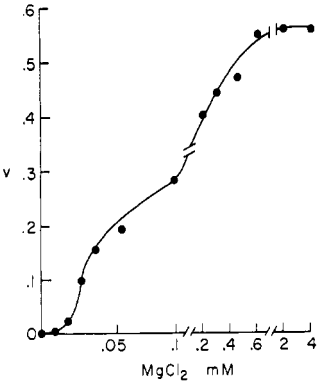

FIGURE 8: Effect of magnesium ion on exoribonuclease activity. Reaction conditions were as in Figure 1, using native enzyme; v is expressed in OD_{260} units per 30 min.

curve for the effects of Mg ion has been found for ribonucleotide diphosphate reductase (Larsson and Reichard, 1966). The Hill equation is the same as for phosphate, with $v = V_{max}$ at $[MgCl_2] = 4$ mM. The Hill coefficient is 2.0 for the first part of the curve at low concentrations of Mg ion and 1.1 for the curve at the higher concentrations. The nonlinearity of the Mg ion effect may be caused by either (1) the effect of ionic interaction with substrate (as was shown for RNA polymerase (Steck *et al.*, 1968)) or (2) ionic interaction with the protein, as has been shown to occur with other allosteric enzymes (Brown *et al.*, 1967; Valentine *et al.*, 1968; Shapiro and Ginsburg, 1968), or (3) a combination of these. It has been demonstrated that the interaction of poly(A) and Mg is nonlinear, as shown by conductivity measurements (Felsenfeld and Huang, 1959). The data in Table I suggest that Mg ion does interact with the enzyme as well as the substrate, poly(A), since the ability of PDS to inhibit exoribonuclease is distinctly enhanced by Mg ion. Moreover, the enhancement by magnesium ion of the ability of PDS to inhibit exoribonuclease activity strongly suggests that the inhibitory effects of PDS on enzymatic activity are not the result of binding of magnesium ion by PDS.

Discussion

Modulation of degradation of nuclear RNA may be a key step in the control of gene action (Harris, 1968). The allosteric nature of nuclear exoribonuclease would provide for potential modulation of its activity *in vivo*. Although PDS is not a naturally occurring substance, polyanionic ribonuclease inhibitors from rat liver cytoplasm have been isolated and characterized (Roth, 1958; Shortman, 1961, 1962; Gribnau *et al.*, 1969). More recently, V. S. Shapot and coworkers (personal communication, 1969) have isolated a ribonuclease inhibitor from rat liver nuclei, and such a nuclear inhibitor would be expected to exert a physiological control on nuclear exoribonuclease. In addition, Stewart and Farber (1968) have recently suggested that the activity of nuclear ribonucleases may be controlled or inhibited *in situ* by DNA.

The number of parameters which control the activity of nuclear exoribonuclease is large, and thus the entire system is of sufficient complexity that a definitive interpretation of the molecular mechanism of action of the various effectors cannot be given at present. Two small ions, namely magnesium and phosphate, would appear to be affecting enzymatic conformation, so that the enzyme is more or less susceptible to cooperative inhibition by PDS. It is not known at present if the cooperative inhibition by PDS involves binding to several different subunits, but it is apparent that the pattern of inhibition caused by PDS is very different from that caused by DNA, which does not inhibit in a cooperative manner.

The present paper has described certain allosteric properties of nuclear exoribonuclease which may contribute to posttranscriptional control of nuclear RNA metabolism. The extent to which such metabolism is controlled *in vivo* by these mechanisms is not known at present, but the potency of the effectors studied would suggest that allosteric control of nuclear exoribonuclease might be of significance in the physiological state.

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Pulse-Labeled Ribonucleic Acid Complexes Released by Dissociation of Rat Liver Polysomes*

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ABSTRACT: Rat liver polysomes, prepared by Mg precipitation and freed of native subunits by differential centrifugation, were dissociated into subunits by various treatments such as EDTA, urea, and 0.5 M KCl at pH 9. In all cases, the pulse-labeled RNA sedimented as heterogeneous material overlapping the two ribosomal subunits. This sedimentation behavior was not significantly altered in a wide range of KCl concentrations. Different centrifugal fractions derived from EDTA-treated polysomes were recentrifuged after further EDTA treatment. A large portion of the labeled RNA showed unchanged sedimentation characteristics. Sodium dodecyl sulfate treatment of these fractions released labeled RNA with considerably smaller *s* values. The range of *s* values of the released RNA was about the same, regardless of the sedimentation range of the fraction from which it was derived.

On the basis of the behavior of the pulse-labeled RNA, it is proposed that the mRNA of polysomes is firmly bound to large amounts of other material, which could serve to bind it to cellular structures. Some radioactivity was present in rRNA. The two rRNA components in the polysomes appeared to be about equally labeled, in spite of the wide difference in the specific radioactivity of the two free ribosomal subunits in the cytoplasm.

Numerous investigations of the nature of mRNA in eukaryotic cells point to its occurrence as nucleoprotein particles. Pulse-labeled RNA in cytoplasmic extracts has been shown to sediment as heterogeneous material in the range of 20–90 S, although treatment with sodium dodecyl sulfate reduces considerably these sedimentation values (Spirin *et al.*, 1964; Nemer and Infante, 1965; Perry and Kelley, 1966; Henshaw and Loebenstein, 1970). Moreover the labeled material was found to exhibit buoyant density values far lower than those of free RNA. Attempts to release pulse-labeled RNA from polysomes by treatment with EDTA have resulted in the appearance of complexes with characteristics similar to those of the free ribonucleoprotein particles (Perry and Kelley, 1968; Cartouzou *et al.*, 1968; Henshaw, 1968). Thus it has been postulated that mRNA is transferred from nucleus to cytoplasm in the form of nucleoprotein particles, and that these nucleoproteins become

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