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ACTIVATION BY ELECTROLYTES AND POLYLYSINE OF POLY(A) SYNTHESIS BY *CLOSTRIDIUM PERFRINGENS* POLYNUCLEOTIDE PHOSPHORYLASE

P. S. FITT*, F. W. DIETZ, JR. AND M. GRUNBERG-MANAGO
Institut de Biologie Physico-chimique, rue Pierre Curie, Paris (France)

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SUMMARY

A modified purification procedure for polynucleotide phosphorylase from *Clostridium perfringens* is described which yields an enzyme with a very low nucleotide contamination. The activation of poly(A) synthesis by polylysine and electrolytes has been studied in detail. Both polylysine and inorganic salts stimulate the rate of formation of poly(A); they differ in that a lag phase exists in the reaction activated by salt, whereas in the presence of polylysine the reaction is linear from the earliest time measured and proceeds at about twice the rate achieved with salt. The effect of polylysine is dependent on pH and ionic strength. While both salt and polylysine lower the K_s for ADP, the former has only a small effect on the v_{max} and the latter increases it markedly. Polylysine forms an insoluble complex with the newly formed poly(A) and the extent of reaction is displaced in the direction of polymer synthesis.

INTRODUCTION

Previous studies of *Clostridium perfringens* polynucleotide phosphorylase (nucleoside diphosphate:polynucleotide nucleotidyl transferase, EC 2.7.7.8) by DOLIN *et al.*[1–3] and KNIGHT, FITT AND GRUNBERG-MANAGO[4] have demonstrated that this enzyme differs considerably from polynucleotide phosphorylase isolated from other organisms[5]. In particular, ADP, and to some extent GDP polymerization were highly stimulated by polylysine, and several other high molecular weight polyamines; CDP and UDP incorporation, on the other hand, were completely inhibited as was the phosphorolysis of poly(A). High concentrations of salts also appeared to stimulate poly(A) synthesis.

Among the few other known cases of the stimulation of an enzymatic activity by a polybase, are the results of KREBS with the salmine and polylysine stimulation of

Abbreviation: Poly(A), polyadenylic acid.

* Present address: Department of Biochemistry, Faculty of Medicine, University of Ottawa, Canada.

glycogen synthesis by polysaccharide phosphorylase[6]. He studied this in the presence of sub-optimal concentrations of AMP and it was found that the K_m for adenylic acid was decreased 10-fold in the presence of basic activators. The mechanism for this activation remains unknown. There have also been several reports of the activation of DNA-dependent RNA polymerase[7]. Recently, KRAKOW[8] has brought forward evidence that the polybase interacts with the RNA produced in the reaction, preventing some form of product inhibition of the enzyme.

An investigation into the mechanism of the stimulation of *C. perfringens* polynucleotide phosphorylase by polybases is in progress in this laboratory with the hope that it may help elucidate the general mechanism of the phosphorylase reaction. The present communication describes a simplified purification procedure for the enzyme and more detailed studies of the effect of polylysine and certain salts on the activity of the enzyme.

MATERIALS AND METHODS

Materials

Poly-L-lysine hydrogen bromide, molecular weight 6000, was obtained from Prof. C. SADRON, and poly-L-lysine hydrogen bromide, molecular weight 3000, was a product of Yeda Research and Development Co., Ltd., Rehovoth, Israel. Poly(A) was prepared with *Aerobacter vinelandii* polynucleotide phosphorylase and had a sedimentation coefficient (s_{20}) of 6.15. *C. perfringens* (A 1844) was kindly supplied by Dr. A. PREVOT. Other materials were products of the following suppliers: bovine serum albumin and unlabelled nucleoside diphosphates, Sigma Chemical Co., St. Louis, Mo.; [^{14}C]nucleoside diphosphates, Schwarz BioResearch Inc., New York; $^{32}P_i$, Commissariat à l'Energie Atomique, Saclay; DEAE-cellulose, Schleicher and Schuell Co., New York.

Enzyme assays

Polynucleotide phosphorylase activity was assayed by the methods described below. One unit of enzyme activity is defined[3] as the amount of enzyme catalyzing the incorporation of 1 μmole of ADP into an acid-insoluble precipitate in 1 h under the conditions of the assay[1]. Specific activity is defined as the number of enzyme units per mg of protein.

(1) Incorporation of [^{14}C]NDP into an acid insoluble precipitate: (a) The basic reaction mixture contained in μmoles/ml: Tris–chloride buffer (pH 8.2), 100; $MgCl_2$, 6; [^{14}C]NDP, 4 (specific activity 20 000–100 000 counts/min per μmole); polylysine where appropriate, and enzyme. After incubation at 37°, the reaction was stopped with an equal volume of 7% perchloric acid. Carrier protein, usually 0.5–1 mg bovine serum albumin, was added and the mixture allowed to stand in ice for at least 10 min. The precipitate was collected on a Millipore filter and washed 3 times with 2 ml of 1% perchloric acid and 3 times with 2 ml of 0.01% perchloric acid. The filters were dried and counted in either a windowless gas flow or thin-window counter. (b) As in (a), but the precipitate, was collected by centrifugation, and washed by re-suspension in the appropriate perchloric acid solutions. The washed precipitate was dissolved in 0.01 M KOH and suitable dilutions were dried and counted.

(2) Release of P_i from NDP: The reaction mixture and the procedure were iden-

tical with those described for Assay 1, except that non-radioactive diphosphates were used. The reaction was followed by determining the P_i liberated, using the first methof of MARSH[9] scaled down 10-fold[3]. (Under these conditions, 0.01 μmole of P_i is equivalent to an absorbance of 0.22 at 310 mμ.) This method could not be used in the presence of polylysine which interferes with the determination of P_i.

(3) Phosphorolysis assay: The reaction mixture (final volume 0.2 ml) contained in μmoles/ml: Tris–chloride buffer (pH 8.2), 100; $MgCl_2$, 6; $^{32}P_i$, 37 (specific activity, about 500 000 counts/min per μmole); poly(A), 1.2 mg/ml; polylysine when appropriate, and enzyme, up to 0.2 units. After 30 min incubation at 37°, an equal volume of 7% perchloric acid was added and the $^{32}P_i$ incorporated into nucleosidediphosphates determined by: (a) the phosphomolybdate–isobutanol extraction procedure[10]; or (b) adsorption of the radioactive nucleotides on activated charcoal[11,12]. Only Method b could be used in the presence of polylysine. The samples containing ^{32}P were counted in a Tracerlab thin-window counter.

Determination of protein

The protein concentration was either determined by the biuret procedure[13], or calculated from the $A_{280\ m\mu}/A_{260\ m\mu}$ ratio[14].

Growth of cells: *C. perfringens* (A 1844) was grown and harvested as previously described[3].

Sucrose density gradients: Solutions of 5% and 20% (w/v) sucrose were made up in 0.02 M Tris, 0.001 M EDTA, 0.007 M β-mercaptoethanol (pH 8.3) (standard buffer *plus* mercaptoethanol). Linear gradients were prepared according to BOCK AND LING[15] and 0.1 ml of sample was layered on 4.6 ml of the gradient and centrifuged in the SW39 head of the Spinco Model L ultracentrifuge. After centrifugation, the bottom of the tube was pierced with a needle and 13-drop fractions were collected.

All pH values quoted for buffers were recorded at room temperature.

RESULTS

Purification of the enzyme

(a) *General considerations*: Two slightly different purification procedures were used during the course of these investigations. In the early phase of these studies,the enzyme was prepared in a manner similar to that previously reported[3], except that a column chromatography on DEAE-Sephadex was substituted for the starch block electrophoresis. This yielded a preparation having a specific activity of 50–90 units/mg when tested with polylysine. Several experiments communicated here were performed with this preparation as is indicated. In each case preliminary experiments have shown that this fraction is identical to the preparation described below in regard to the particular properties under discussion. This will be called Prep. A.

One problem with this early preparation was that it always yielded an enzyme with a very low $A_{280\ m\mu}/A_{260\ m\mu}$ ratio, indicating a substantial contamination by nucleic acids. Therefore the procedure was modified so that more nucleic acid might be removed. In fact, only a very simple modification of the protamine sulfate step was necessary to accomplish this.

Preliminary experiments showed that the formation of the protamine–nucleic acid precipitate was dependent upon concentration and ionic strength. It was found

that with the crude extract, under certain conditions, the addition of protamine sulfate caused no precipitate to form. Subsequent dialysis led to the formation of a precipitate which, on centrifugation, left a supernatant with a $A_{280\ m\mu}/A_{260\ m\mu}$ ratio of 1.1–1.4. These values were raised to 1.5–1.8 by the succeeding steps in the purification. This procedure for the protamine sulfate precipitation of nucleic acids could be better controlled than the old one and no enzyme was lost with the precipitate (Table I).

TABLE I

ENZYME PURIFICATION

Activity for the incorporation of ADP into an acid-insoluble precipitate was assayed as described in MATERIALS AND METHODS, Assay 1; polylysine mol. wt. 3000) when present was at 250 μg/ml. Numbers in parentheses refer to activity tested in the absence of polylysine; the other numbers refer to assays made in the presence of polylysine. $(NH_4)_2SO_4$ concentrations calculated with the nomograph of DIXON[16].

Fraction	*Total protein (g)*	*Total activity (units × 10^3)*	*Specific activity*	*Purification (-fold)*	*Yield*	*$A_{280\ m\mu}/A_{260\ m\mu}$*
(1) Crude extract (106 g bacteria wet wt.)	4.4	7.3 (1.4)	1.70 (0.33)	1	100 (100)	0.53
(2) 45–75% saturated $(NH_4)_2SO_4$		4.0 (0.89)			54 (63)	0.53
(3) Protamine supernatant	1.58	3.72 (0.72)	2.36 (0.28)	1.4	50 (51)	1.10
(4) DEAE-cellulose column eluate concentrated	0.038	2.0 (0.15)	53.5 (3.95)	32	27 (10)	1.50

(b) *Description of a typical purification*: All operations were carried out at 0–5°, including centrifugations.

(1) Crude extract: A crude extract of *C. perfringens* was prepared by grinding 106 g wet weight of the bacteria in a mortar with twice this weight of alumina and suspending the material in 5 vol. of standard buffer (0.02 M Tris–0.001 M EDTA, pH 8.3). The resulting suspension was centrifuged in the Servall rotor type GSA at 9000 rev./min for 45 min and the supernatant carefully decanted.

(2) 0–45% saturated $(NH_4)_2SO_4$ fraction: This extract was made 45% saturated in $(NH_4)_2SO_4$ (ref. 16) by the addition of 0.275 g $(NH_4)_2SO_4$ per ml of crude extract. After standing for 30 min the suspension was centrifuged as above and the precipitate discarded.

(3) 45–75% saturated $(NH_4)_2SO_4$ fraction: The supernatant from Step 2 was brought to 75% saturation with 0.205 g $(NH_4)_2SO_4$ per ml supernatant, and after standing for at least 1 h, the suspension was centrifuged as above. The supernatant was discarded and the precipitate was taken up in standard buffer to give an $A_{260\ m\mu}$ value of 75 units per ml.

(4) Protamine sulfate treatment: As usually prepared (*e.g.* ref. 17) protamine sulfate solutions give variable results in the precipitation of nucleic acids, depending on batch and age of solution. The following procedure seems to give a more reproduci-

ble solution which need not be prepared fresh daily. A suspension of protamine sulfate, 20 mg/ml in glass-distilled water, was acidified with dilute acetic acid until a clear solution was obtained. It was then brought to pH 7 with 1 M NaOH and allowed to stand for several days at room temperature. Any precipitate was removed by centrifugation and the clear solution could be stored at 5° for at least 2 months. In the cold a gelatinous precipitate forms which can be redissolved upon gentle heating of the solution just before its use. This solution gave a biuret reaction equivalent to 28 mg per ml crystalline bovine serum albumin.

100 mg of $(NH_4)_2SO_4$ was added to each of several 1-ml samples of the 45–75% saturated $(NH_4)_2SO_4$ fraction. Various amounts of the protamine solution (0.025–0.3 ml) were mixed with these samples which were then dialyzed overnight against 100 vol. of standard buffer. After dialysis the test fractions were clarified by centrifugation and the absorbance at 280 and 260 mμ recorded. Using these values the $A_{280\ m\mu}/A_{260 m\mu}$ ratios were calculated. It was found that this ratio would increase to a maximum and then, at high protamine concentrations, decrease. The least amount of protamine solution giving a ratio of about 1.1 was used to calculate the volume to be added to the bulk of the fraction. This addition was made after the fraction had been made 100 mg/ml in $(NH_4)_2SO_4$.

Dialysis for 18 h against standard buffer *plus* mercaptoethanol followed. The clean white precipitate (a brownish precipitate signals the loss of protein, perhaps the enzyme, and is to be avoided) was removed by centrifugation.

(5) DEAE-cellulose column chromatography: DEAE-cellulose was washed rapidly and successively with 1 M NaOH, H_2O, 1 M HCl, and finally H_2O to neutrality. During the final water washes the material was allowed to settle for 45 min and the fines were decanted and discarded. A column was poured, 3.4 cm × 12 cm which was washed overnight with standard buffer *plus* mercaptoethanol.

The protamine supernatant was run into the column and the column washed with the same buffer until a relatively constant $A_{280\ m\mu}$ value was reached (0.180). The column was next washed successively with 450 ml each of 12 g/l, 13 g/l, 20 g/l and 30 g/l $(NH_4)_2SO_4$ in standard buffer *plus* mercaptoethanol. The enzyme came off with the final wash which was 0.227 M $(NH_4)_2SO_4$. Other, equally successful preparations have been made, condensing the wash steps to one wash at 20 g/l followed by elution at 30 g/l.

The activity peak at 192 ml was dialyzed for 10 h against 500 ml of saturated $(NH_4)_2SO_4$ in standard buffer *plus* mercaptoethanol, final pH about 7.3. To keep the buffer outside the sac saturated in $(NH_4)_2SO_4$, solid $(NH_4)_2SO_4$ was added after 3 and 6 h of dialysis. The precipitate was collected by centrifugation, dissolved in standard buffer *plus* mercaptoethanol and dialyzed overnight against this same buffer. A slight precipitate was removed by centrifugation, and 8.6 ml of enzyme solution, 4.35 mg/ml in protein was obtained.

Several other fractionation steps were attempted in earlier purifications. These included extraction of an 80% $(NH_4)_2SO_4$ precipitate with neutral solutions of decreasing $(NH_4)_2SO_4$ concentration, DEAE-cellulose and DEAE-Sephadex column chromatography at pH 7.4, calcium phosphate gel–cellulose column fractionation, and starch-block electrophoresis under the previously described conditions[3]. None of these procedures led to any significant purification, and there were serious losses of activity during the column fractionations.

The specific activity of the fraction after DEAE-cellulose chromatography was 54 units/mg in the presence of polylysine with an overall yield of 27%. A subsequent preparation gave an activity of over 100 units/mg. The results of the sucrose density-gradient centrifugation experiment indicate that this preparation may be further purified up to a specific activity of well over 500 units/mg comparable with the most highly purified enzyme from *Escherichia coli*[18]. DOLIN[3] studied the enzyme at two steps of purification, the less pure, an elutate from calcium phosphate gel, had a specific activity of 30 units/mg with an overall yield of 16%; after starch-block electrophoresis of the gel eluate a very dilute solution was obtained whose specific activity could only be estimated to be around 290 units/mg.

Several experiments to be described show that the preparation was essentially free of nuclease or phosphatase activity. It may be stored for at least a month at 3–5° in 0.02 M Tris–0.001 M EDTA–0.007 M β-mercaptoethanol, with little loss of activity.

It has recently been found that at very low enzyme concentrations, less than 0.2 units/ml of incubation, the specific activity of the preparation measured in the presence of salt or polylysine, or in the absence of activator, decreases upon further dilution of the enzyme. However, when the tests are carried out at 15 μmoles in the presence of $MgCl_2$ per ml the specific activity is independent of enzyme concentration. Further experiments, necessary to completely describe the phenomenon are presently under way. It should be noted, however, that most of the experiments to be reported were carried out at an enzyme concentration greater than 0.2 units/ml.

Sucrose density gradient centrifugation of the enzyme preparation after DEAE-cellulose chromatography

Fig. 1 shows the activity profile for polylysine-stimulated ADP polymerization in a sucrose gradient after centrifugation of a sample of the enzyme preparation from the DEAE-cellulose chromatography step. There are two peaks of activity present, with the smaller representing about 15–20% of the total activity. Alcohol dehydrogenase was included with the enzyme sample and its position can also be noted in Fig. 1. Using the method devised by MARTIN AND AMES[19] the molecular weight of these two peaks can be calculated as 190 000 and 62 000.

Sucrose density-gradient centrifugation may also serve as a powerful tool in the purification of the enzyme. Preliminary experiments indicate at least a 5-fold purification of the DEAE-cellulose eluate. This is due to the fact that most of the contaminating proteins are of low molecular weight which allows the heavy peak to be recovered free from contamination. Unfortunately the light material sediments with the bulk of the protein and its further purification is still under study. WILLIAMS AND GRUNBERG-MANAGO[18], and LUCAS-LENARD AND COHEN[20] have also used a sucrose density-gradient step in the purification of polynucleotide phosphorylases.

With regard to experiments to be reported, carried out with fractions not subjected to sucrose density gradient centrifugation, it should be noted that the fraction obtained after DEAE-cellulose column chromatography contains at most 15–20% of the small molecular weight species and therefore the properties of such a preparation should be predominantly those of the high molecular weight form of the enzyme.

Studies of poly(A) synthesis as a function of time

The effect of various activators on the time course of the polymerization of

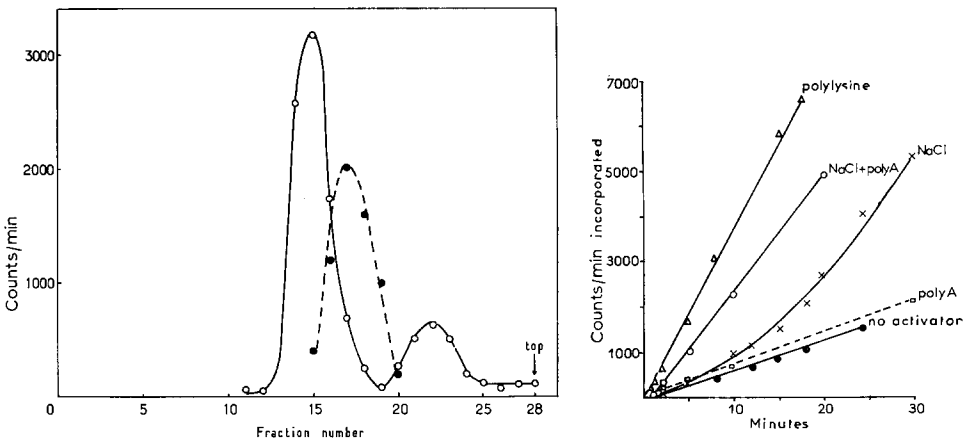

Fig. 1. Distribution of activity for polylysine-stimulated ADP polymerization after sucrose density-gradient centrifugation. The enzyme fraction was mixed with a sample of yeast alcohol dehydrogenase, and 0.1 ml was layered on a 5–20% sucrose density gradient which was centrifuged for 9 h at 36 000 rev./min in the Spinco Model L at 0°. 0.025-ml samples were incubated under the conditions of Assay 1 with polylysine (mol. wt. 3000) present at a concentration of 250 μg/ml for 3.75 h at 37°. The alcohol dehydrogenase was assayed as described by MARTIN AND AMES[19] and the values plotted for the activity *vs.* the changes in absorbance at 340 mμ per 20 sec incubation at room temperature. ○—○, polynucleotide phosphorylase activity; ●—●, alcohol dehydrogenase activity.

Fig. 2. Time curves for poly(A) synthesis in the presence or absence of activators. Assay 1 was used with enzyme Prep. A. Where present the activators were at the following final concentrations: polylysine, mol. wt. 6000, 200 μg/ml; NaCl, 0.2 M; poly(A), 400 μg/ml.

ADP were studied. In the absence of any activator the reaction was preceded by a very short but reproducible lag phase followed by a rapid transition to a linear rate. In the presence of poly(A) this lag was abolished and the reaction was linear from the earliest time measured. This rate was not, however, very different from that ultimately reached in the absence of any addition (Fig. 2).

The effect of NaCl was more complex. Although the rate for the first 5 min was not substantially different from that of the control with no activator, as the reaction continued the rate of polymerization increased progressively until it was substantially higher. When both salt and poly(A) were present, their effects were additive; the lag phase disappeared and the rate of incorporation was several times that of the control.

When the time course of the reaction was studied in the presence of polylysine alone, two effects were evident. First, the lag phase was overcome; and second, there was a much higher rate of incorporation of ADP into polymer.

Effect of salt or polylysine on the kinetic constants

The K_s and the v_{max} were determined by standard procedures without activator, with polylysine, and with NaCl *plus* poly(A); the results are shown in Table II. Previous studies had demonstrated that while polylysine and salt each lowered the K_s for the synthesis of poly(A) from ADP, polylysine increased the v_{max}, while salt had no effect[3]. The present studies confirm the results with polylysine. However, after the effect of poly(A) on the time course of the reaction was recognized, it was decided to look at the kinetic constants in the presence of both salt and poly(A). In this case,

TABLE II

MICHAELIS CONSTANTS AND RELATIVE MAXIMUM VELOCITIES FOR *C. perfringens* POLYNUCLEOTIDE PHOSPHORYLASE

The enzymatic fraction was Prep. A (see RESULTS). The enzyme activity was determined using Assay 1. The concentration of ADP was varied and the activators were present in the concentrations shown.

Activators	*K_s for ADP (mmoles/l)*	*v_{max} (μmoles ADP per h per ml)*	*Relative v_{max}*
None	4.5	0.06	1
Polylysine, mol. wt. 6000 (200 μg/ml)	0.48	0.24	4
0.2 M NaCl + poly(A) (400 μg/ml)	0.53	0.09	1.5

as in the case with salt alone, there was a decrease in K_s but, unlike the results with salt alone, there was a 50% increase in the v_{max} as well.

Effect of pH on ADP polymerization

The formation of poly(A) was measured in 0.1 M Tris–HCl buffer at various pH values with the results shown in Fig. 3. In the presence of polylysine there was a pronounced optimum at pH 8.2. With NaCl as activator there was no optimum, only a more or less linear increase in activity up to pH 9, the highest pH tested. In the absence of activator there was little effect of pH on the polymerization reaction between pH 7 and pH 9.

Effect of polylysine on polymer formation

It was found, in agreement with earlier reports[3] that while polylysine stimulated GDP and ADP polymerization, it inhibited the polymerization of CDP and UDP (*e.g.* the activity of about 11 μmoles/mg per h for UDP polymerization was reduced to zero in the presence of polylysine (140 μg/ml)). The saturating concentration of polylysine under the assay conditions for ADP polymerization varied somewhat, depending on

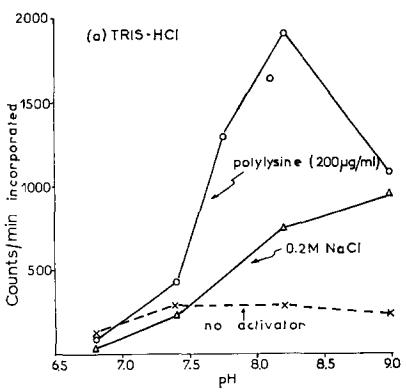

Fig. 3. Effect of pH on the formation of poly(A) in the presence of various activators. Assay, enzyme fraction and polylysine as in Fig. 2.

the purification state of the enzyme, but lay in the region of 140–280 μg/ml, in general agreement with previous reports.

Effect of electrolyte concentration on poly(A) synthesis.

The effect of varying the electrolyte concentration under the conditions of Assay 1 was studied using NaCl, KCl, LiCl and $(NH_4)_2SO_4$ (pH 7) with the results shown in Figs. 4a and b. It can be seen that all salts tested stimulate synthesis while the optimum concentration varied: 0.4 M for KCl and LiCl, 0.2 M for NaCl, and 0.1 M for $(NH_4)_2SO_4$.

In the presence of the optimal polylysine concentration the effect of salt is quite different. It may be seen in Fig. 4 that salts abolish the polylysine stimulation

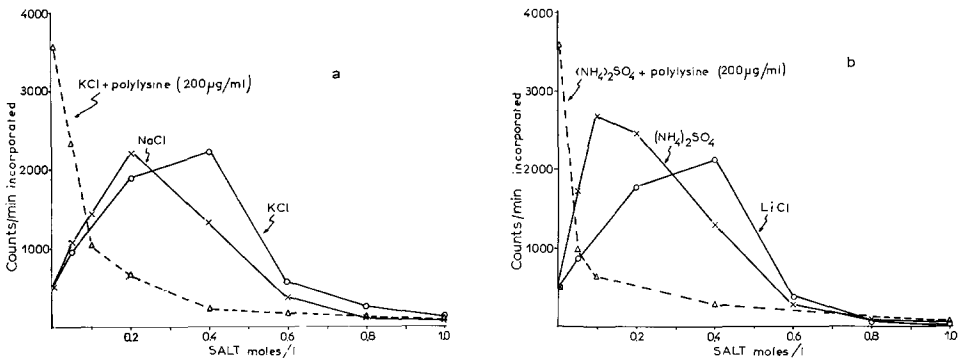

Fig. 4. Effect of inorganic salts on poly(A) synthesis. Assay, enzyme fraction and polylysine as in Fig. 2.

and that this effect is a function of the salt concentration. Indeed, when both salt and polylysine are present together in the incubation mixture, at concentrations which are optimal for each when tested separately, the activity is somewhat less than in the control when neither is added.

Effect of polylysine and NaCl on the extent of poly(A) synthesis

Fig. 5 shows the effect of increasing the polylysine concentration on the extent of the reaction in poly(A) synthesis. In the absence of polylysine, polymerization was followed by the release of P_i, or by measuring the incorporation of [^{14}C]ADP into a perchloric acid insoluble precipitate, with both methods giving identical results. In the presence of polylysine it is impossible to follow the P_i liberation and the reaction was followed by measuring [^{14}C]AMP incorporation. The correspondence between the results from the P_i released and ADP polymerized indicates that the preparation was free of phosphatase activity under these conditions, and the fact that the equilibrium was stable for 48 h, even in the presence of additional enzyme, showed that no nuclease activity was present. In the absence of polylysine, the reaction stopped at 28% polymerization.

It may be seen in Fig. 5 that the extent of reaction is dependent on the polylysine concentration up to about 2500 μg/ml where a level of 68% polymerization is reached. At the optimum NaCl concentration, 47% of the ADP was polymerized. At

all the polylysine concentrations shown, the system was saturated so that the addition of more polylysine did not increase the initial rate. However, the period during which this rate was maintained was progressively lengthened. A visible precipitate began to form at once when polylysine was present, and it was found in separate experiments that it contained 99% of the acid-insoluble product. This precipitate became increasingly difficult to dislodge from the walls of the reaction vessel, or to dissolve in

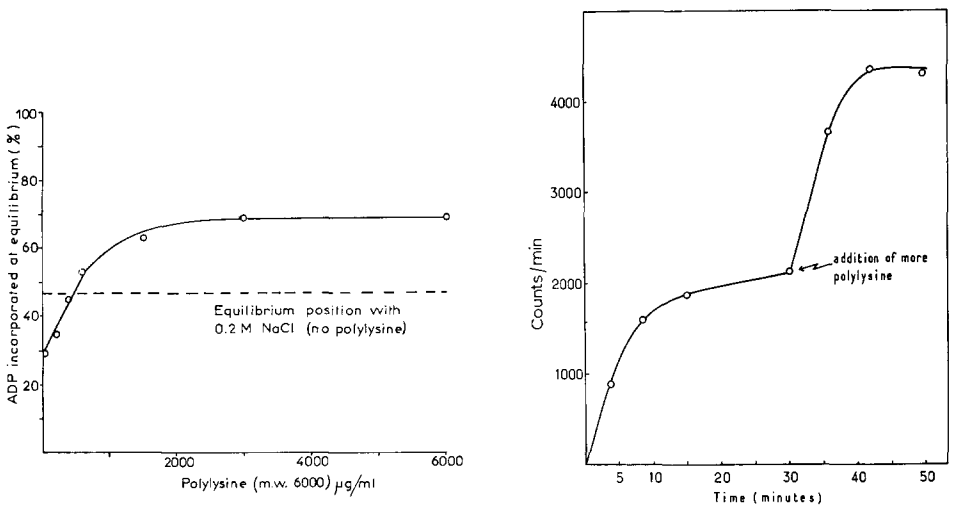

Fig. 5. Effect of polylysine on the extent of poly(A) synthesis. Assay, enzyme fraction and polylysine as in Fig. 2. Both acid-soluble and acid-precipitable counts were determined so that all of the ADP could be accounted for. The equilibrium position in the absence of polylysine was also confirmed by measurement of the release of P_i. (Note: the saturating concentration of polylysine under these conditions is 160 μg/ml.)

Fig. 6. Re-addition of polylysine after establishement of equilibrium. The enzyme fraction after the DEAE-cellulose chromatography was used, incubated under the conditions of Assay 1. Polylysine of mol. wt. 3000 was present at a final concentration of 250 μg/ml. Each point represents a separate 0.150-ml sample incubated for the indicated time. At 30 min the remaining tubes received 0.01 ml of polylysine solution (2 mg/ml).

dilute alkali, as the incubation time lengthened; therefore individual tubes containing aliquots of the reaction mixture were used for each point on the time curve. In the absence of polylysine no precipitate formed until acid was added to stop the reaction.

The factors effecting the extent of reaction were further investigated. When the reaction had come to a halt in the presence of a rather low concentration of polylysine, various components were added; specifically more $MgCl_2$ and ADP, more enzyme, or more polylysine. While the $MgCl_2$ *plus* ADP, and enzyme additions showed little effect, the addition of more polylysine caused the immediate resumption of the polymerization at the maximum rate, as can be seen in Fig. 6, until the reaction once again stopped at a much higher level of incorporation.

These results show clearly that the polylysine affects the extent of reaction since it complexes with poly(A), thus displacing the equilibrium. As a consequence, the polylysine is removed from the solution, and the reaction slows down unless more polylysine is added. It should be noted, however, that the maximum initial rate was

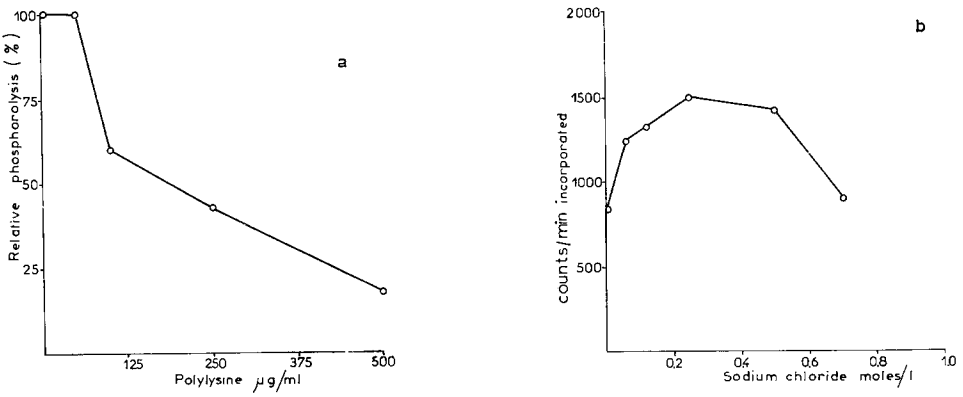

Fig. 7. Effect of salt and polylysine on the phosphorolysis reaction. The enzyme was Prep. A which was tested, in the presence of polylysine by Assay 3b, or in the presence of NaCl by Assay 3a.

attained with a concentration of polylysine which did not profoundly change the extent of the reaction.

Effect of polylysine and NaCl on phosphorolysis of poly(A)

Phosphorolysis of poly(A) was progressively inhibited as the polylysine concentration was raised. Once again a visible precipitate was formed immediately and the inhibition was undoubtedly due to the removal of the substrate in this insoluble complex with polylysine. NaCl, on the other hand, caused a slight stimulation of poly (A) phosphorolysis as seen in Fig. 7.

TABLE III

STUDIES ON THE HEAT INACTIVATION OF *C. perfringens* POLYNUCLEOTIDE PHOSPHORYLASE

The solution contained 25 μmoles of Tris–chloride buffer (pH 8.2); 0.01 ml enzyme fraction, Prep. A; and additions as shown in the table in the following quantitities: $MgCl_2$, 1.5 μmoles; ADP, 1 μmole; NaCl, 50 μmoles; bovine serum albumin, 50 μg; and polylysine, mol. wt. 6000, 50 μg. The medium *minus* the enzyme was preincubated at the desired temperature for 2 min, and the enzyme was then added. The volume of the solution at this stage was 0.24 ml. After a further minute of incubation, the mixtures were chilled in an ice bath. The residual ADP incorporation activity was determined using Assay 1. The other components of the test medium were added in 0.01 ml, and the final volume for the assay was 0.25 ml. Each experiment was carried out in duplicate, together with suitable controls. The residual activity was expressed as a percentage of the activity of the unheated enzyme assayed in the same conditions. In those cases where the enzyme was heated alone or with either $MgCl_2$ or ADP, the activity was determined in the presence of (a) polylysine (200 μg/ml), (b) NaCl (0.02 M), and (c) without activator. It was found that the percentage residual activity was identical in all three cases.

Additions	*Residual activity (%) after 1 min*		
	50°	*55°*	*60°*
None	67	34	3
$MgCl_2$	—	33	—
NaCl	30	5	0
Polylysine	91	86	62
Polylysine + ADP	62	—	—
Poly(A) + $MgCl_2$	—	100	—
Bovine serum albumin	—	99	—
ADP	60	—	—

Effect of heat-treatment on subsequent activity for polymerization of ADP

Table III shows the results of a study on the heat inactivation of the enzyme in the presence of various components of the reaction mixture. Of the three factors studied which affect the reaction, NaCl destabilized the enzyme, while polylysine, and poly(A) *plus* Mg^{2+} protected it. For example in the case of NaCl at 50°, well over two-thirds of the activity was lost compared to one-third for the control. It is interesting to note that while the presence of ADP at concentrations used in the incubation did not protect the enzyme, when present along with polylysine it abolished the protection seen with polylysine alone.

Some doubt is cast on the specificity of the polylysine effect by the observation that bovine serum albumin also protected the enzyme against heating. It is possible that what is observed is just a non-specific action due to the presence of a polypeptide.

The overall heat stability of the enzyme seems to be dependent upon the purification steps used. Thus, a fraction obtained after a phosphate gel step was completely inactivated when heated alone at 60°, and even in the presence of poly(A) with or without Mg^{2+}, there was still a 40% loss of activity.

DISCUSSION

The existence of the enzyme in two forms differing in molecular weight has been the subject of a recent communication[21]. It was found that the catalytic properties of the light and heavy species of the enzyme were very different. The heavy enzyme showed activity for both the synthesis and phosphorolysis of poly(A) in the absence of activator and was stimulated in the synthesis of poly(A) by salt and polylysine. The light enzyme, on the other hand, was incapable of phosphorolysis and was unable to catalyze the formation of poly(A) from ADP in the absence of polylysine; the polylysine requirement could not be replaced by inorganic salts. Since the bulk of the activity in the preparations which have been described in this communication was associated with the high molecular weight enzyme, we are indeed describing a polynucleotide phosphorylase, according to the classical definition[5]. This is rather important, since earlier, DOLIN had purified the polylysine-stimulated ADP polymerizing activity to the point where it was completely dependent upon polylysine for the polymerization and incapable of the phosphorolysis reaction. Because of these properties it seemed questionable at that time whether or not one might call this activity polynucleotide phosphorylase.

The molecular weight of about 190 000 for the heavy component is similar to those reported for polynucleotide phosphorylase isolated from other bacteria[18,22]. In fact, the purification scheme reported is very similar to that developed in this laboratory for the *E. coli* enzyme, again suggesting a general similarity with other polynucleotide phosphorylases. Several other similarities might be catalogued, such as catalysis of the exchange reaction, heat stability, and migration in gel electrophoreses[23]. The effects of salt and polybases on the catalytic properties of this preparation, however, are quite different from those of other known polynucleotide phosphorylases.

The results presented in this paper show that the effect of polylysine on polynucleotide synthesis by *C. perfringens* polynucleotide phosphorylase is complex, and depends on many variable factors such as pH and ionic strength of the medium. It

has also been found that several organic salts are themselves able to affect polymer synthesis markedly. Although polylysine and inorganic salts each stimulate poly(A) synthesis in the absence of the other, they have quite different effects on its phosphorolysis which is inhibited by polylysine and stimulated by NaCl.

That the stimulation of poly(A) synthesis by polylysine is due to some sort of electrostatic interaction, seems to be well documented. The reaction stimulated by polylysine has a sharp pH maximum and is inhibited at high salt concentrations. In fact, polylysine interacts with the product; all of the poly(A) formed during the reaction could be quantitatively recovered as an insoluble precipitate. This affected the extent of reaction so that when polylysine was present, the apparent equilibrium shifted from 28% to 70% polymerization of ADP. It seems likely that the P_i/ADP ratio of 2.3 in the reaction medium was then too high for further synthesis to take place, and it is interesting to note that with other known polynucleotide phosphorylases, equilibrium is usually reached when the P_i/NDP ratio is 1.5 or 2 (ref. 5). The inhibition of poly(A) phosphorolysis by polylysine was likewise due to the formation of an insoluble complex between the polynucleotide and the polybase.

All of the above is in general agreement with previously reported observations on polybase–polynucleotide interactions. It has been established that polynucleotides interact with basic polypeptides, including polylysine[24,25] and in addition, SPITNIK, LIPSHITZ AND CHARGAFF[26] have shown that polylysine and polyvinylamine form complexes with polydeoxyribonucleotides. Recently, LENG AND FELSENFELD[27] have shown that the interaction of polylysine with DNA is dependent upon the base ratio of the polynucleotide[27]. Polylysine preferentially precipitates DNA high in AT. Most recently, OLINS, OLINS AND VON HIPPEL[28] have likewise demonstrated the preferential interaction of polylysine with regions of DNA high in AT. An apparent contradiction to the above has been reported by SOBER *et al.*[29] who, while studying the protection of RNA from nuclease degradation by polylysine, found a specificity for regions of high GC in native Ms 2-RNA. They pointed out, however, that the protectional specificity need not be the same as the specificity for interaction such as seen in the precipitation studies.

The specificity for AT regions seems to be reflected in our work, where, of the four ribonucleotide diphosphates tested, ADP polymerization showed the greatest stimulation by polylysine. However, further extrapolation would lead to the suggestion that polylysine should have no effect on UDP polymerization, while in fact it is almost completely inhibitory[3].

Polylysine may also interact with the substrate of the polymerization reaction. DOLIN[3] observed that ADP could form turbid solutions with polylysine and our observations indicate that all four common nucleoside diphosphates form precipitates with polylysine, both in aqueous solutions and under the conditions of the polymerization reaction.

Finally polybases are undoubtedly capable of interaction with the enzyme. In our early studies of the protamine sulfate precipitation of the nucleic acids in the enzyme preparation we often observed losses which could be attributed to the precipitation of the enzyme. Previously, WILLIAMS AND GRUNBERG-MANAGO[18] described the recovery of *E. coli* polynucleotide phosphorylase from a protamine precipitate[18]. Although precipitation represents an extreme case it is not difficult to envision polybase–protein interactions of a less dramatic nature. The results obtained in the heat-

inactivation experiments where polylysine protected the enzyme, are also suggestive of some form of interaction.

Earlier reports indicated that electrolytes stimulate polymer synthesis by this enzyme, and a more detailed study of this effect was carried out. Several inorganic salts were found to be effective activators of poly(A) synthesis by the *C. perfringens* enzyme, provided polylysine was absent, and in the presence of 0.2 M NaCl the extent of reaction was displaced from 28% to 47% polymerization. The product was not, however, precipitated and NaCl, unlike polylysine, also stimulated poly(A) phosphorolysis.

BEERS[30], and HENDLEY AND BEERS[31] have shown that ADP incorporation and poly(A) phosphorolysis by the *Micrococcus lysodeikticus* enzyme are considerably affected by changes in the ionic strength of the medium, although this effect seems attenuated in a more purified fraction[31]. The results obtained with the *C. perfringens* enzyme are consistent with their observations and confirm that charge effects are important in this system.

Even taking into consideration all of the results so far obtained, the question of the exact point at which polylysine exerts its stimulating action remains unanswered. The fact that polylysine precipitates the product of the reaction and at high concentrations displaces the equilibrium in favor of polymer synthesis does not completely explain the effects on the kinetics of the reaction. It does suggest, perhaps, that if a rate-limiting step in the reaction were the dissociation of the enzyme from a long polymer chain, this dissociation might be facilitated either specifically by the interaction with polylysine, or generally at high salt concentrations.

The change in the apparent equilibrium constant elicited by salt is especially suggestive of such a mechanism. It is difficult to see how the addition of salt can sufficiently change the state of the ADP or the poly(A) to displace the equilibrium, while such a result might be observed if the enzyme were being inhibited by bound product, as, for example, is the case in the RNA polymerase reaction[32]. Evidence from studies on the mechanism of action of polynucleotide phosphorylase isolated from other bacteria indicates that the enzyme has a high binding constant for polynucleotides and, under normal conditions, synthesizes a few long chains rather than many short chains[23,32].

It must be evident from the above discussion that the ultimate solution of this problem will rest not only on further studies of the enzymatic reaction, but equally on the systematic development of a theory of the interaction of polybases and polynucleotides.

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