The Preferential Loss of the Polylysine- or Polyornithine-Stimulated Activity of *Clostridium perfringens* Polynucleotide Phosphorylase during Proteolysis

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1. An improved method for the purification of *Clostridium perfringens* polynucleotide phosphorylase (nucleoside diphosphate-polyribonucleotide nucleotidyltransferase, EC 2.7.7.8) is described. The product was stable and was highly stimulated by polylysine or polyornithine. 2. It migrated as a single enzyme during sucrose-density-gradient centrifugation, and no separation of polymerization and phosphorolytic activities was observed. 3. Trypsin digestion caused a rapid, preferential loss of the polylysine- or polyornithine-stimulated activity, which was prevented by low concentrations of polyornithine. 4. The protection by polyornithine was not specific. 5. It is concluded that charge effects on the clostridial polynucleotide phosphorylase itself are primarily responsible for the stimulation of this enzyme by polylysine or polyornithine.

Previous studies of the polynucleotide phosphorylase (nucleoside diphosphate-polyribonucleotide nucleotidyltransferase, EC 2.7.7.8) of *Clostridium perfringens* (Dolin, Godiniaux & Grunberg-Manago, 1961; Dolin, 1961, 1962; Fitt, Dietz & Grunberg-Manago, 1968a) have shown that this enzyme differs significantly from the well-studied polynucleotide phosphorylases isolated from *Azotobacter vinelandii*, *Escherichia coli* and *Micrococcus lysodeikticus* (Grunberg-Manago, 1963). In particular, the polymerization of ADP or GDP by the clostridial enzyme is markedly stimulated by polybases such as polylysine, polyornithine and polyvinylamine and by inorganic salts such as sodium chloride. In some cases, the partially purified enzyme appeared to have an absolute requirement for the polybase, to be specific for ADP and to lack phosphorolytic activity (Dolin, 1962). Fitt *et al.* (1968a) demonstrated that the equilibrium

could be displaced progressively in the direction of polymer synthesis by raising the concentration of polylysine in the reaction medium. However, the maximum initial velocity was attained with amounts of the polybase that had only a limited effect on the equilibrium position, and no evidence of product inhibition could be obtained. Polylysine protects *Cl. perfringens* polynucleotide phosphorylase against thermal inactivation (Fitt *et al.* 1968a), but bovine serum albumin is equally effective and evidence in favour of the formation

of a specific enzyme-polybase complex is not available. The mechanism of the stimulation by polybases remains uncertain, and the possibility that the enzyme preparations studied so far contained an inhibitor capable of interacting with polybases (Dolin, 1962) has not yet been excluded.

Evidence was presented that *Cl. perfringens* polynucleotide phosphorylase can be separated into two components by calcium phosphate-gel fractionation (Knight, Fitt & Grunberg-Manago, 1963) and sucrose-density-gradient centrifugation (Dietz & Grunberg-Manago, 1967). The two components differed in their degree of stimulation by polylysine, and Dietz & Grunberg-Manago (1967) concluded that their preparation contained two enzymes, one stimulated by polylysine and electrolytes but inhibited by β -mercaptoethanol, the other of lower molecular weight and totally dependent on both polylysine and β -mercaptoethanol.

The present paper describes a study of the effect of trypsin digestion on the activity of *Cl. perfringens* polynucleotide phosphorylase.

EXPERIMENTAL

Materials. Intermediates and enzymes were purchased from the following suppliers: ApA,* Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y., U.S.A.; polyA and poly-DL-ornithine (mol.wt. 3000-6000), as its hydrobromide, Miles Chemical Co., Elkhart, Ind., U.S.A.;

* Abbreviations: ApA, polyA, polyadenylic acid; adenyl-yl-(3',5')-adenosine.

Sephadex G-200 and DEAE-Sephadex (bead form), Pharmacia, Uppsala, Sweden; unlabelled and ^{14}C -labelled ribonucleoside diphosphates, Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.; DL-benzoylarginine *p*-nitro-anilide and polylysine (mol.wt. 3000), as its hydrobromide, Sigma Chemical Co., St Louis, Mo., U.S.A.; carrier-free ^{32}P orthophosphate, The Radiochemical Centre, Amersham, Bucks.; pancreatic ribonuclease, trypsin, soybean trypsin inhibitor and freeze-dried *Cl. perfringens* cells, Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Preparation of *Cl. perfringens* polynucleotide phosphorylase. Polynucleotide phosphorylase was prepared from freeze-dried *Cl. perfringens* (A.T.C.C. 10543) cells, harvested after approx. 18 hr. of growth (personal communication from Worthington Biochemical Corp.), by a modification of the method of Fitt *et al.* (1968a). All operations after the preparation of the crude extract were carried out at 2°. The $(\text{NH}_4)_2\text{SO}_4$ concentrations were calculated by the procedure of Dixon & Webb (1964).

The cells (10 g.) were mixed with cold (−20°) neutral alumina (40 g.) and 0.02 M-tris-HCl buffer, pH 8.2–1 mM-EDTA–5 mM-β-mercaptoethanol (referred to below as 'buffer') (40 ml.), and ground in a mortar at 0° until the bacteria were disrupted (about 20 min.). Buffer (200 ml.) was added and the suspension was stirred for 1 hr. at 0°. The solids were collected by centrifugation and extracted with buffer (150 ml.) for 1 hr. at 0°. The suspension was centrifuged and the supernatants from the two extractions were combined to give the crude extract (total activity with polylysine 18400 units, specific activity 7.1 units/mg.; total activity without polylysine 880 units, specific activity 0.35 unit/mg.). The crude extract was purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation and protamine fractionation as described by Fitt *et al.* (1968a). Sufficient NaCl was added to the protamine supernatant to give a final concentration of 0.1 M, and the solution was passed at 60 ml./hr. through a column (2.5 cm. × 40 cm.) of DEAE-Sephadex equilibrated with buffer containing 0.1 M-NaCl. Elution was performed at 30–40 ml./hr. with successive gradients of 0.1–0.25 M-NaCl (800 ml.) and 0.25–0.4 M-NaCl (1 l.) in buffer. The active fractions (280 ml.) emerged in the middle of the second gradient. They were concentrated to 5–7 ml. by $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by dialysis against buffer. This solution, with sucrose added to a final concentration of 10% (w/v), was passed at 10 ml./hr. through a column (2.5 cm. × 90 cm.) of Sephadex G-200 equilibrated with buffer. The enzyme was eluted with buffer at 10 ml./hr. and the active fractions were concentrated by precipitation and dialysis as described above. Solid NaCl was added to a final concentration of 0.2 M and the solution was passed through a column (1.5 cm. × 26 cm.) of DEAE-Sephadex previously equilibrated with buffer containing 0.2 M-NaCl. Elution was performed at 35–60 ml./hr. with successive gradients of 0.2–0.3 M-NaCl (300 ml.) and 0.3–0.4 M-NaCl (600 ml.) in buffer. The active fractions (56 ml.) were eluted in the second gradient. They were diluted with buffer to give a final NaCl concentration of approx. 0.2 M and were concentrated by adsorption to a column (0.9 cm. × 3 cm.) of DEAE-Sephadex, followed by elution with 0.8 M-NaCl in buffer and dialysis against salt-free buffer. The product was stable for at least 4 months at 2–4° (total activity with polylysine 2850 units, specific activity 355 units/mg.; total activity without polylysine 164 units, specific activity 20.3 units/mg.).

M. lysodeikticus polynucleotide phosphorylase. The purification of the enzyme used for the experiments in Table 3 has been described (Fitt, Fitt & Wille, 1968b).

Sucrose-density-gradient centrifugation. Sucrose-density-gradient centrifugation of *Cl. perfringens* polynucleotide phosphorylase was performed as described by Dietz & Grunberg-Manago (1967).

Polyacrylamide-gel electrophoresis. Analytical polyacrylamide-gel electrophoresis of polynucleotide phosphorylase in 7% gels was carried out as described by Fitt *et al.* (1968b). The gels were sliced in half longitudinally with a Canaco gel slicer and examined for protein distribution and enzyme activity. Protein was stained with Amido Black. For detection of the enzymic activity, the gels were incubated for 5 min. at 37° in the standard incorporation assay mixture (assay A, below), with or without polylysine. Longer incubation times caused the formation of very diffuse polyA bands with this enzyme, possibly because of the small molecular size of the product (Fitt & Wille, 1969). The polyA was stained with 0.2% (w/v) methylene blue in 0.4 M-sodium acetate, pH 4.7 (Peacock & Dingman, 1967). Gels were destained with running tap water.

Assay procedures. Polynucleotide phosphorylase was assayed by the following procedures.

Assay A. This assay depends on the incorporation of ^{14}C ADP into an acid-insoluble form. The reaction mixture (final volume 0.25 ml.) contained: tris-HCl, pH 8.1, 25 μmoles; MgCl_2 , 3.75 μmoles; EDTA, 0.25 μmole; ^{14}C -ADP, 1 μmole (about 5000–10000 c.p.m./μmole); polylysine (mol.wt. 3000), 50 μg., where appropriate; enzyme. Incubation was performed at 37° for 30 min. The reaction was stopped by addition of 7% (w/v) HClO_4 (0.25 ml.) and the mixture was kept in ice for 10 min. The precipitate was collected on a Whatman GF/C glass-fibre filter, washed four times with 1% (w/v) HClO_4 (2 ml.) and once with aq. 50% (v/v) ethanol (2 ml.), dried and counted.

Assay B. This was similar to assay A, except that 1.5 μmoles of MgCl_2 were used.

Assay C. This assay measures the formation of nucleoside ^{32}P diphosphates during the phosphorylation of polyA in the presence of ^{32}P orthophosphate. It was carried out as described for *M. lysodeikticus* polynucleotide phosphorylase by Fitt & Fitt (1967). It was confirmed that this procedure gave the same results as the method previously used for the clostridial enzyme (Fitt *et al.* 1968a).

Radioactivity assay. Radioactivity was determined in a Nuclear-Chicago low-background thin-window counter. Appropriate controls were carried out in all cases and their radioactivity was subtracted from the assay values.

Protein assay. Protein was determined spectrophotometrically (Warburg & Christian, 1942) or by the biuret procedure (Gornall, Bardawill & David, 1949).

RESULTS

Purification of *Cl. perfringens* polynucleotide phosphorylase. The improved method of purification described in the Experimental section gave a stable product with the highest specific activity so far reported for the *Cl. perfringens* polynucleotide phosphorylase. The maximum stimulation by polylysine (200 μg./ml.) in the standard assay conditions only changed slightly as a result of the purification

Table 1. *Effect of polylysine and polyornithine on the rate of ADP polymerization by Cl. perfringens polynucleotide phosphorylase before and after trypsin digestion of the enzyme*

The digestion and control incubations of polynucleotide phosphorylase were carried out as described in Fig. 1. The untreated enzyme, control enzyme and trypsin-digested enzyme were assayed for ADP-polymerization activity (assay A) without polybase, with polylysine (200 $\mu\text{g./ml.}$) and with polyornithine (200 $\mu\text{g./ml.}$). The results are expressed as the ratio of the activities with the corresponding basic poly(amino acid) to those without activator ('Stimulation').

Assay conditions	Stimulation			
	Digestion time (min.)...	0	2	10
Untreated enzyme plus polylysine	17	—	—	
Untreated enzyme plus polyornithine	17	—	—	
Control plus polylysine	—	16	16	
Control plus polyornithine	—	17	17	
Trypsin digest plus polylysine	—	2	0	
Trypsin digest plus polyornithine	—	5	1.7	

(crude extract: 20-fold; final product: 18-fold). In addition to having ADP-incorporation activity with and without polylysine, the purified enzyme was active with CDP and UDP in the absence of polylysine, and with GDP in the presence of the polybase. It was also active in the polyA phosphorylase assay.

The product was examined by analytical polyacrylamide-gel electrophoresis (see the Experimental section) and separated into two closely spaced major protein bands and a third, faint, faster-moving component. The activity, both with and without polylysine, appeared to be associated with the faster-moving of the two major bands of protein. However, even after short periods of incubation of the gels in the reaction medium for the formation of polyA from ADP, the single band due to the polyA produced was usually somewhat diffuse, possibly because of the small molecular size of the product formed in these conditions (Fitt & Wille, 1969).

Effect of trypsin digestion on the activity of Cl. perfringens polynucleotide phosphorylase. Fig. 1 shows the effect with time of trypsin digestion on the ADP-incorporation activity of *Cl. perfringens* polynucleotide phosphorylase assayed (i) without activators, (ii) with ApA, (iii) with polylysine and (iv) with 0.6 M-sodium chloride (the optimum sodium chloride concentration for this enzyme preparation; Fitt & Wille, 1969). Each point on the curves represents the residual activity of the digested enzyme expressed as a percentage of the activity of a similar control to which soya-bean trypsin inhibitor was added at zero time as well as trypsin. The polylysine- or sodium chloride-stimulated activity was lost very rapidly compared with the activity in the absence of these activators. The loss of polylysine-stimulation was not due to any effect of trypsin on polylysine during the assay,

Fig. 1. Time-course of the proteolysis of *Cl. perfringens* polynucleotide phosphorylase by trypsin. The digestion medium (final volume 3 ml.) contained: tris-HCl buffer, pH 8.1, 10 μmoles ; trypsin, 10 $\mu\text{g.}$; polynucleotide phosphorylase, 40 $\mu\text{g.}$ Incubation was at 37°. Samples (0.3 ml.) were withdrawn at the indicated times and added to ice-cold soya-bean trypsin inhibitor (0.2 ml.; 0.5 mg./ml.). The control contained, in addition, soya-bean trypsin inhibitor (1 mg.) and the corresponding samples were added to cold water (0.2 ml.). Both digested and control samples were assayed for ADP-polymerization activity by the standard assay A (i) without activator (○), (ii) with polylysine (200 $\mu\text{g./ml.}$) (●), (iii) with ApA (500 $\mu\text{g./ml.}$) (△) and (iv) with 0.6 M-NaCl (▲). The residual activity of the digested samples is expressed as a percentage of the corresponding control.

despite the presence of a large excess of soya-bean trypsin inhibitor, because the controls incubated in the presence of both trypsin and the inhibitor had lost no activity when assayed with or without

Fig. 2. Effect of trypsin digestion on the stimulation of *Cl. perfringens* polynucleotide phosphorylase by polylysine or ApA. The digestion and assays were carried out as described in Fig. 1. ●, Stimulation by polylysine (200 μ g./ml.); Δ, stimulation by ApA (500 μ g./ml.).

activators (see also the experiments with polyornithine below). In Fig. 2, the effect of trypsin digestion on the degree of stimulation of ADP-incorporation by either polylysine or ApA is shown. The effect on the polylysine stimulation was very rapid, whereas the loss of the small initial ApA stimulation was more gradual. After 20 min. of digestion, the enzyme was inhibited by both polylysine and ApA, and it was inhibited by sodium chloride after only 5 min.

Effect of β -mercaptoethanol on the activity of *Cl. perfringens* polynucleotide phosphorylase before and after trypsin digestion. It has been shown (Klee, 1968; Fitt *et al.* 1968b) that the large increase in primer requirement in *M. lysodeikticus* polynucleotide phosphorylase-catalysed polymer synthesis caused by treatment of the enzyme with trypsin (Fitt & Fitt, 1967; Klee, 1967; Klee & Singer, 1967) is eliminated by the presence of 1 mM- or 8 mM- β -mercaptoethanol in the assay mixture. We therefore examined the effect of β -mercaptoethanol on the activity of *Cl. perfringens* polynucleotide phosphorylase (i) before trypsin digestion, (ii) after trypsin digestion for 5, 10 and 20 min. in the conditions described in Table 1, and (iii) after incubation with trypsin and soya-bean trypsin inhibitor for 5, 10 and 20 min. as in (ii). No significant differences were found between the ADP-incorporation activi-

Fig. 3. Sucrose-density-gradient centrifugation of *Cl. perfringens* polynucleotide phosphorylase. The enzyme solution was diluted 100-fold with 10 mM-tris-HCl buffer, pH 8.1, and 0.1 ml. of the diluted solution was layered, at room temperature, on a 5–20% (w/v) sucrose gradient (4.5 ml.) in 20 mM-tris-HCl buffer, pH 8.3–1 mM-EDTA–7 mM- β -mercaptoethanol. The sample was centrifuged for 15 hr. at 33 000 rev./min. in a Spinco SW 39 rotor at 0–5°. The tube was pierced and fractions (approx. 0.16 ml.) were collected from the bottom. The fractions were assayed for ADP-polymerization activity with and without polylysine (assay A) and for phosphorylase activity (assay C). The top of the gradient is at the right. ●, ADP-polymerization with polylysine; Δ, ADP-polymerization without polylysine; ○ phosphorylase.

ties of any of the samples in the absence of β -mercaptoethanol and their corresponding activities in the presence of 1 mM-, 2 mM-, 5 mM-, 10 mM- or 50 mM- β -mercaptoethanol. The polylysine-stimulated activity lost during trypsin digestion could not therefore be restored by the presence of β -mercaptoethanol in the assay medium over a wide range of concentrations.

Sucrose-density-gradient-centrifugation studies. Dietz & Grunberg-Manago (1967) described the separation of the polynucleotide phosphorylase isolated from early-exponential-phase cells of *Cl. perfringens* strain A1844 (Institut Pasteur, Paris, France) into two components by sucrose-density-gradient centrifugation. They found one component to be specific for ADP-incorporation and totally dependent on both polylysine and β -mercaptoethanol. The results discussed above could be partly explained if trypsin destroyed such a polylysine-dependent enzyme preferentially. Six separate sucrose-density-gradient centrifugations

Table 2. *Effect of polyornithine on the digestion of Cl. perfringens polynucleotide phosphorylase by trypsin*

The incubation mixtures (final volume 0.15 ml.) contained: tris-HCl buffer, pH 8.1, 1.5 μ moles; polynucleotide phosphorylase, 1.04 μ g.; trypsin, 0.5 μ g.; polyornithine, as indicated. Incubation was at 37° for 10 min. The digestion was stopped by the addition of soya-bean trypsin inhibitor (0.1 ml.; 0.5 mg./ml.) and the solutions were cooled in ice. The controls contained in addition soya-bean trypsin inhibitor (50 μ g.), and water (0.1 ml.) was added at the end of the incubation. Portions (0.1 ml.) of the digested and control samples were assayed for ADP-polymerization activity (assay A) in the presence of polyornithine (200 μ g./ml.). (The polyornithine already present in the portions was insufficient to affect the results and was ignored.) The activity of the digests is expressed as a percentage of the activity of the corresponding controls.

Polyornithine (μ g.)	Residual activity (%)
0.00	0
0.05	12
0.10	63
0.50	89
1.00	100

were therefore carried out as described by Dietz & Grunberg-Manago (1967) with the final product from two different preparations of enzyme from the A.T.C.C. 10543 strain of *Cl. perfringens*. In all cases, the results were similar to those shown in Fig. 3. No evidence could be obtained either for the presence of a polylysine- and β -mercapto-ethanol-dependent enzyme or for the separation of the ADP-incorporation and polyA phosphorylase activities, even with enzyme prepared from the A.T.C.C. 10543 strain of *Cl. perfringens* by the method of Fitt *et al.* (1968a). However, since the polylysine-stimulation observed with *Cl. perfringens* polynucleotide phosphorylase falls markedly if the enzyme concentration is too high (Dolin, 1962), spurious double peaks of activity were obtained if the volume of each fraction used in the assay with polylysine was not adjusted to ensure proportionality. The difference between these results and those of Dietz & Grunberg-Manago (1967) may be due to the difference in the strains of *Cl. perfringens* or to the use of cells harvested at different stages in the growth cycle.

The effects of trypsin on fractions 3 and 8 (Fig. 3) were compared. In both cases, results identical with those described above were obtained. Further, no evidence for the separation of the enzyme into two components was observed during the Sephadex G-200 gel-filtration step of the purification. It is therefore unlikely that the loss of polybase-stimulation during proteolysis is explained by the presence in the preparation of two enzymes with different

Table 3. *Effect of polyornithine on the digestion of M. lysodeikticus polynucleotide phosphorylase by trypsin*

The incubation mixtures (final volume 1.2 ml.) contained: tris-HCl buffer, pH 8.1, 12 μ moles; *M. lysodeikticus* polynucleotide phosphorylase, 1.6 μ g.; trypsin, 0.04 μ g.; polyornithine, as indicated. Incubation was at 37° for 15 min. The digestion was stopped by the addition of soya-bean trypsin inhibitor (5 μ l.; 0.2 mg./ml.) and the samples were cooled in ice. The controls contained in addition soya-bean trypsin inhibitor (1 μ g.), and water (5 μ l.) was added at the end of the incubation. Portions (40 μ l.) of the samples were assayed for ADP- and CDP-polymerization activity as described by Fitt & Fitt (1967). The reaction mixture (final volume 0.1 ml.) contained: tris-HCl buffer, pH 9, 15 μ moles; MgCl₂, 1 μ mole; EDTA, 0.04 μ mole; bovine serum albumin, 5 μ g.; [¹⁴C]nucleoside diphosphate, 4 μ moles (about 5000–10000 c.p.m./mole); enzyme sample. Incubation was at 37° for 30 min. The reaction was stopped by the addition of 0.1 ml. of 7% (w/v) HClO₄ and the precipitates were washed, dried and counted as described in assay A (see the Experimental section). The activity of the digested enzyme is expressed as a percentage of the activity of the corresponding control.

Polyornithine (μ g.)	Residual activity (%)	
	Substrate.....	
	ADP	CDP
0	40	14
0.4	86	86
4.0	100	100

susceptibilities to proteolysis, although this explanation cannot be entirely eliminated until a pure enzyme preparation is available.

Protection of Cl. perfringens polynucleotide phosphorylase against trypsin digestion by polyornithine. The results in Table 1 show that poly-DL-ornithine (mol.wt. 3000–6000) was as effective as poly-L-lysine (mol.wt. 3000) in stimulating polyA synthesis by *Cl. perfringens* polynucleotide phosphorylase. The polyornithine-stimulated activity was also rapidly destroyed by trypsin digestion of the enzyme. Since polyornithine is not a substrate for trypsin (Brand, Plentl & Erlanger, 1949; Erlanger, 1957), the possibility that it might protect polynucleotide phosphorylase against proteolysis was studied.

It was confirmed by pH-stat titration (Waley & Watson, 1953), with a Radiometer Titrimeter, that no detectable hydrolysis of poly-DL-ornithine by trypsin occurred at pH 8 in conditions where poly-L-lysine was hydrolysed rapidly. (The pH-stat titrations were performed by Mr J. H. Seely, whose assistance is gratefully acknowledged.) Further experiments were undertaken to show that polyornithine was not an inhibitor of trypsin. The hydrolysis of DL-benzoylarginine *p*-nitroanilide

(Erlanger, Kowosky & Cohen, 1961) by trypsin at pH 8.1 was examined in the presence and absence of polyornithine. The polybase had no effect on the rate of the reaction when present at 100 times the concentration of the proteolytic enzyme.

The effect of poly-DL-ornithine on the hydrolysis of *Cl. perfringens* polynucleotide phosphorylase by trypsin was then studied over a range of polyornithine concentrations (Table 2). The results show that a concentration of polyornithine (1 µg./0.15 ml.) that has little effect on the rate of polyA synthesis in the standard assay (Fitt & Wille, 1969) protects the polynucleotide phosphorylase completely in the conditions used. Since polyornithine is neither a substrate nor an inhibitor of trypsin in these conditions, an interaction between the polybase and the polynucleotide phosphorylase must occur. Although the effect is not totally non-specific, since serum albumin (5 µg./0.15 ml. in the digestion medium) did not protect the enzyme, *M. lysodeikticus* polynucleotide phosphorylase, which is not stimulated by polybases (Singer & O'Brien, 1963), was protected against proteolysis by polyornithine (Table 3). Similarly it was observed that polyornithine can protect pancreatic ribonuclease against proteolysis. In this latter experiment, trypsin soya-bean inhibitor could not be used to stop the proteolysis, since it was found to inhibit ribonuclease as well as trypsin, and further digestion could occur during the assay of the residual ribonuclease activity. However, in conditions where 55% of the ribonuclease activity (measured by the method of Kalnitsky, Hummel & Dierks, 1959) was destroyed in the absence of polyornithine, only 25% was lost in its presence.

DISCUSSION

The rapid loss of the polybase-stimulated ADP-polymerization activity of *Cl. perfringens* polynucleotide phosphorylase during trypsin digestion in conditions where the activity measured in the absence of the activator is affected more slowly is best explained by one of the following alternatives. Either trypsin partially degrades the polynucleotide phosphorylase in such a way that it is no longer capable of interacting with the polybase, or the enzyme preparation contains two enzymes capable of catalysing the polymerization of ADP, one dependent on the presence of polybase and also more susceptible to degradation than the other. The possibility that two such enzymes might exist is suggested by the results of Knight *et al.* (1963) and Dietz & Grunberg-Manago (1967). Our results favour the first explanation, since the polynucleotide phosphorylase used in this work could not be separated into two components either by sucrose-density-gradient centrifugation by the

method of Dietz & Grunberg-Manago (1967) or by Sephadex G-200 gel filtration, and fractions from either side of the peak of activity in a density-gradient experiment did not differ in their sensitivity to trypsin digestion.

Dolin (1962) suggested that *Cl. perfringens* might contain an inhibitor of polynucleotide phosphorylase that could be bound selectively by polybases. Our results eliminate this possibility: it is difficult to see why a brief treatment with trypsin, which caused either (i) a change in the enzyme that rendered it incapable of binding such an inhibitor or (ii) the inactivation of the inhibitor itself, should destroy the activity in the presence of polybase without effect on the activity in its absence. Further, removal of basic groups from the enzyme, in accordance with the specificity of trypsin, should decrease rather than increase its ability to bind the hypothetical inhibitor, which should be acidic since it is supposedly bound by bases. Similarly, if the stimulation by polybases were due to their known ability to interact with the substrate and product of the reaction (Fitt *et al.* 1968a), digestion of the enzyme should not destroy the polybase-stimulated activity preferentially.

If it is accepted that the stimulation is due to an enzyme-polybase interaction, the mechanism could either be due to the formation of a specific complex or be merely a non-specific charge effect involving some part of the enzyme molecule susceptible to digestion by trypsin. The protection of the *Cl. perfringens* enzyme against trypsin digestion by concentrations of polyornithine that have little effect on either the rate of ADP polymerization or the activity of trypsin confirms that an interaction between the polynucleotide phosphorylase and the polybase is possible, as was previously shown by the thermal-inactivation studies of Fitt *et al.* (1968a). However, a specific interaction is probably not involved, because *M. lysodeikticus* polynucleotide phosphorylase and pancreatic ribonuclease were also protected against proteolysis by polyornithine. It seems unlikely that the non-specific interactions observed in the absence of substrate and Mg^{2+} both in this work and the earlier thermal-inactivation studies could be responsible for the formation of a specific enzyme-polybase complex in the reaction conditions.

We therefore conclude, in the absence of any evidence for the formation of an enzyme-activator complex, that relatively non-specific ionic effects on the enzyme itself are primarily responsible for the stimulation of *Cl. perfringens* polynucleotide phosphorylase by polybases. This conclusion is further supported by the facts that inorganic salts stimulate the enzyme (Dolin, 1962; Fitt *et al.* 1968a), that the salt-stimulated activity is destroyed at least as rapidly by trypsin as the base-stimulated

activity, and that the activation by bases is not specific with respect to either their nature or their stereochemistry (Fitt & Wille, 1969).

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REFERENCES

- Brand, E., Plentl, A. A. & Erlanger, B. F. (1949). *Abstr. Pap. Amer. chem. Soc., 116th Meet., Atlantic City*, p. 27c.
- Dietz, G. W., jun. & Grunberg-Manago, M. (1967). *Biochem. biophys. Res. Commun.* **28**, 146.
- Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd ed., p. 40. New York: Academic Press Inc.
- Dolin, M. I. (1961). *Biochem. biophys. Res. Commun.* **6**, 11.
- Dolin, M. I. (1962). *J. biol. Chem.* **237**, 1626.
- Dolin, M. I., Godiniaux, E. & Grunberg-Manago, M. (1961). *Bact. Proc.* **61**, 180.
- Erlanger, B. F. (1957). *J. biol. Chem.* **224**, 1073.
- Erlanger, B. F., Kowosky, N. & Cohen, W. (1961). *Arch. Biochem. Biophys.* **95**, 271.
- Fitt, P. S., Dietz, G. W., jun. & Grunberg-Manago, M. (1968a). *Biochim. biophys. Acta*, **151**, 99.
- Fitt, P. S. & Fitt, E. A. (1967). *Biochem. J.* **105**, 99.
- Fitt, P. S., Fitt, E. A. & Wille, H. (1968b). *Biochem. J.* **110**, 475.
- Fitt, P. S. & Wille, H. (1969). *Biochem. J.* **112**, 497.
- Gornall, A. G., Bardawill, C. W. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
- Grunberg-Manago, M. (1963). *Progr. Nucleic Acid Res.* **1**, 93.
- Kalnitsky, G., Hummel, J. P. & Dierks, C. (1959). *J. biol. Chem.* **234**, 1512.
- Klee, C. (1967). *J. biol. Chem.* **242**, 3579.
- Klee, C. (1968). *Fed. Proc.* **27**, 295.
- Klee, C. & Singer, M. F. (1967). *Biochem. biophys. Res. Commun.* **29**, 356.
- Knight, E., jun., Fitt, P. S. & Grunberg-Manago, M. (1963). *Biochem. biophys. Res. Commun.* **10**, 488.
- Peacock, A. C. & Dingman, C. W. (1967). *Biochemistry*, **6**, 1818.
- Singer, M. F. & O'Brien, B. M. (1963). *J. biol. Chem.* **238**, 328.
- Waley, S. G. & Watson, J. (1953). *Biochem. J.* **55**, 328.
- Warburg, O. & Christian, W. (1942). *Biochem. Z.* **310**, 384.