The Effect of Trypsin Digestion on the Activities of Polynucleotide Phosphorylase

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1. Treatment of *Micrococcus lysodeikticus* polynucleotide phosphorylase (nucleoside diphosphate-polynucleotide nucleotidyltransferase) with trypsin causes a preferential loss of its cytidine diphosphate and uridine diphosphate polymerization activities. 2. The phosphorolytic activity of the enzyme towards polycytidylic acid is unaffected in conditions in which the cytidine diphosphate-polymerization activity without added primer is virtually abolished. 3. The treated enzyme retains its altered pattern of activities when purified fivefold by gel filtration. 4. The effect on the cytidine diphosphate-polymerization activity is due, in part, to a large increase in primer requirement as a result of proteolysis, and is qualitatively independent of the state of purity of the polynucleotide phosphorylase. 5. The enzyme is protected from trypsin degradation by nucleic acids, polynucleotides and nucleoside diphosphates. 6. A similar, but less marked differential effect, is caused by α -chymotrypsin.

Polynucleotide phosphorylase (EC 2.7.7.8, nucleoside diphosphate-polynucleotide nucleotidyltransferase) occurs in many bacteria (Grunberg-Manago, 1963), and the enzymes isolated from *Azotobacter agilis* (Grunberg-Manago, Ortiz & Ochoa, 1956), *Micrococcus lysodeikticus* (Beers, 1956) and *Escherichia coli* (Littauer & Kornberg, 1957) have been studied in detail. The main reactions catalysed by all bacterial polynucleotide phosphorylases are known to be: (a) polyribonucleotide synthesis from ribonucleoside diphosphates, with release of orthophosphate; (b) the phosphorolysis of polyribonucleotides by orthophosphate with formation of ribonucleoside diphosphates (the reverse of a); (c) the exchange of the terminal phosphate group of a ribonucleoside diphosphate with orthophosphate. The enzymes have an absolute requirement for magnesium, are specific for ribonucleotides and are believed to be unable to catalyse the formation or phosphorolysis of dinucleotides. Synthesis of polymers occurs by addition of ribonucleoside monophosphate units to the free 3'-hydroxyl group of a pre-existing dinucleotide unit (Grunberg-Manago, 1963).

Although polynucleotide phosphorylases from different bacteria have a general similarity, there are unexplained variations in their detailed primer requirements and optimum nucleoside diphosphate/magnesium ratios. Furthermore, the evidence that they are single enzymes, and not a mixture of

enzymes each specific for a different nucleoside diphosphate, is not conclusive (Grunberg-Manago, 1963). Olmsted & Lowe (1959a,b) claimed that partial purification of the polynucleotide phosphorylase from *M. lysodeikticus* after treatment of the crude enzyme with crude trypsin gave an enzyme specific for ADP. They found that their product did not catalyse the polymerization of CDP or UDP, or the phosphorolysis of poly C.* They suggested that either (a) polynucleotide phosphorylase was a mixture of enzymes and their procedures destroyed all but the ADP-specific enzyme, or (b) the enzyme contained more than one active site and proteolysis destroyed the site involved in CDP polymerization. They concluded that the second explanation was unlikely, because treatment of the crude extract with five widely differing enzymes (trypsin, papain, hyaluronidase, crude snake venom, cellulase) or with various inhibitors (copper, zinc or barium ions, or iodoacetate) had similar effects; however, their results with inhibitors could not be confirmed (Singer & Guss, 1962).

In this paper, studies of the action of crystalline trypsin and α -chymotrypsin on partially purified *M. lysodeikticus* polynucleotide phosphorylase are described.

* Abbreviations: poly A and poly C, polyadenylic acid and polycytidylic acid; NDP, nucleoside diphosphate; ApA, adenylyl-(3'-5')-adenosine; s-RNA, transfer RNA.

EXPERIMENTAL

Materials. Intermediates and enzymes were purchased from the following suppliers: unlabelled ribonucleoside diphosphates and yeast s-RNA, Calbiochem, Los Angeles, Calif., U.S.A.; calf thymus DNA, yeast ribosomal RNA and crystalline bovine serum albumin, Sigma Chemical Co., St Louis, Mo., U.S.A.; [^{14}C]ribonucleoside diphosphates, Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.; carrier-free [^{32}P]orthophosphate, The Radiochemical Centre, Amersham, Bucks.; crystalline lysozyme, crystalline trypsin, crystalline α -chymotrypsin, soya bean trypsin inhibitor, pancreatic trypsin inhibitor, and spray-dried *Micrococcus lysodeikticus* cells, Worthington Biochemical Corp., Freehold, N.J., U.S.A.; polyadenylic acid and polycytidylic acid, Miles Chemical Co., Elkhart, Ind., U.S.A.; adenylyl-(3'-5')adenosine, Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y., U.S.A.

Preparation of polynucleotide phosphorylase. Polynucleotide phosphorylase was prepared from *M. lysodeikticus* spray-dried cells by three different procedures. All operations after the initial lysis of the cells were carried out at 2°. Ammonium sulphate saturations were calculated according to Dixon & Webb (1964).

Method A. The enzyme was isolated and purified tenfold with respect to phosphorolytic activity by the procedure of Singer & Guss (1962). The final specific activity of such preparations was approx. 1.6 units/mg. Unless otherwise mentioned, this is the material used in the present studies.

Method B. Lysis of the cells with lysozyme and two $(\text{NH}_4)_2\text{SO}_4$ fractionations were carried out as in method A. The NaCl concentration of the active fraction was adjusted to 0.1 M, and it was subjected to chromatography on DEAE-cellulose. The enzyme was eluted with a linear gradient of NaCl (0.2–1.0 M) and the active fractions, which came off the column at a concentration of about 0.3 M-NaCl, were purified further by $(\text{NH}_4)_2\text{SO}_4$ precipitation. The enzyme was precipitated between 35% and 55% saturation. It was dissolved in 0.1 M-tris-HCl, pH 8.7, and dialysed free from $(\text{NH}_4)_2\text{SO}_4$ against 0.01 M-tris-HCl (pH 8.2)–1 mM-EDTA–1 mM- β -mercaptoethanol (referred to as 'tris buffer' below). It was then passed through a column (2.5 cm. $\times$ 45 cm.) of Sephadex G-200, equilibrated with 'tris buffer'. The flow rate was 10 ml./hr. with a maximum hydrostatic head of 10 cm. To concentrate the product, the enzyme was adsorbed on to a small DEAE-cellulose column (0.9 cm. $\times$ 1.0 cm.) and eluted with 1 M-NaCl. It was dialysed twice against 250 ml. of tris buffer and stored at 2° or –20°. This method gave an overall purification of at least 100-fold: specific activity 17 units/mg.

Method C. The crude extract, prepared as in method A, was fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation. The fraction insoluble between 30% and 60% saturation was passed through a DEAE-cellulose column, in the conditions described by Thanassi & Singer (1966), to remove nucleic acids. The enzyme was precipitated by addition of $(\text{NH}_4)_2\text{SO}_4$ to the active fractions to a final concentration of 55% saturation. The protein was dissolved in 0.1 M-tris-HCl, pH 8.7, and dialysed against tris buffer. The product, which was purified fourfold, was used for the experiments described in Fig. 6.

Assay procedures. Polynucleotide phosphorylase activity was determined by the following assays in the conditions described by Singer & Guss (1962). If the protein concentra-

tion of the enzyme solution was below 0.1 mg./ml., 5 μg . of bovine serum albumin was included in the assay medium.

Assay A. This assay measures the formation of [^{32}P]nucleoside diphosphates during phosphorolysis of polynucleotides in the presence of [^{32}P]orthophosphate. The reaction mixture, final volume 0.1 ml., contained: tris-HCl, pH 8.2, 10 μmoles ; MgCl_2 , 0.5 μmole ; EDTA 0.03 μmole ; poly A or poly C, 50 μg ; [^{32}P]orthophosphate, 1 μmole (about 1×10^5 counts/min./ μmole); enzyme. Incubation was performed at 37° for 15 min. The reaction was stopped by the addition of 1 ml. of a mixture containing 1 part of an aqueous 10% (w/w) suspension of acid-washed Norit A and 10 parts of 2.5% (v/v) HClO_4 . The charcoal suspension was allowed to stand for at least 10 min. at 0°, with occasional mixing, and was then collected on a 2.4 cm. diam. Whatman GF/C glass-fibre filter. The charcoal was washed four times with 10 ml. of cold water. The filter was then placed upside-down in a planchet, dried and counted. The specific activity of [^{32}P]orthophosphate was determined by counting a suitable dried portion of the solution over which a GF/C filter had been placed. One unit of enzyme activity was defined as the amount of enzyme catalysing the incorporation of 1 μmole of [^{32}P]orthophosphate into charcoal-adsorbable material in these conditions. All specific activities mentioned in this paper are in terms of units of phosphorolysis activity per mg. of protein.

Assay B. This assay depends on the incorporation of [^{14}C]nucleoside diphosphates into an acid-insoluble form. The reaction mixture, final volume 0.1 ml., contained: tris-HCl, pH 9.0, 15 μmoles ; MgCl_2 , 1 μmole ; EDTA, 0.04 μmole ; [^{14}C]NDP, 4 μmoles (about $5\text{--}10 \times 10^3$ counts/min./ μmole); enzyme. Incubation was at 37° for 30 min. The reaction was stopped by addition of 0.1 ml. of 7% (v/v) HClO_4 and the mixture was allowed to stand at 0° for 10 min. The precipitate was collected on a Whatman GF/C filter, washed four times with 1% (v/v) HClO_4 , once with 50% (v/v) ethanol, dried and counted.

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

Protein assay. Protein was determined spectrophotometrically by the procedure of Warburg & Christian (1942).

RESULTS

Preliminary studies of the effect of trypsin digestion on the crude *M. lysodeikticus* polynucleotide phosphorylase showed that there was a marked differential effect on the ADP- and CDP-polymerizing activities. It was also shown that soya bean trypsin inhibitor alone, and trypsin together with, at least, a twofold excess of the inhibitor, had no effect on the polynucleotide phosphorylase assays with ADP and CDP as substrates. The soya bean inhibitor did stimulate UDP polymerization by the crude enzyme significantly, but this effect was not observed with the partially purified polynucleotide phosphorylase preparations. The lack of effect of the appropriate mixture of trypsin and inhibitor on the assays was confirmed in all the experiments described below.

Fig. 1. Time-course of the proteolysis of *M. lysodeikticus* polynucleotide phosphorylase by trypsin. The digestion medium, pH 8.0, contained: 5 mM-tris-HCl buffer, pH 8.2; polynucleotide phosphorylase (sp. activity 1.6 units/mg.; prepared by method A), 2 mg./ml.; trypsin, 0.5 mg./ml. Incubation was at 37°. At the indicated times, 0.1 ml. portions were withdrawn and added to 0.1 ml. of ice-cold trypsin soya bean inhibitor (1 mg./ml.). The samples were stored in ice and assayed as soon as possible for ADP-, CDP- and UDP-polymerization activity in the standard polynucleotide phosphorylase assay B, described in the Experimental section. The residual activity is expressed as a percentage of the activity of the untreated polynucleotide phosphorylase assayed in the same conditions. ●, ADP; □, CDP; △, UDP.

Detailed studies with tenfold-purified polynucleotide phosphorylase were then undertaken. Fig. 1 shows the time-course of digestion with trypsin. The residual activity is expressed as a percentage of the activity of the untreated enzyme assayed in the same conditions. It can be seen that the CDP- and UDP-polymerization activities fell rapidly, and together, whereas the ADP-incorporating activity was destroyed more slowly.

Further experiments with enzyme preparations purified from 20- to over 100-fold (method B) indicated that the results obtained were qualitatively independent of the purity of the preparation. These experiments also showed that the enzyme became more sensitive to trypsin as a result of purification. Since the crude enzyme used in the preliminary studies contained much nucleic acid, the effect of nucleic acids, polynucleotides, ADP, CDP, magnesium and serum albumin on the trypsin digestion of the partially purified enzyme was examined. The data in Table 1 show that nucleic acids and synthetic polynucleotides protected both the ADP- and CDP-incorporation activities significantly. Surprisingly, DNA, which is not a substrate,

Table 1. Effect of nucleic acids, synthetic polynucleotides, serum albumin, nucleoside diphosphates and magnesium ions on the proteolysis of *M. lysodeikticus* polynucleotide phosphorylase by trypsin

Individual tubes containing 20 μ l. of the digestion mixture described under Fig. 1, in which the compounds indicated in the Table were included as appropriate, were incubated at 37° for 5 min. Ice-cold trypsin soya bean inhibitor (1 mg./ml.; 20 μ l.) was added, and the samples were assayed for ADP- and CDP-polymerization activity in the standard assay B after the addition of the other components of the assay medium. The residual activity is expressed as a percentage of the activity of the untreated enzyme assayed in the same conditions.

Additions	Residual activity (%)	
	ADP	CDP
None	32	0.8
DNA (10 μ g.)	77	20
RNA (ribosomal, 10 μ g.)	67	10
s-RNA (10 μ g.)	84	21
poly A (10 μ g.)	79	9
poly C (10 μ g.)	74	4
Serum albumin (10 μ g.)	31	—
ADP (4 μ moles)	76	—
CDP (4 μ moles)	—	9
MgCl ₂ (1 μ mole)	45	—

was as effective as ribosomal RNA, s-RNA and the polynucleotides, which are substrates. However, the effect was not non-specific, because bovine serum albumin did not protect the enzyme. Magnesium ions did not protect the enzyme to any great extent.

The ADP- and CDP-incorporation activities were protected significantly by ADP and CDP respectively. The effect of ADP on the hydrolysis of tosylarginine methyl ester by trypsin (Hummel, 1959) was then studied, with the same relative concentrations of ADP and trypsin as used in the experiments described in Table 1. In these conditions, ADP had no effect on the activity of trypsin. Thus, it appears that the nucleoside diphosphate specifically protected polynucleotide phosphorylase against proteolysis, in agreement with the studies of Trayser & Colowick (1961), who have shown that hexokinase is protected by its substrate, glucose, against the action of trypsin. It seems therefore that the effects observed during trypsin treatment of *M. lysodeikticus* polynucleotide phosphorylase are due to some direct action on the latter, and not to the formation of inhibitors from protein impurities, since both ADP and CDP protected the enzyme significantly.

A study was made of the loss of polynucleotide phosphorylase activity during a 5-min. digestion as a function of trypsin concentration. Fig. 2 shows

Fig. 2. Effect of changes in trypsin concentration on the proteolysis of *M. lysodeikticus* polynucleotide phosphorylase. The digestion medium, pH 8.0, contained: 5 mM-tris-HCl buffer, pH 8.2; polynucleotide phosphorylase (sp. activity 1.6 units/mg.; prepared by method A), 0.27 mg./ml.; trypsin as indicated. The experiment was conducted as described in Table 1, and the residual activities are expressed as a percentage of that of the untreated enzyme. ●, ADP; □, CDP.

Fig. 3. Changes in primer requirement of *M. lysodeikticus* polynucleotide phosphorylase during trypsin digestion. The digestion medium, pH 8.0, contained: 5 mM-tris-HCl buffer, pH 8.2; polynucleotide phosphorylase (sp. activity 1.6 units/mg.; prepared by method A), 0.27 mg./ml.; trypsin, 5 μg./ml. Incubation was at 37°. At the indicated times, portions were withdrawn and added to an equal volume of ice-cold trypsin soya bean inhibitor (1 mg./ml.). The samples (40 μl.) were assayed in the standard assay B for ADP- and CDP-polymerization activity with and without ApA, 0.35 mg./ml. The residual activity is expressed as a percentage of the activity of the untreated enzyme assayed in the same conditions. ●, ADP; ○, ADP + ApA; △, CDP; □, CDP + ApA.

that the concentration of trypsin is critically important if the maximum differential effect is to be obtained. In this case, it was possible to destroy over 80% of the CDP-incorporating activity whilst retaining virtually all the ADP-incorporating activity with a trypsin to enzyme ratio approx. 1:50 (w/w). The ratio of trypsin to enzyme in the experiments described in Fig. 1 was 1:16 (w/w).

Olmsted & Lowe (1959b) did not observe any change in the primer requirement of their partially purified enzyme compared with that of the crude polynucleotide phosphorylase. However, they only tried poly C as a primer, and suitable small oligonucleotides are now readily available. A study of the primer requirements for ADP and CDP polymerization by the treated and untreated enzyme was therefore undertaken. The data presented in Fig. 3 show that there was a marked change in primer requirement during trypsin digestion. The ADP-polymerizing activity of the untreated enzyme preparation used for these experiments was unaffected by the addition of ApA (0.35 mg./ml.) to the assay medium; the CDP-incorporating activity was stimulated threefold. During the digestion with trypsin, the residual ADP-incorporating activity became inhibited by ApA and the observed inhibition increased slowly with the time of treatment. However, after digestion for 10 min., the stimulation of the CDP-incorporating activity increased markedly to 25-fold. The differential effect on the CDP-incorporating activity therefore appeared much less marked when the assays were carried out in the presence of the primer.

The change in stimulation by ApA of poly A and poly C synthesis was studied further as a function of (i) trypsin concentration with a constant time of digestion (Fig. 4) and (ii) time at a constant, low trypsin concentration (Fig. 5). (The enzyme preparations used are described in the legends under the Figures. In this case poly A synthesis by the untreated enzyme was stimulated about 11% by ApA.) In both Figures it can be seen that the stimulation of poly C synthesis by ApA increased rapidly, whereas there was little or no change in the primer requirement for poly A synthesis. Fig. 4 shows that the primer requirement in CDP polymerization increased rapidly, and linearly, with trypsin concentration when the time of incubation was maintained constant: thus the effect was not caused by incubation at 37°. Fig. 5 shows that a similar effect was obtained by altering the time of incubation when the amounts of trypsin and inhibitor used were constant, so that it was not due to changes in the amount of either trypsin-inhibitor complex or free inhibitor in the final assay.

The primer requirement in the CDP-polymerization reaction increased upon purification of the enzyme, but this change was small compared with

Fig. 4. Effect of changes in trypsin concentration during proteolysis of polynucleotide phosphorylase on change in primer requirement of the latter in polynucleotide synthesis. The experiment was carried out as described under Fig. 2 with an enzyme prepared by method C (sp. activity 0.4 unit/mg.). The residual ADP- and CDP-polymerization activities were determined by assay B with and without ApA, 1 mg./ml. The results are expressed as the ratio of the activity with ApA to that without primer for each substrate. ●, ADP; ○, CDP.

Fig. 5. Changes in primer requirement of polynucleotide phosphorylase with time during trypsin digestion. The enzyme used was fraction 12, Fig. 6(a) (sp. activity 2 units/mg.). The digestion medium, pH 8.0, contained: 5 mm-tris-HCl, pH 8.2; polynucleotide phosphorylase, 0.37 mg./ml.; trypsin, 0.05 $\mu\text{g./ml.}$ The incubation was at 37° and portions were withdrawn at the indicated times and added to an equal volume of cold trypsin soya bean inhibitor (2 $\mu\text{g./ml.}$). The samples were assayed by assay B, with and without ApA (1 mg./ml.), on 50 $\mu\text{l.}$ portions. Results were expressed as in Fig. 4. ●, ADP; ○, CDP.

the change due to proteolytic digestion. It was also found that changes in the primer requirement for CDP polymerization took place during prolonged storage of polynucleotide phosphorylase either at 2° or at -20°. These changes became more noticeable as the purity of the preparation increased. To obtain consistent results, it was important that the enzyme preparation be less than 4 weeks old.

It is known that crystalline trypsin may contain pancreatic ribonuclease (Polatnick & Bachrach, 1961). The amount present in the trypsin used in this work was determined by the ribonuclease assay of Kalnitsky, Hummel & Dierks (1959), modified for use at pH 9 and with poly C as substrate. It was found that 1 mg. of the trypsin contained sufficient nucleases to liberate a maximum of 0.15 μmole of CMP during a 30 min. incubation at 37°. In the assays used to determine polynucleotide phosphorylase activity after trypsin digestion in, e.g., the experiments described in Fig. 3, the final amount of trypsin in the assay medium (final volume 0.1 ml.) was 0.1 $\mu\text{g.}$ Therefore sufficient nucleases were present to liberate 1.5×10^{-6} μmole of CMP from poly C in conditions where the untreated polynucleotide phosphorylase catalysed the incorporation of 2×10^{-2} μmole of CDP into poly C in the

absence of a primer. Thus the amount of polymer destroyed by nucleases present in the trypsin could not have exceeded 0.1% of the expected theoretical yield in these experiments, a difference undetectable by the assay procedure. It is therefore impossible that the results described in this paper could have been affected by the nucleases in the trypsin, since their concentration in the polymerization assays was negligible. This conclusion is further confirmed by the following facts: (a) the data in Fig. 5 demonstrate that the change in primer requirement in poly C synthesis depended solely on the time of trypsin digestion when a constant concentration of trypsin (and, necessarily, of contaminants) was used, and all other conditions, including those of the assay, were constant; (b) as previously stated, the simultaneous addition of the appropriate amounts of trypsin and trypsin inhibitor to the polynucleotide phosphorylase assay system did not affect the incorporation of ADP, CDP or UDP by partially purified enzyme preparations (this was always confirmed in all experiments); (c) poly A, DNA and

Fig. 6. Sephadex G-200 filtration of polynucleotide phosphorylase before and after digestion with trypsin. (a) *M. lysodeikticus* polynucleotide phosphorylase (5 ml.; sp. activity 0.4 unit/mg.; prepared by method C) in 0.01 M-tris-HCl (pH 8.2)-0.001 M-EDTA-0.001 M-mercaptoethanol was passed through a column, 2.5 cm. $\times$ 40 cm., of Sephadex G-200. Elution was carried out with the same buffer at 10 ml./hr. with a hydrostatic head of 10 cm.; 4 ml. fractions were collected. The fractions were assayed for protein, ADP- and CDP-polymerization activity (assay B), with and without ApA (1 mg./ml.). (b) A mixture of polynucleotide phosphorylase (5 ml. of enzyme solution used in a) and trypsin (25 μ l. of 1 mg./ml. solution) were incubated at 37°. After 5 min., 50 μ l. of trypsin soya bean inhibitor (1 mg./ml.) was added. The solution was mixed well and cooled in ice, and 5 ml. was then passed through the Sephadex G-200 column as in (a). The fractions were assayed as in (a). $\square$, Protein; $\circ$, ADP; $\bullet$, ADP + ApA; $\triangle$, CDP; $\blacktriangle$, CDP + ApA.

Table 2. Comparison of the relative activities of untreated and trypsin-digested polynucleotide phosphorylase before and after gel filtration

The enzyme preparations were: (a) untreated and treated enzyme as used in Fig. 6; (b) untreated, after Sephadex G-200 gel filtration, fraction 12, Fig. 6(a); (c) treated, after Sephadex G-200 gel filtration, fraction 13, Fig. 6(b). The activities were determined in the standard assay B system in the presence of ApA (1 mg./ml.) where indicated. The results are expressed as a ratio of the ADP- to CDP-polymerization activities either with or without primer.

Ratio of activities	Un-treated enzyme	Un-treated enzyme (G-200)	Treated enzyme	Treated enzyme (G-200)
ADP/CDP	3.4	6.4	28	35
ADP(+ ApA)/CDP(+ ApA)	1.2	1.2	2.3	2.3

CDP, none of which is a substrate for pancreatic ribonuclease, were more effective than poly C in protecting the CDP-polymerization activity of polynucleotide phosphorylase during trypsin treatment (Table 1).

Fig. 6 shows the results of gel-filtration experiments with untreated polynucleotide phosphorylase (Fig. 6a) and the same enzyme after trypsin digestion (Fig. 6b). The untreated enzyme had been purified fourfold by method C. Both the treated and untreated enzymes were purified a further fivefold with respect to their ADP-incorporating activities, assayed with ApA as primer, by passage through a column (2.5 cm. $\times$ 40 cm.) of Sephadex G-200. The treated enzyme retained the modified pattern of activities after partial purification (Table 2).

An attempt was then made to separate a mixture (1 ml.) of the two peak fractions from the gel-filtration experiments described above by passage, at 10 ml./hr., through a column (2.5 cm. $\times$ 90 cm.) of Sephadex G-200; 2 ml. fractions were collected. A clear separation could not be obtained, but the activity was eluted in a peak with broad shoulders both on the leading and trailing edges; it is therefore probable that trypsin treatment caused some significant, physical change in the polynucleotide phosphorylase molecule, even though the data are not conclusive. Since *M. lysodeikticus* polynucleotide phosphorylase is a large molecule, and is believed to have a molecular weight in excess of 200,000 (Singer & O'Brien, 1963), considerable

Fig. 7. Time-course of reactions catalysed by polynucleotide phosphorylase after gel filtration of the untreated and trypsin-digested enzyme. The polynucleotide phosphorylase preparations used were in the peak tubes from Fig. 6(a) (no. 12) and Fig. 6(b) (no. 13). The assay procedures (cf. assays A and B in the Experimental section) were as follows. (A) Phosphorolysis assay. The reaction mixture, total volume 1 ml., contained: tris-HCl, pH 8.2, 100 μ moles; $MgCl_2$, 5 μ moles; EDTA, 0.3 μ mole; poly A or poly C, 500 μ g.; [^{32}P]orthophosphate, 10 μ moles (approx. 1×10^5 counts/min./ μ mole); 0.1 ml. of appropriate enzyme fraction. Incubation was at 37°. Portions (50 μ l.) were withdrawn at the indicated times and added to 0.5 ml. of a mixture containing one part of aq. 10% (w/w) suspension of acid-washed Norit A and 10 parts of 2.5% (v/v) $HClO_4$. The charcoal suspension was allowed to stand at 0° for at least 10 min., with occasional mixing, and was then collected on a Whatman GF/C filter. The charcoal was washed four times with 10 ml. of cold water. The filter was then placed upside-down in a planchet, dried and counted. (B) Polymerization assay. The reaction mixture, total volume 1 ml., contained: tris-HCl, pH 9.0, 150 μ moles; $MgCl_2$, 10 μ moles; EDTA, 0.4 μ mole; [^{14}C]ADP or [^{14}C]CDP, 40 μ moles (approx. $5-10 \times 10^3$ counts/min./ μ mole); ApA, 1 mg. (where indicated); 0.1 ml. of the appropriate enzyme fraction. Incubation was at 37°. Portions (50 μ l.) were withdrawn at the indicated times and added to an equal volume of 7% (v/v) $HClO_4$. The mixture was allowed to stand at 0° for at least 10 min. The precipitate was then collected on a Whatman GF/C filter, washed four times with 1% (v/v) $HClO_4$ and once with 50% (v/v) ethanol, dried and counted. (a) ●, Phosphorolysis of poly A or poly C, treated and untreated enzyme; △, CDP, untreated enzyme; ○, CDP, treated enzyme. (b) ●, ADP+ApA, untreated enzyme; ■, ADP+ApA, treated enzyme; ○, ADP, untreated enzyme; □, ADP, treated enzyme. (c) ●, CDP+ApA, untreated enzyme; ○, CDP+ApA, treated enzyme.

degradation would have to occur before the treated and untreated enzyme became separable by gel filtration.

The activities of the peak fractions of the treated and untreated enzymes were compared in more detail. Fig. 7(a) shows the time-course of poly A and poly C phosphorolysis, and of poly C synthesis in the absence of primer with both the treated and untreated polynucleotide phosphorylase. The treated and untreated enzymes both had a specific activity of poly A phosphorolysis of 2 units/mg., so that the phosphorolytic activity was unchanged by the trypsin treatment. Furthermore, the data show that the phosphorolytic activity was independent of the substrate used, and identical results were obtained with either poly A or poly C before and after trypsin digestion. On the other hand, the untreated enzyme catalysed CDP polymerization at a steady rate, after a short lag phase, whereas the

treated enzyme had a very low activity in these conditions. Incubation was continued for 48 hr. (not shown) and the poly C formed by the treated enzyme was only 5% of that synthesized by the untreated enzyme in the same time. However, as can be seen from Fig. 7(c), the treated enzyme had considerable CDP-polymerization activity in the presence of saturating amounts of ApA, although this was still somewhat lower than with the untreated enzyme. (It will be noted that saturating amounts of ApA did not entirely eliminate the lag phase in CDP polymerization.) It can also be seen, from Fig. 7(b), that the trypsin digestion caused a limited loss of ADP-polymerization activity measured in the presence of ApA. The primer requirement in poly A synthesis was increased somewhat by the proteolysis, but the effect was small compared with that on the CDP-incorporation activity.

Fig. 8 shows the effects of digestion of *M. lyso-*

Fig. 8. Effect of α -chymotrypsin on *M. lysodeikticus* polynucleotide phosphorylase. The digestion medium contained: 5 mM-tris-HCl buffer, pH 8.2; polynucleotide phosphorylase (sp. activity: 0.7 unit/mg.; prepared by method C), 0.75 mg./ml.; α -chymotrypsin, 2.5 μ g./ml. The incubation temperature was 37°. Portions (0.1 ml.) were withdrawn at the indicated times and added to 0.05 ml. of ice-cold pancreatic trypsin inhibitor (0.01 mg./ml.). The samples were assayed (assay B) for ADP- and CDP-polymerization activity, with and without ApA, 0.35 mg./ml. The residual activity is expressed as a percentage of the activity of the untreated enzyme assayed in the same conditions. ●, ADP + ApA; △, CDP; ○, CDP + ApA.

deikticus polynucleotide phosphorylase by α -chymotrypsin on the ADP- and CDP-polymerization activities in the presence and absence of primer. They are somewhat similar to those observed with trypsin, although the loss of CDP-polymerization activity and the increase in the primer requirement in poly C synthesis are both less marked than those observed in the experiments with trypsin.

Preliminary studies with the *Azotobacter agilis* and *Escherichia coli* polynucleotide phosphorylases (with Mr D. Natta and Mr A. Gajda) have shown that trypsin has effects on these enzymes similar to those described above.

DISCUSSION

The results show that during trypsin digestion of *M. lysodeikticus* polynucleotide phosphorylase there is a rapid loss of the CDP- and UDP-polymerization activities, while the ability of the enzyme to catalyse the formation of poly A declines more slowly. Preliminary studies with the *E. coli* and *A. agilis* polynucleotide phosphorylases show that the effect is a general one, not restricted to the *M. lysodeikticus* enzyme. Proteolysis of the latter with α -chymotrypsin has a similar, but less marked, effect on these

activities. Insufficient pancreatic ribonuclease was present in the trypsin used for it to have a detectable effect on the results.

Nucleic acids and polynucleotides protect polynucleotide phosphorylase against proteolysis by trypsin, whereas serum albumin and Mg^{2+} ions do not. Nucleoside diphosphates also protect the enzyme, and since they do not inhibit trypsin, this protection appears to be specific.

The size of the differential effect observed with any particular preparation of polynucleotide phosphorylase depends on the ratio of trypsin to polynucleotide phosphorylase used, because the latter becomes more sensitive to proteolysis during purification. This increase in sensitivity is probably due to the removal of nucleic acids.

Contrary to the results of Olmsted & Lowe (1959a, b), it was found that the differential effect was due, in part, to a large increase in the primer requirement of the enzyme in poly C synthesis. However, the addition of saturating amounts of ApA to the reaction medium did not completely eliminate the differential effect. Furthermore, the present studies show that the phosphorolytic activity of the polynucleotide phosphorylase towards either poly A or poly C was stable in the conditions used. Thus the major effect of trypsin was on the CDP- and UDP-polymerization activities.

No method is yet available for the preparation of a physically homogeneous polynucleotide phosphorylase from any source. However, the effects observed were qualitatively independent of the state of purity of the polynucleotide phosphorylase and the treated enzyme retained its modified pattern of activities when purified fivefold by gel filtration. These results, together with the fact that the enzyme is significantly protected against trypsin action by its substrates, make it unlikely that the differential effects could be caused by specific inhibitors formed from impurities in the polynucleotide phosphorylase preparation.

It is unlikely that the increase in primer requirement in CDP polymerization could be caused by the release of an existing, bound oligonucleotide from the polynucleotide phosphorylase. Such a primer would have to be highly specific, and no evidence has been found of a base specificity in primer action (Grunberg-Manago, 1963). Furthermore, such a primer would have to be inactive unless attached to the polynucleotide phosphorylase since it would have been present in solution in all assays, except those with the purified, treated enzyme, and free ApA was an effective primer in these conditions.

Several authors have shown that many of the amino acid residues of certain enzymes, up to two-thirds for papain, may be removed from either end of the protein molecule by exopeptidases without effect on the catalytic activity. Such studies include

the work of Hill & Smith (1956) on papain, Kalnitsky & Rogers (1956) on ribonuclease, and Nylander & Malmström (1959) on enolase. However, few cases are known in which proteolysis of an enzyme with more than one distinguishable activity has been shown to cause a change in the relative activities. Anan (1958a,b) and Radhakrishnan and his collaborators (Radhakrishnan, Raghupathy & Sarma, 1963; Radhakrishnan & Sarma, 1965) have studied the effect of trypsin, α -chymotrypsin and pepsin on catalase, and have demonstrated that significant changes in the relative catalatic and peroxidatic activities take place during proteolysis. Juni & Heym (1966) have described studies in which a proteolytic enzyme from yeast was shown to increase the ability of pyruvate decarboxylase to catalyse the reaction between pyruvate and acetaldehyde in conditions which led to the destruction of its acetaldehyde-forming ability. The nature of the structural changes responsible for these alterations in relative activity is unknown, but Radhakrishnan & Sarma (1965) were able to isolate from the peptic digest of catalase a peroxidatically active product which had a molecular weight only 12% that of catalase. These authors showed that the loss of catalatic activity was not simply due to the disassociation of catalase into sub-units during the digestion, and they suggested that certain fragments essential for catalatic activity were removed in these conditions.

The present results are consistent with *M. lysodeikticus* polynucleotide phosphorylase being either one enzyme or a mixture of enzymes. However, it should be emphasized that, although polynucleotide phosphorylases have yet to be isolated in a physically homogeneous state, no separation of activities has been found during partial purification of the *E. coli*, *A. agilis* or *M. lysodeikticus* enzymes (Grunberg-Manago, 1963). Even if polynucleotide phosphorylase were a mixture of enzymes, or one enzyme with multiple sites either on one peptide chain or on different sub-units, the need would remain to explain the large effect on the CDP-polymerization activity in conditions in which the phosphorolytic activity towards poly C is unchanged. Since it is accepted that phosphorolysis and synthesis of any given polymer are merely different aspects of the same overall catalytic function, some change must have taken place in the detailed

structure of the active site, either directly or as a result of changes in the overall conformation of the enzyme molecule. One possibility is that the active site consists of the combination of a catalytic site together with one of several sites responsible for binding the substrates, and that there is a differential destruction of some of the binding sites.

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