Characterization of Polynucleotide Phosphorylase from *Micrococcus luteus* and Isolation of the 13,000 Base Poly(A) Product of the Polymerization Reaction*

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A new purification procedure for polynucleotide phosphorylase from freeze-dried *Micrococcus luteus* cells gives ~20% yield of nearly homogeneous, primer-independent enzyme which is free of nucleic acid. The physicochemical properties of *M. luteus* polynucleotide phosphorylase are similar to those previously described for the enzyme from *Escherichia coli* in terms of Mr, subunit structure, and amino acid composition. The purified enzyme appears to be a trimer composed of three identical subunits (M, 92,000), but it probably does not exist as such in the cell. Ferguson plot analyses of enzyme in cell extracts indicate that prior to purification the enzyme exists in oligomeric forms characterized by both higher charge and greater Mr. Changes in size and charge of oligomers which occur during purification are probably due to the dissociation of proteins and/or nucleic acids. Dissociation of the oligomers is achieved by dilution and electrophoresis, but reassociation does not occur after concentration.

The poly(A) product of the initial polymerization stages migrates as a single band on both nondenaturing and urea-agarose gels. It is 13,000 ± 2,000 nucleotides long, as measured by electron microscopy, and 8,000 nucleotides long by gel electrophoretic analysis. This poly(A) product remains bound to the enzyme after synthesis, yet can be easily obtained free of protein by proteinase K digestion.

Polynucleotide phosphorylase from *Micrococcus luteus* has been isolated in two forms: a primer-independent form in which polymerization activity is stimulated only 1.1- to 2-fold by small oligonucleotides (Form I), and a primer-dependent form generated by partial proteolysis in which polymerization activity is stimulated up to 20-fold by oligonucleotide primer (Form T) (1-5). Because of its key role in the synthesis of model nucleic acids (6, 7), there is a need for a preparative procedure which provides enzyme of defined structure and function, free of contaminating activities. However, in procedures published to date, both the yields of enzyme and degree of proteolysis (and thus primer stimulation) have been variable, primarily due to an extreme sensitivity to proteolytic breakdown. This has kept the structure of *M. luteus* polynucleotide phosphorylase surrounded by uncertainty as exemplified by multiple electrophoretic polypeptide bands corresponding to a size range from 59,000 to 70,000 for the primer-dependent enzyme and 67,000 to 100,000 for the primer-independent form (2, 8). With the present procedure, we are able to reproducibly isolate enzyme in milligram amounts (8 mg/100 gm of freeze-dried cells) with minimal proteolysis as evidenced by gel electrophoresis. This has allowed us to conduct studies on the chemical and physical characterization of intact *M. luteus* polynucleotide phosphorylase and to examine the nature of higher level aggregates of the enzyme apparently present in the cell. The structure of the *M. luteus* enzyme is very similar to that of the enzyme from *Escherichia coli*. However, the poly(A) synthesized by the *M. luteus* enzyme is large (~13,000 nucleotides by electron microscopy) compared to the previously reported values of 200 to "greater than" 700 bases for *M. luteus* poly(A) (4, 9) or 200 bases for the poly(A) synthesized by the *E. coli* enzyme (10).

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Properties of Purified Polynucleotide Phosphorylase**

The purification procedure described in the miniprint section gives polynucleotide phosphorylase with a specific activity of 45 units/mg (see footnote to Table I in the miniprint) in a yield of 18%. The enzyme is primer-independent, showing only a 1.1- to 2-fold stimulation when assayed for polymerization in the presence of (AP)~A. Analysis of the protein by SDS gel electrophoresis had shown (11) that 90% of the protein co-migrated with the σ subunit of RNA polymerase (Mr = 85,000 to 92,000 (40)). The remaining 10% has a slightly lower molecular weight, Mr = 85,000, which has been attributed to proteolysis (41).

Under native conditions, gel electrophoresis of the purified enzyme reveals a single zone of activity composed of a set of multiple bands (see gels 2 and 3, Fig. 3 of Ref. 11). When the propagation rate of the "front" moving boundary was investigated by guest on March 25, 2020http://www.jbc.org/Downloaded from
increased in order to spread the gel pattern across the entire gel length (buffer system 2860.15.VII.) (see "Methods"), and resolution was thereby sharpened, there were two major bands with $M_\text{r} = 220,000$ and $200,000$, respectively. These are "size isomers" (19), i.e., they differ in size rather than net charge, since they share a common y-intercept ($Y_0$) on a Ferguson plot (see below).

This heterogeneity of the purified enzyme is seen in the activity and protein staining patterns in gel electrofocusing (Fig. 1). Both activity (Fig. 1A) and protein patterns (Fig. 1, B to E) show a dominant broad zone reminiscent of the "sets" of enzyme apparent in gel electrophoresis. These zones can extend over 2 pH units, and even at the lowest load (Fig. 1E), the major band extends over an entire pH unit (6-7). These zones can show minor contaminant bands. When isoelectric focusing was done in the presence of 8 M urea (Fig. 1F), the enzyme was resolved into a major band at pH 6.1 and a minor band at pH 6.8. (The ratio of the two components corresponds approximately to that of the two subunits detected by SDS gel electrophoresis (11).)

Physical and Chemical Properties of Polynucleotide Phosphorylase—The molecular weight of the purified enzyme was determined by sedimentation equilibrium as shown in Fig. 2. It was found that the best fit was obtained by assuming two noninteracting components of $M_r$ values of 237,000 $\pm$ 24,000 and 830,000 $\pm$ 200,000, respectively. 92% of component 1 and 8% of component 2 are present. The errors cited are those attributable to fitting and do not take into account possible errors in molecular weight determination due to uncertainties in the value of the partial specific volume.

The molecular weight of the major component suggests that the enzyme in the native conformation is a trimer. This was confirmed by SDS gel electrophoresis on the enzyme cross-linked with dimethyl suberimidate according to Davies and Stark (25). The three components seen upon gel electrophoresis in SDS corresponded to the monomer, dimer, and trimer of the 90,000-dalton subunit (Fig. 3). The trimer was the predominant species when cross-linking of Form I enzyme was done in the absence of primer. When the cross-linking was

**Fig. 1.** Isoelectric focusing of purified polynucleotide phosphorylase (Fraction VII). Gels A to E were subjected to isoelectric focusing under non-denaturing conditions. Gel A (10 $\mu$g) was electrophoresed for 24 h (see "Methods") and stained for activity with ethidium bromide (61). Gels B to E (gel B, 65 $\mu$g; gel C, 32 $\mu$g; gel D, 16 $\mu$g; gel E, 6.5 $\mu$g) were electrophoresed for 6.5 h and stained for protein by the method of Vesterberg (62). Parallel gels without protein were run simultaneously, cut into 1-mm slices, extracted in 0.5 ml of 0.025 M KCl, and the pH of the eluates measured. The pH span of the stained protein was 6 to 8 for gel B and 6 to 7 for the remaining gels. The pH 6 boundary is at the lower end of the staining pattern. Gel F shows the pattern obtained after isoelectric focusing in 8 M urea. Fraction VII (65 $\mu$g) was dialyzed under $N_2$ overnight at room temperature against 8 M urea, 0.1 mM EDTA, and 0.1 mM dithiothreitol. Electrophoresis was for 6 h at 6 °C (see "Methods"). The gel was stained for protein as described above. The pH values corresponding to the major protein bands are shown at the right. Gels are cut at both origin and bottom.

**Fig. 2.** Sedimentation equilibrium of native polynucleotide phosphorylase. Centrifugation was at 8000 rpm for 48 h at 56 °C in the following buffer: 0.02 M Tris-Cl, pH 8.1, 0.1 M NaCl, 0.001 M MgCl₂, and 0.1 mM dithiothreitol. Prior to centrifugation, the samples were dialyzed for 24 h against the same buffer. Upper panel, deviation of the data points from the fitting line; RMS error = 0.036 fringes. Lower panel, concentration distribution as a function of radial position at equilibrium.

**Fig. 3.** SDS gel electrophoretic pattern of polynucleotide phosphorylase after cross-linking with dimethyl suberimidate (25). The protein samples (40 $\mu$g in 40 $\mu$l of 0.2 M triethylamine-HCl buffer (pH 8.5) were mixed with 10 $\mu$l of a 25 mg/ml solution of dimethylsuberimidate in the above buffer. When present (+), (Ap)A was added prior to the reagent to give a final concentration of 0.13 mM. The reaction was allowed to proceed for 30 min at 30 °C, after which it was stopped by addition of 6 $\mu$l of 10% SDS, 1 $\mu$l of β-mercaptoethanol, and 3 min of boiling. The denatured samples were then incubated at 37 °C for 3 h. Samples of 20 $\mu$g were applied to 3.5% polyacrylamide gels and subjected to electrophoresis in SDS-containing buffer as described by Davies and Stark (25). Gels 1 and 2, primer-independent polynucleotide phosphorylase (1); gels 3 and 4, trypsin-treated enzyme (T) after incubation for 10 min in the presence of 0.14 M β-mercaptoethanol (55); gels 5 and 6, trypsin-treated enzyme after incubation for 10 min in the presence of 2 mM N-ethylmaleimide (55). Molecular weight standards are shown on the left.
performed in the presence of primer, which has been shown to interact with the primer-independent enzyme (11), a reduction of the amount of trimeric species was consistently observed together with a concomitant increase in monomeric species.

The enzyme obtained by limited proteolysis with trypsin also showed three components after cross-linking corresponding to the monomer, dimer, and trimer of this polypeptide. In this case, however, the monomeric species was predominant, and the presence of primer during cross-linking did not affect the pattern. Treatment with thiol or N-ethylmaleimide which affects the de novo polymerization properties of the partially proteolyzed enzyme (2), also had no effect on the cross-linking pattern (Fig. 3).

Amino Acid Composition—The amino acid composition of polynucleotide phosphorylase is shown in Table I. With the exception of the tryptophan content, the values agree with those previously published (8). Minor differences are probably due to proteolysis in the original procedure as evidenced by the previously reported heterogeneity of subunit size (8). The tryptophan content of the protein was determined both indirectly as described by Edelhoch (42) and directly by the method of Simpson et al. (29). Both methods gave similarly low values (Table I). The values of tyrosine and tryptophan content are in good agreement with the UV-absorption spectrum of the protein in 6 M guanidine-HCl and that of a mixture of the N-acetyl-tyrosinamide, N-acetyl-tryptophanamide, and N-acetyl-phenylalaninamide in amounts equimolar to that present in the protein (42). With the exception of a small red shift (data not shown), the correspondence of the two spectra between 260 and 300 nm was excellent.

End Group Analysis—In contrast to the E. coli enzyme, neither the primer-independent form of M. luteus polynucleotide phosphorylase nor the primer-dependent form of the enzyme obtained by limited proteolysis with trypsin contain a detectable free amino end group (Table II). This suggests that proteolysis removes a peptide at the carboxyl end of the molecule. It is also another indication of the homogeneity of the product and low level of proteolysis that characterizes this purification procedure.

Spectroscopic Properties—The electrophoretic heterogeneity mentioned above, as opposed to the apparently homogeneous subunit composition, could be explained if oligonucleotides were tightly bound to the enzyme. The UV-absorption spectrum of polynucleotide phosphorylase is shown in Fig. 4. An extinction coefficient of $e_{278}^{1 M} = 4.80$ was calculated when the protein concentration was measured by the method of Lowry et al. (43) using bovine serum albumin as a standard. This value is similar to that previously reported (2). The ratio of absorbancies at 280 and 260 nm of 1.75 indicates a very low content of nucleic acid. Contamination with 1 mol of adenine-nucleotide/mol of protein subunit should increase the absorbance at 260 nm of a 1 mg/ml solution of enzyme by 0.167 and would give a 280/260 ratio of 1.08. The absence of contaminating nucleic acid was confirmed by measurement of the UV-absorption spectrum of the enzyme in the presence of 6 M guanidine hydrochloride before and after gel filtration on Sephadex G-25 to remove small size UV-absorbing material. As shown in Fig. 4, the absorption of the enzyme at 260 nm...
Polycrylamide gel electrophoresis at various gel concentrations was carried out as described under "Methods." Molecular weights were obtained from $K_v$ values, assuming sphericity of unknowns and standards, partial specific volumes of 0.74 and zero hydration, using the following $M_r$ standards: bromphenol blue (670), angiotensin (1,287), bacitracin (1,411), insulin B chain (3,400), glucagon (3,485), cytochrome c (12,400), human growth hormone (21,500), soybean trypsin inhibitor (22,700), ovalbumin (43,500), bovine serum albumin monomer (67,000), transferrin (74,000), bovine serum albumin dimer (134,000), and acetylcholinesterase (240,000).

was not significantly decreased by this treatment, indicating the absence of bound oligonucleotide. The UV-difference spectrum shown in the inset of Fig. 4 suggests that 6 tyrosyl residues and maybe 1 tryptophanyl residue are buried in the interior of the protein and become exposed upon treatment with guanidine (42). The remaining tyrosyl and tryptophanyl residues are exposed and can be detected by treatment with cetyltrimethylammonium bromide (44) (data not shown).

### Oligomeric Forms of Polynucleotide Phosphorylase in Less Purified Fractions

In contrast to the purified enzyme which is greater than 90% of one size class, enzyme in crude extracts exhibited a high degree of electrophoretic heterogeneity. The multiple components were investigated by combining gel electrophoresis under non-denaturing conditions with a specific in situ assay for enzymatic activity (see "Methods"). Coupling this assay method with Ferguson plot analyses has enabled us to determine the number of active components present and to obtain estimates of their size and shape as a function of the degree of purification.

Three fractions of widely different degrees of purity were analyzed by this method: the crude extract (Fraction I), the Sepharose 6B fraction, and the hydroxylapatite fraction (Fraction VII) as described in the miniprint section. Table III

| Fraction Set | Individual band | Set average |
|--------------|-----------------|-------------|
| Crude extract | 4.6 0.19 360,000 320,000 |             |
| II           | 4.4 0.22 420,000 420,000 |             |
| Sepharose 6B  | 3.4 0.17 270,000 220,000 |             |
| II           | 3.1 0.15 210,000 220,000 |             |
| III          | 2.8 0.14 180,000 180,000 |             |
| Hydroxylapatite | 4.6 0.25 530,000 510,000 |             |

- Activity stain, 0.05 unit/gel.
- Activity stain, 0.03 unit/gel.
- Protein stain, 10 µg/gel.

### Ferguson plot analysis of fractions of polynucleotide phosphorylase

Polynucleotide phosphorylase activity was assayed by a method described elsewhere (27). Molecular weights of partial fractions were estimated by sedimentation equilibrium analysis. The method relies on the assumption that the protein so that in spite of its being derived from an early stage in the purification procedure, it had only two minor contaminants visible on SDS gels (data not shown).

![Fig. 5. Electrophoretic properties of partially purified polynucleotide phosphorylase.](http://www.jbc.org/Downloadedfrom)
summarizes the data obtained from Ferguson plot analyses. The enzyme species are clustered into a maximum of three sets (11) (see Fig. 5, top and Fig. 3 of Ref. 11). Set I is present at all the purification stages of the enzyme, while Sets II and III are detected only on the gel patterns of the crude extract or the semipurified Sepharose 6B fraction (Table III). Ferguson plot analysis also shows that the sets of enzyme detected at a particular purification stage are size isomers, ("K<sub>R</sub>-Y<sub>0</sub> ellipses") are horizontally displaced against one another (Fig. 6, lower panel). Furthermore, Set I varies in size and charge at different stages of purification (Fig. 6, upper panel) (Table III). Set I in the crude extract is the largest and the most highly charged. Set I of the purified species is the smallest. The Sepharose 6B fraction (selected for its large M<sub>r</sub>) is more heterogeneous. It contains not only a Set I similar in size and charge to that of purified enzyme, but a Set II present in crude extract and an additional set of larger M<sub>r</sub> (Set III). Considering the assumptions in the calculation of the M<sub>r</sub> values (23), the molecular size of Set I measured by Ferguson plot analysis (210,000) and that measured by sedimentation equilibrium (237,000) are in good agreement.

Partial resolution of these sets could be obtained by centrifugation in a 0 to 20% neutral sucrose gradient. Although the enzyme activity appeared as a single peak, aliquots across this peak analyzed by gel electrophoresis showed that the relative amount of each set varied as a function of the sedimentation rate. Slowly sedimenting fractions were essentially composed of Set I, while rapidly sedimenting fractions were enriched in Sets II and III (data not shown). The enzyme thus appears to exist as a mixture of multimers which can be partially resolved by sedimentation and are, therefore, not in rapid equilibrium.

In order to study further the multimeric structure of the enzyme, the Sepharose 6B fraction was subjected to electrophoresis under nondenaturing conditions. The gel was sliced, the enzyme eluted, and the fractions assayed for activity. Phosphorolysis and polymerization assays of sequential gel fractions gave three peaks (Sets I to III) with <i>R</i><sub>F</sub> values identical to those seen when the whole gel was assayed directly by staining (Fig. 5, upper panel). When aliquots of fractions corresponding to Set I were then reelectrophoresed, the patterns generated were similar to those of the parent set and had bands with <i>R</i><sub>F</sub> values which corresponded precisely to those of Set I in the parental species (Fig. 5, lower panel). On the other hand, aliquots from Set II, when reelectrophoresed, exhibited both Set I and Set II activity bands, and again in the parental ratio of 65:35 (Set I:Set II) as determined by densiometer analysis. The <i>R</i><sub>F</sub> values were identical in each case to those of parental enzyme, although there was some enrichment in trailing or leading species as a function of the origin of the slice. Attempts to reverse the process and to generate Set II from Set I using purified enzyme were unsuc-

![Fig. 5](https://example.com/fig5.png)

**Fig. 5.** Joint 95% confidence limits of <i>K</i><sub>R</sub> (descriptive of molecular size) and <i>Y</i><sub>0</sub> (descriptive of free electrophoretic mobility) for the components of polynucleotide phosphorylase. Upper panel, the 95% confidence envelopes of the components of Set I at three stages of purification. Ellipse overlap indicates significant identity and non-overlap significant distinction between species. Horizontal displacement of ellipses signifies differences preponderantly in molecular size ("size isomerization"); vertical displacement signifies preponderantly charge differences (63). Lower panel, non-identity and size isomerization of Sets I, II, and III of the Sepharose 6B fraction. These data were derived from Ferguson plots of the various enzyme species using a set of computer programs (The "Page-Pack") obtainable from Biomedical Computing Technology Information Center, Vanderbilt Medical Center, Nashville, Tennessee 37232.

![Fig. 7](https://example.com/fig7.png)

**Fig. 7.** Radioactivity profiles of commercial and synthesized [14C]poly(A) on 5% acrylamide gels. Gels were cut into 2-mm slices, solubilized, and counted. Left, [14C]poly(A) synthesized by polynucleotide phosphorylase during a 2-min incubation at 37 °C was subjected to electrophoresis as described under "Methods" (solid line). [14C] commercial poly(A) (0.2 μCi) was electrophoresed on a separate 5% gel (dashed line). Right: A, 15 μg of commercial poly(A) electrophoresed on a 5% gel; B, a polynucleotide phosphorylase reaction mixture incubated 60 min at 0 °C before gel electrophoresis (see "Methods"). After electrophoresis, both gels were incubated in the presence of ADP and Mg<sup>2+</sup> and stained with acridine orange. The diagrams to the right of each gel outline the staining pattern.
cessful, even when the enzyme was concentrated up to 8.4 mg/ml prior to electrophoresis.

The Poly(A) Product of Polynucleotide Phosphorylase

When poly(A) is synthesized from ADP by polynucleotide phosphorylase under the conditions described under "Methods," and the reaction mixture immediately subjected to gel electrophoresis under non-denaturing conditions, two sharp acridine orange staining bands are seen in the separating gel (Fig. 7, gel B). This gel was stained for polynucleotide after incubating it with ADP. Thus, any poly(A) formed prior to electrophoresis will be stained, as well as any enzyme component on the gel that can generate poly(A) during the incubation of the gel after electrophoresis. The two bands formed are characterized by very reproducible $R_F$ values (Table IV), with a coefficient of variation of 5%. The relative amounts of each band vary with the temperature and duration of incubation. Table IV, (column a) shows that Band 1 is predominant at early times (2 to 5 min), especially when the incubation is done at 0 °C (data not shown). However, after 7 min at 37 °C or 60 min at 0 °C, Band 2 predominates. If the gels are stained only for poly(A) synthesized prior to electrophoresis by omitting the ADP prior to staining, then Band 2 predominates at 2 min and by 20 min is essentially the only species present (Table IV, column b).

These sharply defined species are contrasted with the heterogeneous electrophoretic pattern of commercial poly(A). Both the staining pattern and the distribution of radioactivity of the commercial preparation show a high degree of polydispersity (Fig. 7, gel A). In contrast, poly(A) or [14C]poly(A) synthesized by the purified enzyme, as described in this study, contain only 1 to 2 components, both when examined by staining or by radioactivity measurement (Fig. 7).

To get a clearer idea of the composition of the acridine orange-stained components observed on polyacrylamide gels, we compared gel patterns of reaction mixtures with and without proteinase K treatment before electrophoresis. The gels, for convenience, have been aligned at the origin which, because of the differences in staining procedure, has caused an apparent lack of correspondence between protein and activity patterns (Fig. 8, gels A and B); the $R_F$ values are identical, however (11). At zero time, in the absence of proteinase K treatment, most of the enzyme is observed at the position of free enzyme (Fig. 8, gels A and B). However, some poly(A) is already detectable at the top of the stacking gel, indicating that a small amount of polymerization had already occurred within the few seconds required for pipetting, even at 0 °C (gel B). However, after 2 min at 37 °C in a gel stained for protein (Fig. 8, gel C), almost all the enzyme is at the top, unable to enter the stacking gel. A gel incubated for activity (gel D) shows that, in addition, there are two acridine orange staining bands in the resolving gel. A gel incubated to detect only poly(A) synthesized before electrophoresis (gel E) shows the usual two bands in the resolving gel with a marked predominance of Band 2. When the sample was treated with proteinase K prior to electrophoresis (gel F), only one of the three components seen on gel D remains, and that is Band 2 as indicated by its $R_F$ value. Thus, Band 2 is the free poly(A) synthesized by polynucleotide phosphorylase. The material which does not enter the stacking gel and Band 1 presumably is different forms of the poly(A)-enzyme complex. The fact that no new bands of poly(A) are released from the enzyme-poly (A) complex indicates that essentially only one size class of poly(A) exists.

Proteinase K treatment confers stability to the poly(A) product by eliminating the polynucleotide phosphorylase-catalyzed "transnucleotidation" reactions, which can result in a variety of sizes of poly(A) (45, 46). These reactions cause a breakdown of the single poly(A) component after storage and freeze-thawing of untreated reaction mixtures, to give a pattern somewhat similar to that of the commercial poly(A) seen in Fig. 7A. The proteinase K-treated sample, however, showed no evidence of breakdown after similar treatment (data not shown).

Size Determination of Poly(A) Synthesized de Nova—Proteinase K-treated and untreated aliquots of a polymerization mixture were sedimented on isosinokinetic sucrose gradients (47). The proteinase K-treated material moved on the gradient coincident with the 18 S marker RNA while the untreated polynucleotide phosphorylase-poly(A) mixture sedimented with a value of 23 S (data not shown). When examined on a 5 M urea-0.8% agarose gel, proteinase K-treated poly(A) migrated coincidentally with 28 S tRNA precursor which is 8,000 bases long (48). Proteinase K-treated poly(A), prepared for electron microscopy by the cytochrome-formamide Kleinschmidt technique (49), gave a mean value of 13,000 ± 2,000 nucleotides, using φX174 as an internal standard (Fig. 9).

![Fig. 8. Formation of poly(A)-enzyme and free poly(A) by M. luteus polynucleotide phosphorylase.](http://www.jbc.org/)

| Table IV |
| --- |

**Formation of poly(A)-enzyme (band 1) and poly(A) (band 2) as a function of duration of incubation.**

| Time (min) | Band 1 | Band 2 |
| --- | --- | --- |
| 2 | 1" | 1" |
| 7 | 1 | 2 |
| 20 | 1 | ND* |

* ND, not done.

**Notes:** $R_F = 0.073 ± 0.0045$ (11); band 2, $R_F = 0.090 ± 0.0048$ (20). $M_a$ values are the mean ± S.D. for the number of gels given in parenthesis.
FIG. 9. Electron micrograph of poly(A) synthesized by M. luteus polynucleotide phosphorylase. The molecules were spread and photographed (49) with φX174 as size marker.

DISCUSSION

The purification procedure described here provides significant improvements in yield and integrity of structure of polynucleotide phosphorylase from M. luteus compared with previously published methods. The overall procedure, as well as individual steps, have been repeated several times and are very reproducible in terms of yield (~20%), pattern on gel electrophoresis, primer-dependence (1.1- to 2-fold), and specific activity (50 phosphorolysis units/mg).

The subunit of the primer-independent enzyme is similar in size to the σ subunit of RNA polymerase when they are electrophoresed together on SDS gels (11). Similar observations have also been made concerning the subunit of polynucleotide phosphorylase from E. coli (50, 51). Although the $M_r$ of the σ subunit has not been definitively established (40, 52, 53), the best fit for the $M_r$ of polynucleotide phosphorylase using other $M_r$ markers was 92,000, in agreement with the most recent value for the σ subunit (40).

The cross-linking experiments indicate clearly that the enzyme is trimeric, as has been shown to be the case for the E. coli enzyme (54). The cross-linked trimer of the independent forms of both enzymes is easily obtained, whereas the primer-dependent form (Form T) of M. luteus polynucleotide phosphorylase is more resistant to cross-linking. Although treating this latter form with a sulfhydryl reagent restores its primer independence in terms of its enzyme activity (2, 55), it does not change the cross-linking pattern. This indicates that the groups which react with dimethylsuberimidate are either eliminated during the trypsin treatment, or that there has been a change in subunit orientation. Treatment of the primer-independent form with (Ap)$_3$A changes the structure so that fewer cross-linked trimers are formed and more monomer remains. Previously, a relatively large conformational change induced by oligonucleotide binding was deduced from the dramatic change in gel electrophoretic pattern of primer-independent enzyme after incubation with oligonucleotides (11). On the other hand, no changes in gel patterns were seen with Form T enzyme when it was incubated with oligonucleotide, in agreement with the lack of effect of oligonucleotides on the cross-linking patterns.

This trimeric structure does not appear to be the only form of the enzyme in crude extracts since at early stages of purification, significant amounts (30 to 40%) of higher molecular weight components (Sets II and III) were always observed as indicated by Ferguson plots and sucrose gradient centrifugation. The cause of this association is not yet clear. Since we were unable to generate these forms with more purified preparations, it is possible that an oligonucleotide or an unidentified protein is required.

The difference in Set I $M_r$ between the more purified fractions (220,000 and 210,000) and the crude filtrate (320,000) is especially interesting in view of the report that the β form of the E. coli enzyme has a subunit structure αβ, and a $M_r$ of 365,000 (56). It is possible that a similar heteropolymer exists in M. luteus, but that the β subunit is lost during purification. It is also possible that oligo or polynucleotides are bound to the M. luteus enzyme in the crude extract and are contributing to the size, since (Ap)$_4$A changes the gel pattern of the purified enzyme back to that found in the least purified fractions (11). The αβ form of the E. coli enzyme has a 280/260 ratio of 1.0, indicating that it does contain bound nucleic acid (56).

The amino acid composition of the E. coli and M. luteus enzymes are similar. Although in one report the cysteine content was 2 to 4 times higher for the E. coli enzyme (12

5 E. K. Barbehenn and C. B. Klee, unpublished observations.
which accompanies early stages of polymerization. They suggested that poly(A) chains attached to one enzyme molecule might subsequently become attached to other enzyme molecules to yield a three-dimensional, cross-linked network.

Several observations from their work and that of others support the idea of such a matrix: 1) no oligonucleotides are released by its isolation on electrophoresis and its adsorption to Millipore filters (1, 10, 59).

It is difficult to obtain a precise size for the poly(A) synthesized by M. luteus polynucleotide phosphorylase. Although 5 M urea-0.8% agarose gels yield a value of 8,000 bases, electron microscopy shows a distribution with a mean of 13,000 ± 2,000 bases. Since the urea gels may not be completely denaturing for poly(A), it seems likely that the electron microscopy may be closer to the true size. A high weight average degree of polymerization was predicted by Feller and Barnett (60) to be an intermediate stage in the unprimed synthesis of poly(A) as a result of the more favorable kinetics for adding a residue in contrast to reinitiating another polymer chain. However, it remains to be found by what mechanism polynucleotide phosphorylase is able to terminate synthesis in a pseudo-synchronous fashion yielding a very homogenous polynucleotide.

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Polynucleotide phosphorylase and its Poly(A) Product

**Experimental Materials**

- Material, grade of manufacture:
  - Polynucleotide phosphorylase and its Poly(A) product.

- General information:
  - The procedure was described in the distinctive section of the protocol used to prepare the Poly(A) product.
  - The poly(A) product was obtained by electrophoresis on a 5% polyacrylamide gel.

- Procedures:
  - The poly(A) product was purified by gel electrophoresis and subsequently analyzed by amino acid analysis.
  - The analysis was performed on a Waters 660 Amino Acid Analyzer.

- Results:
  - The poly(A) product was found to have an amino acid composition of 0.25 mol/mol.

**Discussion**

- The results indicate that the poly(A) product is a highly purified form of polynucleotide phosphorylase.

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1. **Note:** The citation is incomplete and requires further context to be fully understood. The research was conducted by the National Institutes of Health, Bethesda, MD 20010. The full reference is likely needed for further investigation.
Characterization of polynucleotide phosphorylase from Micrococcus luteus and isolation of the 13,000 base poly(A) product of the polymerization reaction.
E K Barbehenn, J E Craine, A Chrambach and C B Klee

J. Biol. Chem. 1982, 257:1007-1016.

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