UNPRIMED INTERDEPENDENT POLYMERIZATION OF ITP AND CTP BY RNA POLYMERASE OF PSEUDOMONAS PUTIDA

Thesis for the Degree of M. S.

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KATHLEEN M. ROSE

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ABSTRACT

UNPRIMED INTERDEPENDENT POLYMERIZATION OF ITP AND CTP BY RNA POLYMERASE OF PSEUDOMONAS PUTIDA

By

Kathleen M. Rose

After a lag, RNA polymerase of Pseudomonas putida catalyzed the unprimed interdependent polymerization of ITP and CTP to form TCA insoluble material which was primarily homopolymers. After fractionation of the enzyme into two components (PC I and PC II) by chromatography on cellulose phosphate, each component was able to catalyze the synthesis of homopolymers from ITP and CTP. However, the length of the lag for the reaction catalyzed by PC I was at least 2.5 times greater than the length of the lag for the reaction catalyzed by PC II. The steady state rate of polymer synthesis was proportional to $[E]^n$, where n equaled 2 for the reaction catalyzed by PC II and n approached 3 for the reaction catalyzed by PC I. PC I and PC II also catalyzed the unprimed interdependent synthesis of homopolymers from ATP and UTP. Again, the length of the lag for the reaction catalyzed by PC I was

longer (2 times) than the corresponding lag for the reaction catalyzed by PC II.

Electrophoresis of PC I and PC II on polyacrylamide gels (Tris-glycine, pH 9.0) each showed two major protein bands, both of which were active in the unprimed reactions catalyzed by RNA polymerase. Electrophoresis of the polypeptide chains derived from PC I and PC II (SDS-sodium phosphate, pH 7.1) indicated PC I had five major polypeptide chains, one of which was a contaminant, and PC II had three polypeptide chains.

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By

Kathleen M. Rose

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INTRODUCTION

RNA polymerase (nucleoside triphosphate: RNA nucleotidyltransferase, EC 2.7.7.6) can catalyze two types of reactions. One type is the template directed synthesis of polyribonucleotides. RNA polymerase catalyzes the synthesis of polyribonucleotides with a base sequence complementary to the template (1-5). The other type of reaction, which is termed unprimed, is the synthesis of ribopolymers in the absence of added template.

RNA polymerase catalyzes two unprimed reactions. One is the interdependent polymerization of ATP and UTP to form homopolymers by RNA polymerase from Escherichia coli, Azotobacter vinelandii and Pseudomonas putida (6-9). The other reaction involves the unprimed interdependent polymerization of ITP and CTP to form polymers in which inosine and cytidine are in alternating sequence. RNA polymerase from E. coli and A. vinelandii has been reported to catalyze this reaction (10).

The unprimed reactions catalyzed by RNA polymerase have been shown to have certain requirements (6-10). Synthesis of TCA insoluble material from ATP and UTP or CTP and ITP occurs only

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1. In the presence of RNA polymerase.
2. In the presence of Mn^{+2}. Mg^{+2} will not substitute as the divalent ion.
3. If both nucleoside triphosphates are present (the exception here is a small amount of ATP incorporation by the P. putida enzyme in the absence of UTP).

The Watson-Crick analogue of ITP, GTP, will not substitute for it in the reaction utilizing ITP and CTP. Synthesis of product in both unprimed reactions occurs only after a lag. The rate of formation of product in the reaction involving ATP and UTP (P. putida enzyme) is related to the square of the enzyme concentration (9).

Polyacrylamide gel electrophoresis of RNA polymerase from E. coli and A. vinelandii has been performed (11, 12). Depending on the structural form, RNA polymerase migrates as a single band (E. coli) or as multiple bands. The A. vinelandii enzyme migrates as two bands, the major band being the more slowly moving component. An in situ assay for RNA polymerase activity in the unprimed reactions has been developed by Krakow (12). In this assay, ribopolymers synthesized by the enzyme which had migrated into the gel during electrophoresis are stained with ethidium bromide. In both unprimed reactions with A. vinelandii RNA polymerase polymers were synthesized by both the major and minor protein bands.

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Polyacrylamide gel electrophoresis of E. coli RNA polymerase in 8 M urea or sodium dodecyl sulfate (SDS) and β-mercaptoethanol has been performed (11, 13). Depending upon the manner of purification various patterns are seen. In its most complete structural form ($\alpha_2\beta\beta'\sigma$). four polypeptides are observed in the SDS gels with molecular weights estimated at 165,000 (β), 155,000 (β'), 95,000 (σ) and 40,000 (α) (14).

RNA polymerase from P. putida has been fractionated into two components by chromatography on cellulose phosphate (J. C. Johnson, private communication). This report describes (1) the unprimed interdependent polymerization of ITP and CTP by each of these components, (2) the unprimed interdependent polymerization of ATP and UTP, (3) polyacrylamide gel electrophoresis (Tris-glycine, pH 9.0) with analysis of enzymatic activity using the in situ assay, and (4) SDS-polyacrylamide gel electrophoresis of the polypeptide chains derived from P. putida RNA polymerase.

MATERIALS AND METHODS

Purification of RNA Polymerase

RNA polymerase was purified by the method of J. C. Johnson and J. A. Boezi (private communication). The purified enzyme was eluted from a cellulose phosphate column as a single peak or as two peaks depending upon the elution procedure. When the material on the column was eluted with a linear gradient of two column volumes from 0.0 to 0.6 M KCl, RNA polymerase was eluted in a single peak, referred to in RESULTS simply as RNA polymerase. However, when a linear gradient of six column volumes from 0.0 to 0.4 M KCl was used, the enzyme was eluted from the column in two peaks, designated PC I (phosphocellulose I) RNA polymerase and PC II RNA polymerase. The enzyme isolated as a single peak was used in only two experiments, that testing the effect of KCl concentration and in the nearest neighbor analysis. PC II RNA polymerase contained twice as much protein as PC I RNA polymerase. PC II RNA polymerase was isolated as an essentially pure protein. PC I RNA polymerase was estimated to be 50-75% pure, containing, in addition to the enzyme, a single protein component (presumably a contaminant) which was inactive in RNA synthesis.

Although the overall specific activity of PC I in the gh-1 DNA-directed synthesis of RNA was the same as that of PC II, the specific activity of the enzymatically active component in PC I was twice that of PC II.

Radioactive Assay for the Incorporation of CMP and/or IMP from H3-CTP and/or ^{14}C-ITP Into Polymers by RNA Polymerase

This assay measured the incorporation of ^{3}H-CTP and/or ^{14}C-ITP into material which was insoluble in trichloroacetic acid (TCA). The standard reaction mixture contained 0.02 M Tris-HOAc, pH 8.1, 2.0 mM $Mn(OAc)_2$, 0.1 M KCl, 0.44 mM ^{3}H-CTP, 0.44 mM ITP (nonradioactive or ^{14}C-labeled) and 0-250 µg RNA polymerase/ml. Any enzyme dilutions which were necessary were made in buffer containing 0.05 M Tris-HOAc, pH 8.1, 1 mM $Mn(OAc)_2$ and 0.01 M dithiothreitol. The reaction was started by the addition of RNA polymerase and incubated for up to five hours at 30°C. During the incubation samples were withdrawn and five ml of cold 10% TCA were added to each sample to stop the synthesis of polymer. Approximately 200 µg of carrier DNA (salmon sperm) were then added. The sample was allowed to remain at 0-4°C for 15 minutes and then the precipitate was collected by filtering on a nitrocellulose membrane. Each sample tube and filter was rinsed with three 5 ml portions of 10% TCA. The filter was allowed to air dry several minutes, then placed in a scintillation

vial and dried at 90°C. Five ml of a solution containing 4 g BBOT per liter of toluene were added to each sample. The sample was then counted in a Packard TriCarb liquid scintillation spectrometer with gain and window settings as indicated: ^{3}H alone, gain 65%, window discriminator 50-1000; ^{3}H and ^{14}C in same sample, ^{3}H channel, gain 65%, window 50-300, ^{14}C channel, gain 15%, window discriminator 250-1000. The overlap of ^{14}C into the ^{3}H channel was 17%. No overlap of ^{3}H into the ^{14}C channel was observed.

Radioactive Assay for Polymer Formation from ^{3}H-ATP and UTP by RNA Polymerase

The standard reaction mixture for polymer formation from ATP and UTP was the same as that for polymerization of CTP and ITP except that 0.44 mM ^{3}H-ATP and 0.44 mM UTP were used as the nucleoside triphosphates. Incubation and analysis were carried out as described previously.

Nearest Neighbor Analysis

The reaction mixture for the nearest neighbor analysis contained 0.02 M Tris-HOAc, pH 8.1, 2 mM $Mn(OAc)_2$, 0.1 M KCl, 0.44 mM CTP, 0.44 mM ITP, 5 μg α-^{32}P-CTP/ml and 100 μg RNA polymerase/ml. Nearest neighbor analyses were also performed for polymers substituting 0.44 mM ATP and 0.44 mM UTP and using α-^{32}P-ATP in the above reaction mixture. The reaction mixtures were incubated six hours at 30°C. The turbid solutions were precipitated

with five ml of 10% TCA and filtered through Whatman GF/C glass fiber filters using a Millipore filtering apparatus. The tube and filter were then washed with an additional 15 ml of 10% TCA. After several minutes of air drying, the filters were placed into vials. Three ml of 0.3 N KOH were added to the vials containing the filters. The hydrolysis mixtures were incubated 15 hours at 30°C. After removing the filters, the solutions were centrifuged 10 minutes at 10,000 x g. The supernatant solutions were decanted and the pH of these solutions adjusted to 7 with Dowex 50 in the H^+ form. The Dowex was filtered off and the solutions were frozen and lyophilized to dryness. The residue was dissolved in 0.5 ml of water.

The samples were analyzed via descending paper chromatography on Whatman, No. 41, paper using isobutyric acid: ammonium hydroxide: water, 66:1:33, v/v/v, as the solvent. Ten µg samples of the standards, 5' IMP and 2'(3') CMP or 2'(3') AMP and 2'(3') UMP were chromatographed separately to identify the products. Fifteen µl of the hydrolysates were also placed on the spots as markers which, after development of the chromatogram, could be located with the use of an ultraviolet lamp. After preliminary location of the radioactivity with a Packard radiochromatogram scanner, the paper strips were cut into 1.0 x 1.3 cm rectangles and placed in vials.

Five ml of BBOT-toluene (4 g/l) were added as fluor. The samples were counted in a Packard TriCarb liquid scintillation spectrometer (gain 4%, window 50-1000).

Disc Electrophoresis on Polyacrylamide Gels

Two types of electrophoresis buffers were employed using the polyacrylamide gel system. The first utilized Tris-glycine buffer, pH 9.0. The second involved the use of the sodium dodecyl sulfate (SDS)-sodium phosphate buffer, pH 7.1, system of Shapiro (15). In both cases the acrylamide gels were 5% cross-linked.

The solutions used for the Tris-glycine system as adapted from B. J. Davis (16) were

A. 1 N HCl 48 ml
   Tris 36.3 g
   Tetramethylethylenediamine 0.23 ml
   H_2O to 100 ml. The pH of the solution was adjusted to 9.0 with 1 N HCl.

C. Acrylamide 20 g
   Bis-acrylamide 0.735 g
   H_2O to 100 ml.

G. Ammonium persulfate 0.14 g
   H_2O to 100 ml.

The solutions were combined, 1A:2C:1H_2O:4G, and immediately 1.2 ml were poured into 4 mm x 70 mm glass tubes. Care was taken to avoid air bubbles. The tubes

were then filled with water and allowed to polymerize 40 minutes at 26°C. After polymerization the gels were allowed to equilibrate at 0-4°C for 30 minutes. All subsequent procedures were performed at 0-4°C. A standard analytical disc electrophoresis apparatus such as described by Davis (16) was employed. The upper and lower buffers were 0.025 M Tris-HCl, 0.2 M glycine, pH 9.0. The upper buffer contained 5 x 10^{-5}% bromophenol blue as the tracking dye. Protein was applied to each tube by use of a syringe; 20 µg samples of protein, containing 5-10% glycerol, were layered on the gels. The running time for the gels was 110 minutes at 2.5 mamps/gel. Protein migrated toward the cathode.

Two staining techniques were applied; one, utilizing Coomaasie Brilliant Blue, to stain for protein, the other utilizing ethidium bromide to stain for polynucleotides formed by RNA polymerase, <u>in situ</u>, after the appropriate reaction.

For the protein stain, the gels, after removal from the electrophoresis tubes, were fixed for 20 minutes in 7.5% HOAc made 5% in methanol (MeOH) and then stained for at least two hours in MeOH:HOAc:H_2O, 5/1/5, v/v/v, made 0.25% in Coomaasie Brilliant Blue. Gels were either destained electrophoretically with 7.5% HOAc, 5% in MeOH, or by equilibration for several days with 5-10 volume changes of the same solution.

To test for RNA polymerase activity in the gels, after completion of electrophoresis and removal from the glass tubes, the gels were rinsed for ten minutes in 0.01 M Tris-HCl, pH 7.8, 0.05 M NaCl, 0.001 M EDTA (TNE) and then immersed in 5 ml reaction mixtures containing 2 mM ITP (or ATP), 2 mM CTP (or UTP), 2 mM $Mn(OAc)_2$, 0.04 M Tris-HOAc, pH 8.1, and 0.015 M β-mercaptoethanol. Reactions were allowed to incubate at 30°C for 20-24 hours. The gels were rinsed again in TNE and then stained for at least six hours in ethidium bromide, 100 μg/ml in TNE. Excess dye was removed by equilibration in TNE overnight. The presence of fluorescent orange bands indicated the formation of ribopolymer-ethidium bromide complexes.

In the SDS-sodium phosphate, pH 7.1, gel system, solution A was replaced by A-SDS which contained 0.8 M sodium phosphate, pH 7.1, 0.8% SDS, 0.23 ml tetramethylethylenediamine and H_2O to a final volume of 100 ml. Gels were poured as before, except that all procedures for the SDS-sodium phosphate, pH 7.1, gels were performed at room temperature. The electrophoresis buffer contained 0.1 M sodium phosphate, pH 7.1, made 0.1% in SDS. Before layering standard proteins on the gels, the protein solutions were denatured for three hours at 37°C in 0.1 M sodium phosphate, pH 7.1, 1% SDS and 1% β-mercaptoethanol and then dialyzed overnight against

0.01 M sodium phosphate, pH 7.1, containing 0.1% SDS and 0.1% β-mercaptoethanol. Protein solutions were then made 2-4% in sucrose to facilitate layering on the gel. Since RNA polymerase had been dialyzed as the last step in the purification procedure, dialysis treatment was not performed, but protein was applied directly to the gels after the denaturing treatment. In all cases 20 µg of protein was applied to the gel. Electrophoresis was carried out at 7.5 mamp/gel for two hours. Protein migrated toward the cathode. Fixing and staining the gels were conducted as described previously.

In both electrophoresis systems, pre-electrophoresis of the gels for 30 minutes at 10 mamps/tube to remove the ammonium persulfate did not alter the results.

General Methods and Materials

Salmon sperm DNA, type III, used as carrier in the radioactive assay was purchased from Sigma Chemical Company. Bact-T-Flex nitrocellulose membrane filters, type B-6, were obtained from Carl Schleicher and Schuell Company. The unlabeled nucleoside triphosphates were from P. L. Laboratories, Inc.

All radioactively labeled nucleoside triphosphates were purchased from Schwarz Bioresearch, Inc. ^{3}H-CTP had a specific activity of 1.3 c/mmole, 500 µc/ml. ^{14}C-ITP had a specific activity of 24.4 mc/mmole, 10 µc/ml. Before use the labeled triphosphates were

lyophilized to dryness in order to remove all EtOH and H_2O was added back to restore the original volume. Radioactive mixes of ^{14}C-ITP or ^{3}H-CTP were made by addition of the appropriate unlabeled nucleoside triphosphate so the final specific activity of the solution was approximately 1000 cpm/nmole. Appropriate volumes of the mixes were then added to the reaction mixture to give the desired molarity. α-^{32}P-CTP and α-^{32}P-ATP were used in the nearest neighbor analyses as water solutions after lyophilization but without dilution with the unlabeled nucleoside triphosphates.

The acrylamide and bis-acrylamide used in gel electrophoresis were obtained from Canalco and were recrystallized in acetone as described by U. E. Loening (17). Coomaasie Brilliant Blue was purchased from Colab Laboratories, Inc. Bovine hemoglobin, rabbit muscle aldolase and yeast pyruvate kinase were gifts of Dr. Clarence Suelter. Bovine serum albumin was purchased from Armour Pharmaceutical Company. Egg white lysozyme was obtained from Sigma Chemical Company.

RESULTS

Characteristics of the Interdependent Polymerization of ITP and CTP by RNA Polymerase in the Absence of Added Template

Requirements for the Reaction

RNA polymerase catalyzed the interdependent polymerization of ITP and CTP into TCA insoluble material in the absence of added template. In addition to the enzyme, polymerization required the presence of both nucleotide triphosphates and Mn^{+2}. Mg^{+2} did not substitute as the divalent ion. GTP would not substitute for its analogue, ITP. These results are summarized in Table 1 for PC I RNA polymerase and for PC II RNA polymerase.

The Time Course for the Incorporation of ^{3}H-CMP and ^{14}C-IMP Into Polymers by RNA Polymerase and the Effect of KCl

The kinetics of synthesis of polymers from CTP and ITP by RNA polymerase are presented in Figure 1. Detectable synthesis of polymer occurred after a lag and then proceeded at a steady state rate. The difference between the upper and lower sets of curves reflects an effect of the KCl concentration. KCl shortened the lag

TABLE 1.--Requirements for the incorporation of ^{3}H-CMP and ^{14}C-IMP into TCA insoluble material.

	PC I RNA Polymerase		PC II RNA Polymerase	
	nmoles ^{3}H-CMP/ml	nmoles ^{14}C-IMP/ml	nmoles ^{3}H-CMP/ml	nmoles ^{14}C-IMP/ml
Complete system	30	31	77	94
RNA polymerase omitted	0.01	0.01	0.01	0.01
CTP omitted	-	0.01	-	0.02
ITP omitted	0.01	-	0.01	-
ITP omitted, GTP added	0.01	-	0.01	-
$Mn(OAc)_2$ omitted	0.01	0.01	0.01	0.01
$Mn(OAc)_2$ omitted, $Mg(OAc)_2$ added	0.01	0.01	0.01	0.01

The standard reaction mixtures were as described in MATERIALS AND METHODS. The enzyme concentration was 100 µg/ml. GTP was added to a final concentration of 0.44 mM. $Mg(OAc)_2$ was 2 mM. Results are expressed as nmoles nucleoside monophosphate incorporated/ml after three hours of incubation at 30°C.

Figure 1.--The time course of the incorporation of ^{3}H-CMP and ^{14}C-IMP by RNA polymerase. The reaction mixtures were as described in MATERIALS AND METHODS. The enzyme concentration in the reaction mixtures was 100 µg/ml. O—O ^{3}H-CMP incorporation with the standard reaction mixture (0.1 M in KCl); Δ---Δ ^{14}C-IMP incorporation also in the presence of 0.1 M KCl; ●—● ^{3}H-CMP and ▲---▲ ^{14}C-IMP incorporation when KCl was omitted from the reaction mixture.

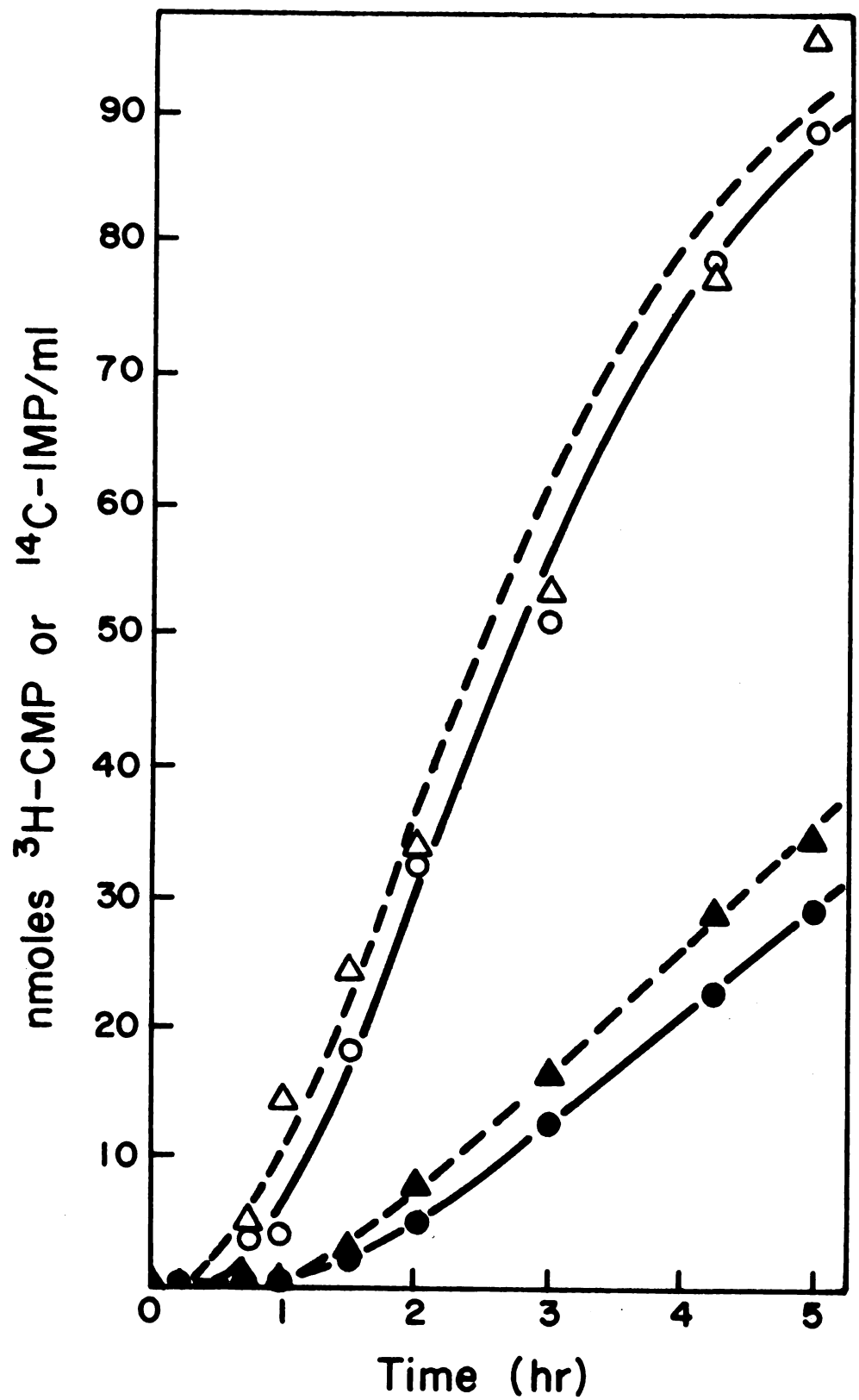

nmoles ^{3}H-CMP or ^{14}C-IMP/ml
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
Time (hr)

Figure 1

and increased the steady state rate. (The rate was calculated from the straight line portion of the ascending curve and expressed as nmoles nucleoside monophosphate (NMP)/ml/hr. The length of the lag was estimated by extrapolating the rate curve to the abscissa.) The kinetics of incorporation of ^{3}H-CMP relative to ^{14}C-IMP in each reaction were similar. The rates and lengths of lags calculated from Figure 1 are presented in Table 2.

TABLE 2.--Effect of 0.1 M KCl upon the kinetics of the incorporation of ^{3}H-CMP and ^{14}C-IMP by RNA polymerase.

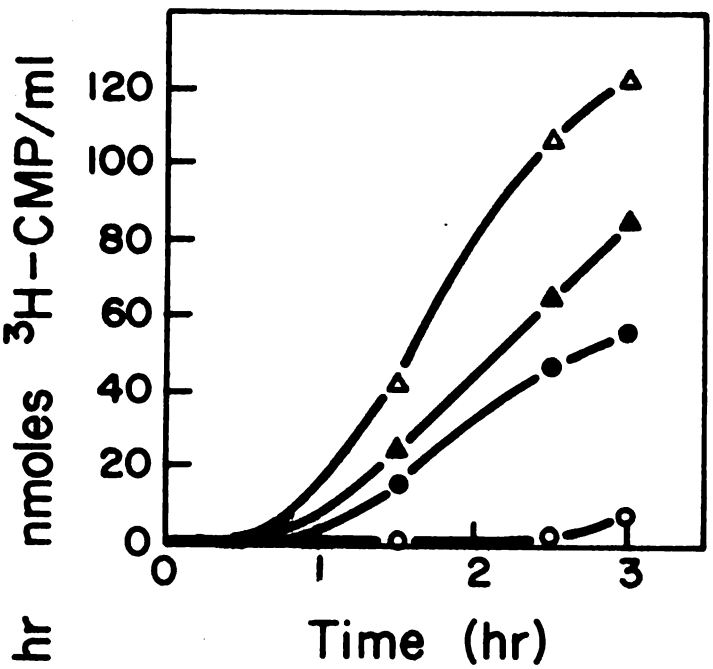
	KCl Omitted	0.1 M KCl
Rate (nmoles ^{3}H-CMP/ml/hr)	9	25
Rate (nmoles ^{14}C-IMP/ml/hr)	10	26
Lag (min)	90	45

The effect of KCl concentration upon ^{3}H-CMP incorporation into TCA insoluble material is shown in Figures 2 and 3. In Figure 2 the effect of KCl upon the time course of ^{3}H-CMP incorporation is presented for various KCl concentrations. For all concentrations tested, KCl increased the rate of the reaction. In Figure 3 the amount of ^{3}H-CMP incorporated after three hours is plotted versus the KCl concentration. The maximum incorporation occurred when the reaction mixture was 0.1 M in KCl.

Figure 2.--The effect of the KCl concentration upon the incorporation of ^{3}H-CMP. The enzyme concentration in the reaction mixture was 100 µg/ml and the reaction mixture as described in MATERIALS AND METHODS with the KCl concentration as indicated. O—O no KCl present; ▲—▲ 0.05 M KCl; Δ—Δ 0.1 M KCl; ●—● 0.2 M KCl.

Figure 3.--The incorporation of ^{3}H-CMP at three hours as an effect of the KCl concentration. The nmoles of ^{3}H-CMP incorporated after three hours (data taken from Figure 2) are shown versus the KCl concentration.

Figure 2



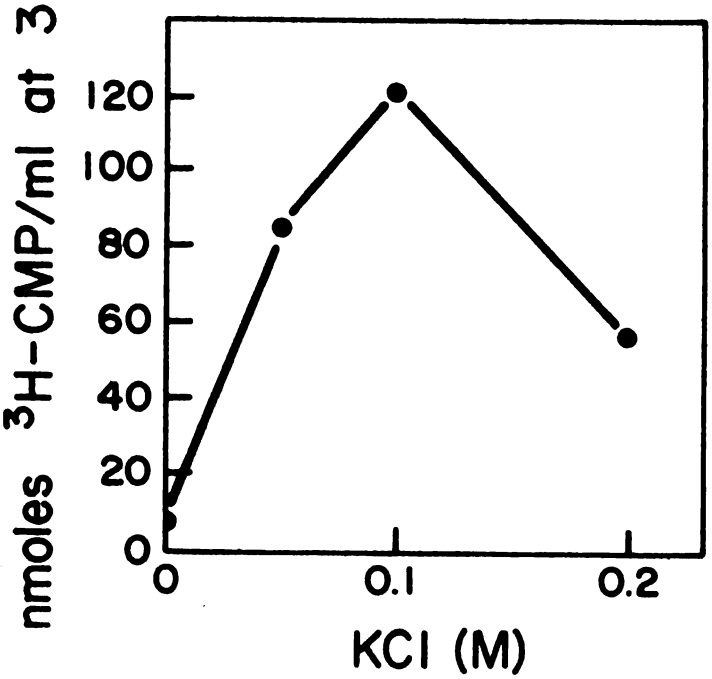


Figure 3

Figure 4.--A time course of the incorporation of ^{3}H-CMP into TCA insoluble material by PC I RNA polymerase and by PC II RNA polymerase in the presence of 0.1 M KCl. The reaction mixtures were as described in MATERIALS AND METHODS. The enzyme concentration was 100 µg/ml in both cases. O—O PC I. Δ—Δ PC II.

Figure 5.--A time course of the incorporation of ^{14}C-IMP into TCA insoluble material by PC I and PC II. The reaction mixtures and enzyme concentrations were as described for Figure 4. O—O PC I. Δ—Δ PC II.

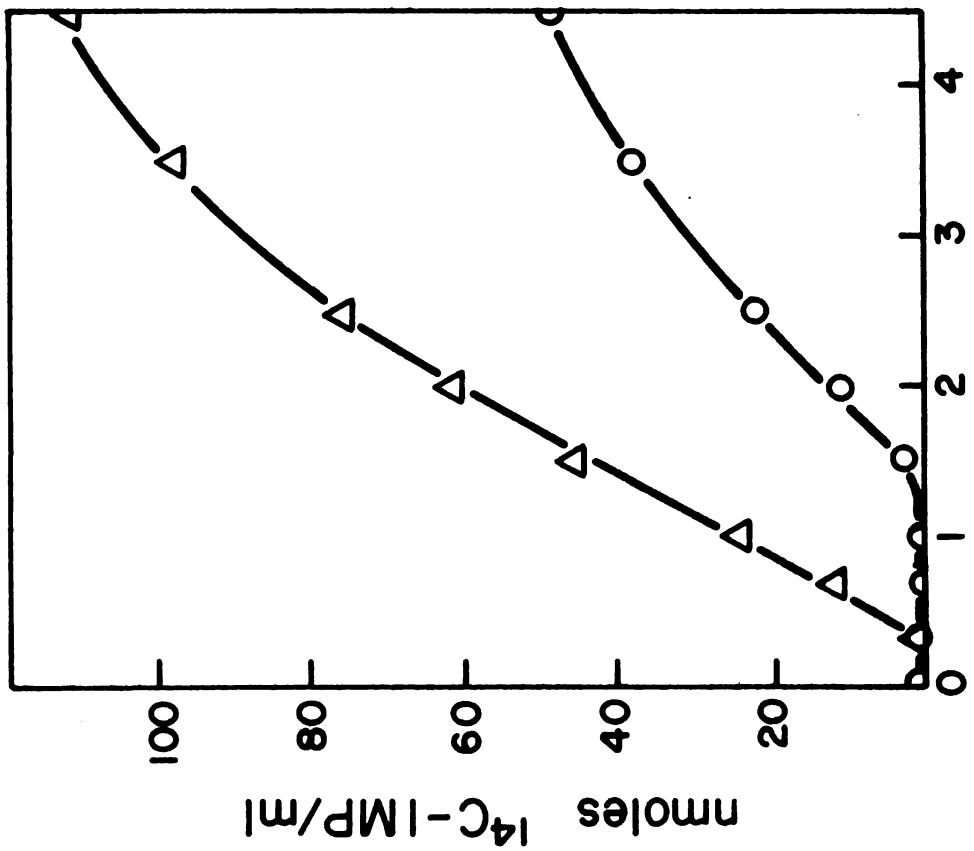


Figure 5

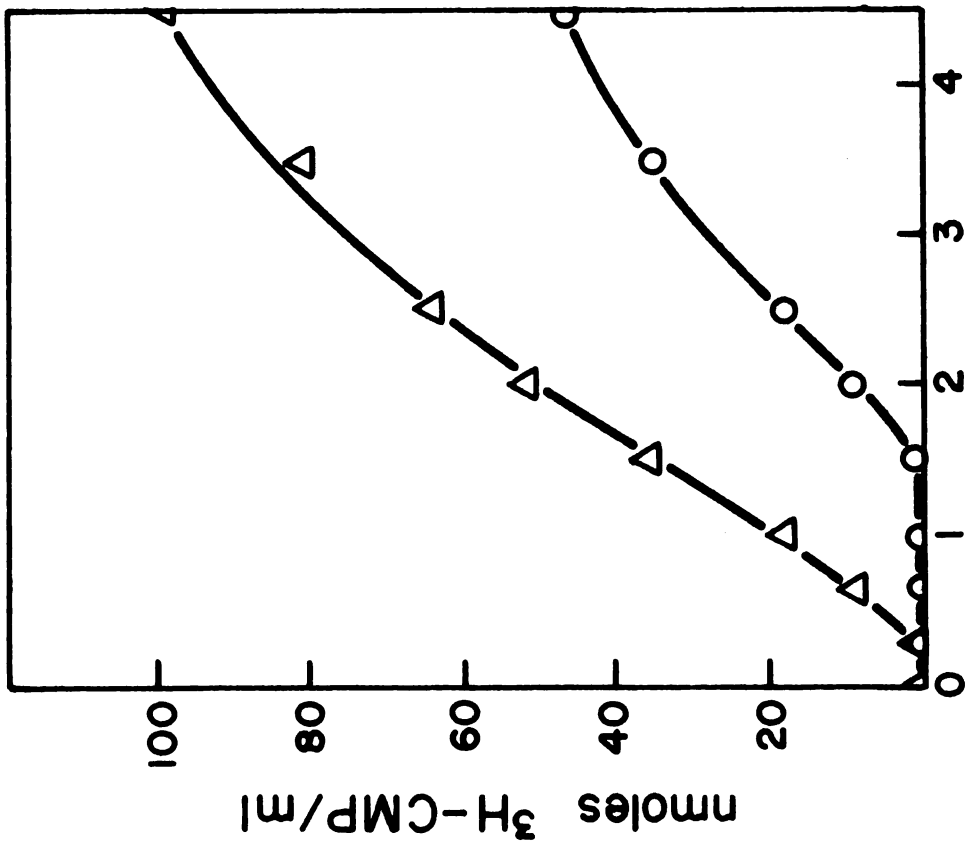


Figure 4

PC I RNA Polymerase and PC II RNA Polymerase; Incorporation of ^{14}C-IMP and ^{3}H-CMP Into TCA Insoluble Material

Since RNA polymerase could be fractionated into two components by chromatography on cellulose phosphate, the capacity of these components to catalyze the unprimed interdependent polymerization of CTP and ITP was investigated. As shown in Figures 4 and 5, the kinetics of incorporation of ^{14}C-IMP relative to ^{3}H-CMP for the reaction catalyzed by PC I RNA polymerase were similar. Likewise, the kinetics of incorporation of ^{14}C-IMP relative to ^{3}H-CMP for the reaction catalyzed by PC II RNA polymerase were similar. By comparison of the reactions catalyzed by the two components of RNA polymerase in 0.1 M KCl, the length of the lag for the reaction catalyzed by PC I was 90 minutes, whereas the length of the lag for the reaction catalyzed by PC II was 25 minutes (averaging the data for CMP and IMP incorporation). The rate of the reaction catalyzed by PC II was greater than that catalyzed by PC I (34 nmoles NMP/ml/hr versus 18 nmoles NMP/ml/hr, again averaging the data for both substrates). Similar kinetics of the reactions catalyzed by PC I and PC II were observed when KCl was omitted from the reaction mixture. The results are presented in Figures 6 and 7. The lag for the reaction catalyzed by PC II in this case was 50 minutes and for PC I, 140 minutes. The rate of incorporation of the nucleoside monophosphates in the

Figure 6.--A time course of the incorporation of ^{3}H-CMP into TCA insoluble material by PC I RNA polymerase and by PC II RNA polymerase in the absence of KCl. The reaction mixtures were as described in MATERIALS AND METHODS, omitting KCl. The enzyme concentration was 100 µg/ml in both cases. O—O PC I RNA polymerase. Δ—Δ PC II RNA polymerase.

Figure 7.--A time course of the incorporation of ^{14}C-IMP into TCA insoluble material by PC I and PC II. The reaction mixtures and enzyme concentrations were as for Figure 6. O—O PC I. Δ—Δ PC II.

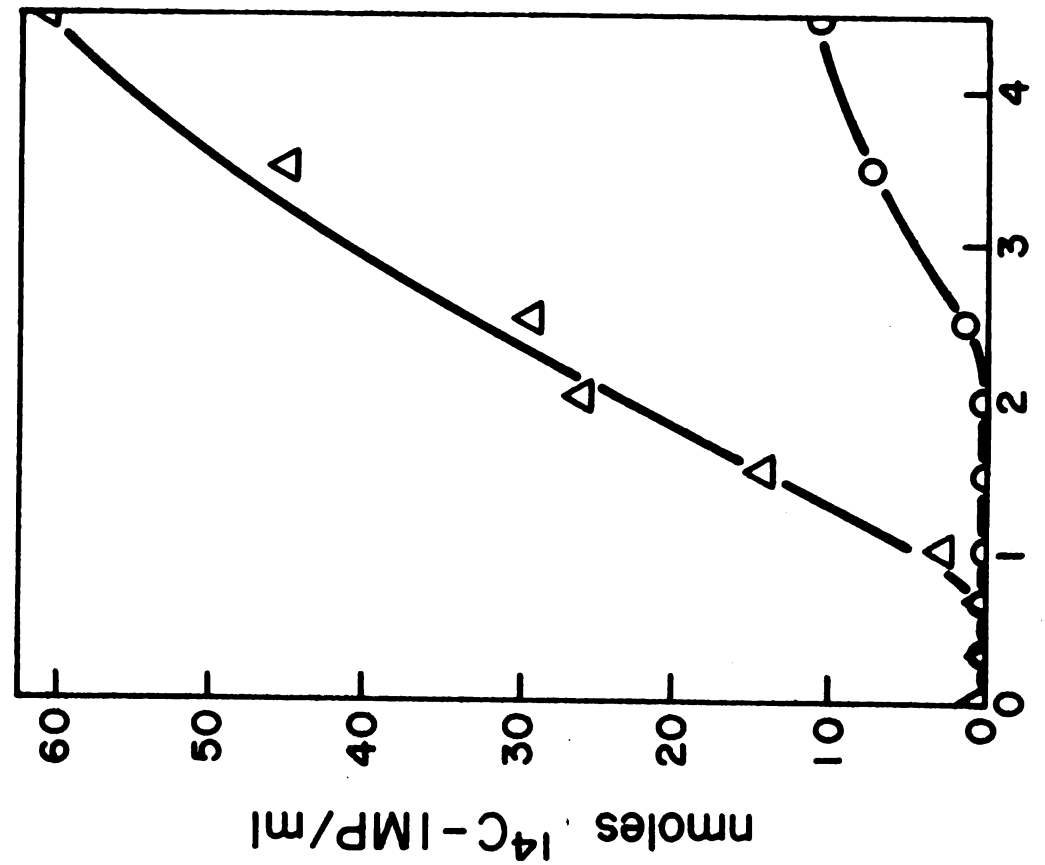


Figure 7

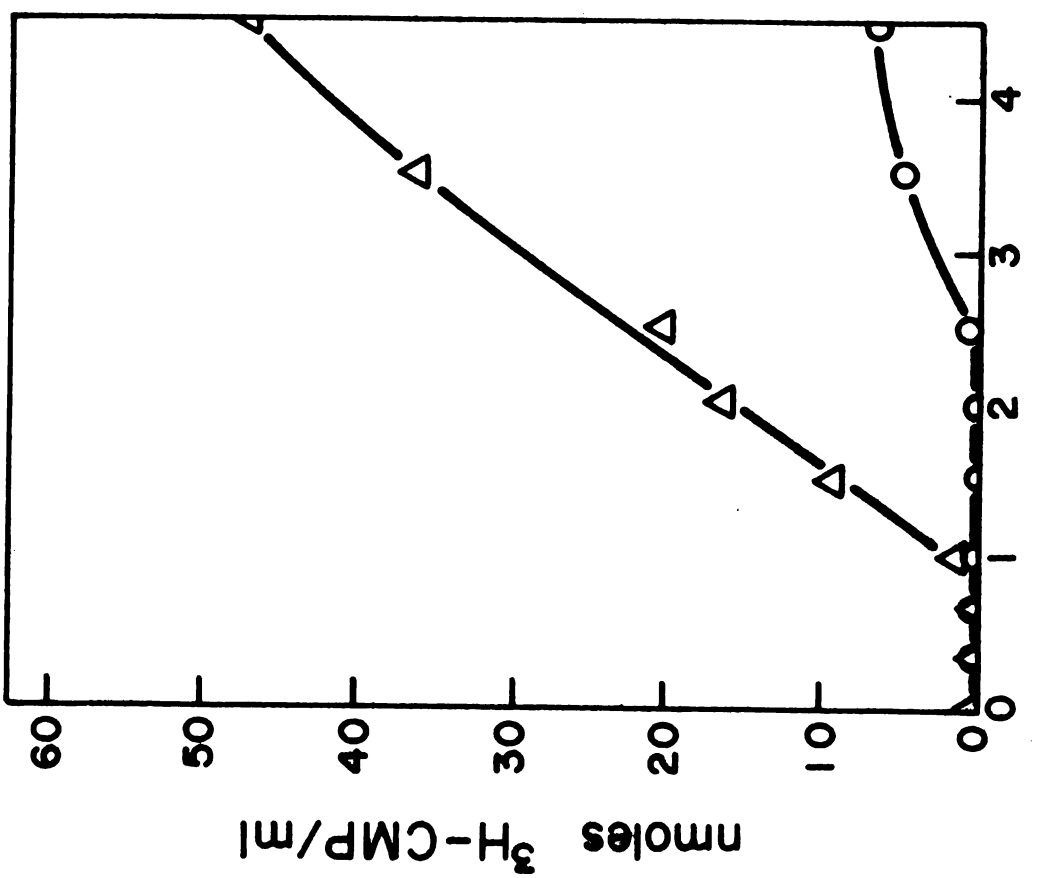


Figure 6

absence of KCl was 17 nmoles NMP/ml/hr for the reaction catalyzed by PC II and 5 nmoles NMP/ml/hr for PC I.

Nearest Neighbor Analysis of the Polymer Formed by RNA Polymerase with α-^{32}P-CTP and ITP as Substrates

Nearest neighbor analyses were conducted using RNA polymerase, PC I RNA polymerase and PC II RNA polymerase with α-^{32}P-CTP and ITP as substrates. The results, which are presented in Table 3, indicate at least 95% (average = 98%) of the ^{32}P cochromatographed with the 2'(3') CMP standard. Thus the sequence CpC occurred an average of 98% of the time, while the sequence IpC occurred less than 2% of the time. The polymer product must then contain long stretches of cytidine. Since the ratio of incorporation of inosine to cytidine was close to one for all three forms of the enzyme (see Figures 1, 4, 5, 6, 7) the polymer product must also contain long stretches of inosine. These results are consistent with the product being mostly homopolymers of cytidine and of inosine or being blocks of inosine connected to blocks of cytidine. Nearest neighbor analyses were carried out both in 0.1 M KCl and in the absence of KCl for PC I and PC II. No significant differences in the products were detectable.

TABLE 3.--Nearest neighbor analysis of the polymer formed from α-^{32}P-CTP and ITP.

	^{32}P-IMP (cpm)	^{32}P-CMP (cpm)	% CpC
	Sequence IpC	Sequence CpC	
RNA polymerase (0.2 M KCl)	16	2,635	100
PC I (0.1 M KCl)	100	5,190	98
PC II (0.1 M KCl)	201	3,890	95
PC I (KCl omitted)	20	793	98
PC II (KCl omitted)	141	4,291	97

The reaction mixtures were as described in MATERIALS AND METHODS with the KCl concentration as indicated in parentheses. The enzyme concentration was 100 µg/ml in all cases. The cpm were corrected for background radioactivity.

The Effect of Enzyme Concentration Upon the Incorporation of ^{3}H-CMP and ^{14}C-IMP Into TCA Insoluble Material; PC I

The kinetics of the incorporation of ^{3}H-CMP and ^{14}C-IMP into TCA insoluble material at various PC I concentrations are presented in Figures 8 and 9. By comparison of Figure 8 with Figure 9, the kinetics of ^{3}H-CMP incorporation for any enzyme concentration were similar to the kinetics of incorporation of ^{14}C-IMP. The steady state rate and the lengths of the lags calculated from the data presented in Figures 8 and 9 are shown in Table 4. As the enzyme concentration increased the steady state rate increased. At high enzyme concentrations the length of the lag was 80 minutes. The effects of enzyme concentration upon the rates of incorporation of CMP and IMP are presented in Figures 10 and 11. The dependence of rate upon enzyme concentration was not a linear function but was exponential in form. Plotting the log of the rate versus the log of the enzyme concentration gives a line whose slope is representative of the power of the reaction with regard to enzyme concentration. The slope of the line thus plotted (not shown) approached 3, indicating the rate may be a function of the $[\text{enzyme}]^{3}$.

Figure 8.--The effect of PC I concentration on the incorporation of ^{3}H-CMP into TCA insoluble material. Standard reaction mixtures, which contained 0.1 M KCl were as described in MATERIALS AND METHODS. PC I concentration as indicated. O—O 42 µg/ml; Δ—Δ 84 µg/ml; ●—● 97 µg/ml; ▲—▲ 126 µg/ml; ■—■ 168 µg/ml; □—□ 210 µg/ml.

Figure 9.--The effect of PC I concentration on the incorporation of ^{14}C-IMP into TCA insoluble form. Reaction mixtures and enzyme concentrations were as for Figure 8.

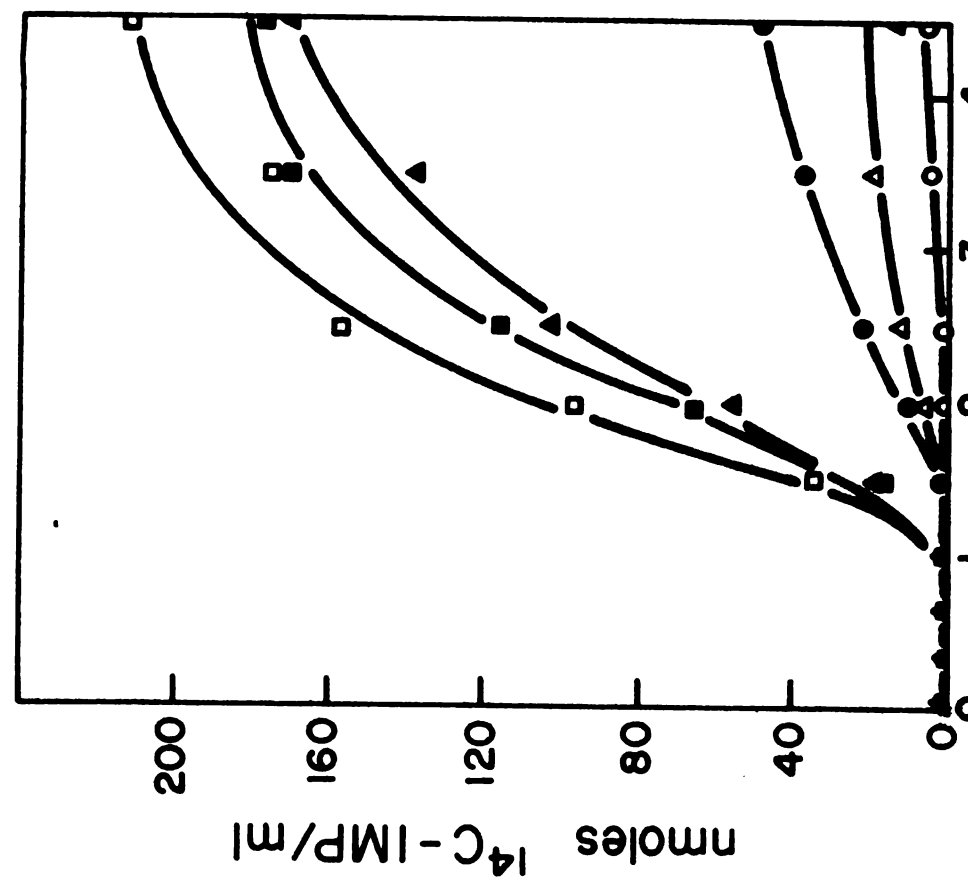


Figure 9

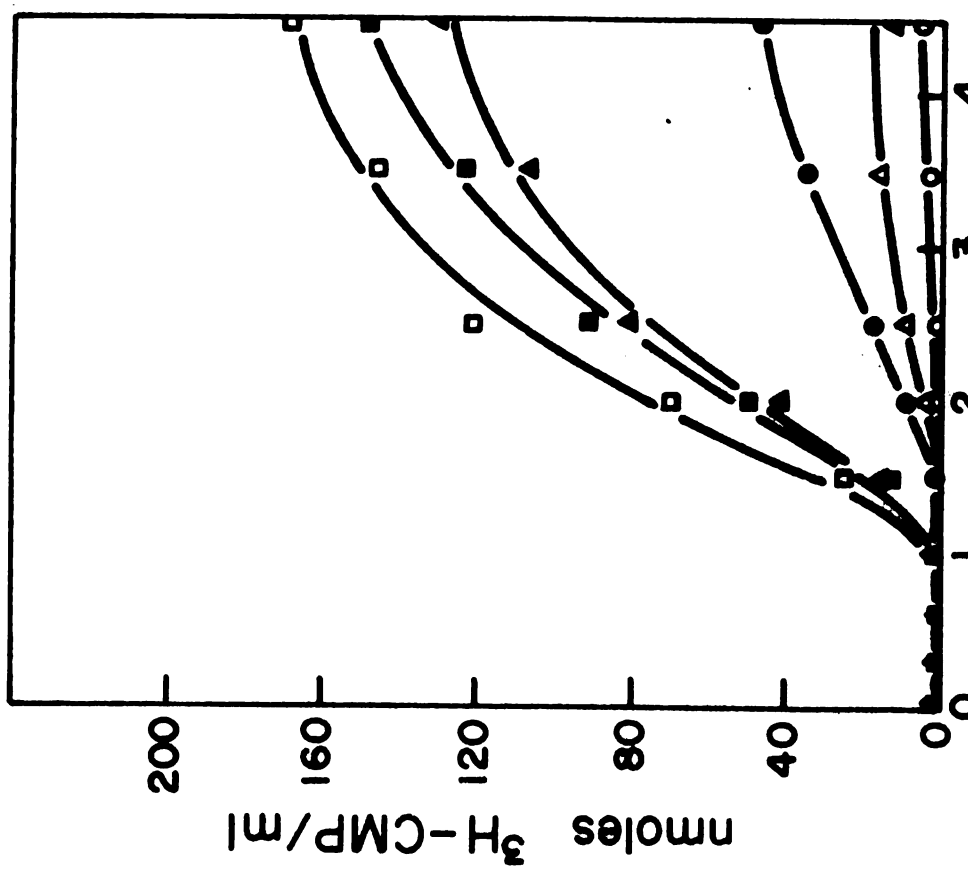


Figure 8

TABLE 4.--PC I RNA polymerase.

Concentration (μg/ml)	Rate (nmoles ^{3}H-CMP/ml/hr)	Rate (nmoles ^{14}C-IMP/ml/hr)	Lag (min)
42	3	3	120
84	9	11	90
97	18	18	90
126	60	81	80
168	72	105	80
210	112	125	80

Reaction mixtures were as described in MATERIALS AND METHODS. Steady state rates and lengths of lags were determined from the data presented in Figures 8 and 9.

Figure 10.--The effect of PC I concentration upon the rate of incorporation of ^{3}H-CMP into TCA insoluble material. Data is taken from Table 4.

Figure 11.--The effect of PC I concentration upon the rate of incorporation of ^{14}C-IMP into TCA insoluble material. Data is taken from Table 4.

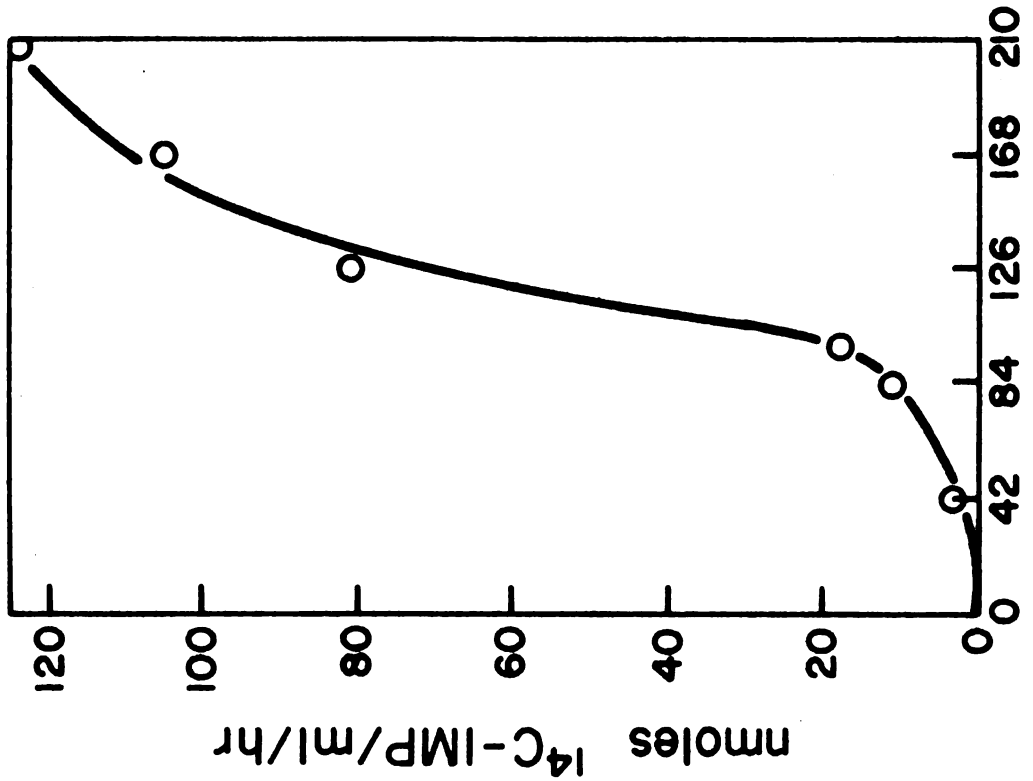


Figure 11

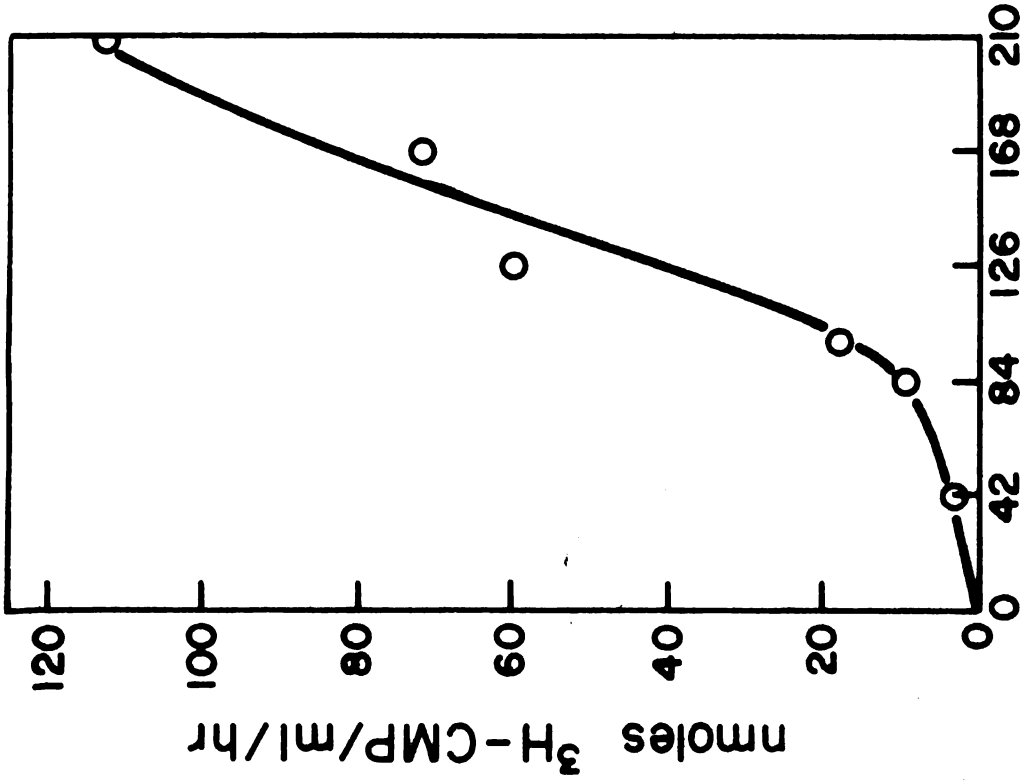


Figure 10

The Effect of Enzyme Concentration Upon the Incorporation of ^{3}H-CMP and ^{14}C-IMP Into TCA Insoluble Material; PC II

The kinetics of the incorporation of ^{3}H-CMP and ^{14}C-IMP into TCA insoluble material at various PC II concentrations are presented in Figures 12 and 13. As for PC I, ^{14}C-IMP incorporation in the reaction catalyzed by PC II was similar to the ^{3}H-CMP incorporation at any enzyme concentration (comparing Figure 12 with Figure 13). Table 5 presents the steady state rates and lengths of the lags determined from the data presented in Figures 12 and 13. As the enzyme concentration increased, the steady state rate of the reaction increased. The length of the lag was 25 to 40 minutes. The steady state rates are plotted versus the protein concentration in Figures 14 and 15. As for PC I, the rate of the reaction catalyzed by PC II was not a linear function of enzyme concentration, but the two parameters were exponentially related. Plotting the log of the rate versus the log of the enzyme concentration (not shown) yielded a straight line with a slope of 2, indicating the rate of the reaction varied with the square of the enzyme concentration.

By comparison of the reactions catalyzed by PC I and PC II (Tables 4 and 5) the length of the lag for the reaction catalyzed by PC I was 50-80 minutes longer than the corresponding lag for the reaction catalyzed by PC II.

Figure 12.--The effect of PC II concentration upon the incorporation of ^{3}H-CMP into TCA insoluble material. Reaction mixtures were as described in MATERIALS AND METHODS. PC II concentration as indicated. O—O 40 µg/ml; Δ—Δ 80 µg/ml; ●—● 98 µg/ml; ▲—▲ 120 µg/ml; ■—■ 160 µg/ml; ▯—▯ 200 µg/ml.

Figure 13.--The effect of PC II concentration upon the incorporation of ^{14}C-IMP into TCA insoluble material. Reaction mixtures and enzyme concentrations were as for Figure 12.

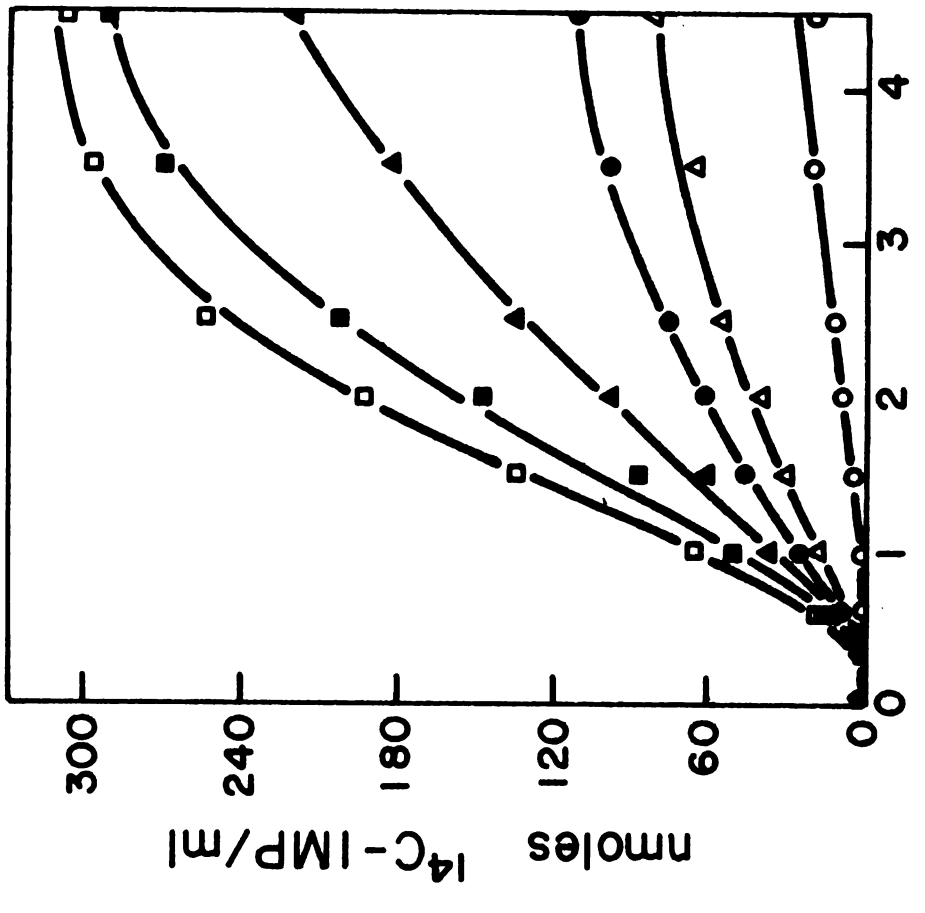


Figure 13

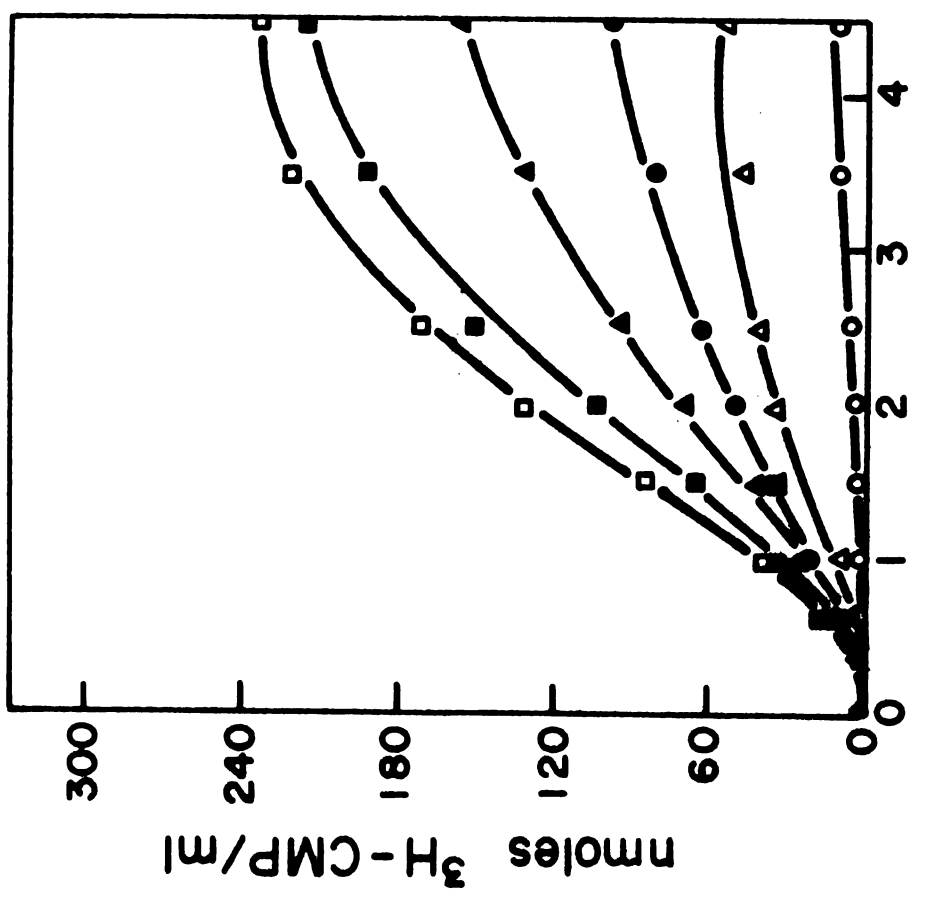


Figure 12

TABLE 5.--PC II RNA polymerase.

Concentration (μg/ml)	Rate (nmoles ^{3}H-CMP/ml/hr)	Rate (nmoles ^{14}C-IMP/ml/hr)	Lag (min)
40	5	8	40
80	18	28	30
98	30	38	25
120	53	62	30
160	91	100	30
200	100	138	30

Reaction mixtures were as described in MATERIALS AND METHODS. Steady state rates and the lengths of the lags were determined from Figures 12 and 13.

Figure 14.--The effect of PC II upon the rate of incorporation of ^{3}H-CMP into TCA insoluble material. Data is taken from Table 5.

Figure 15.--The effect of PC II upon the rate of incorporation of ^{14}C-IMP into TCA insoluble material. Data is taken from Table 5.

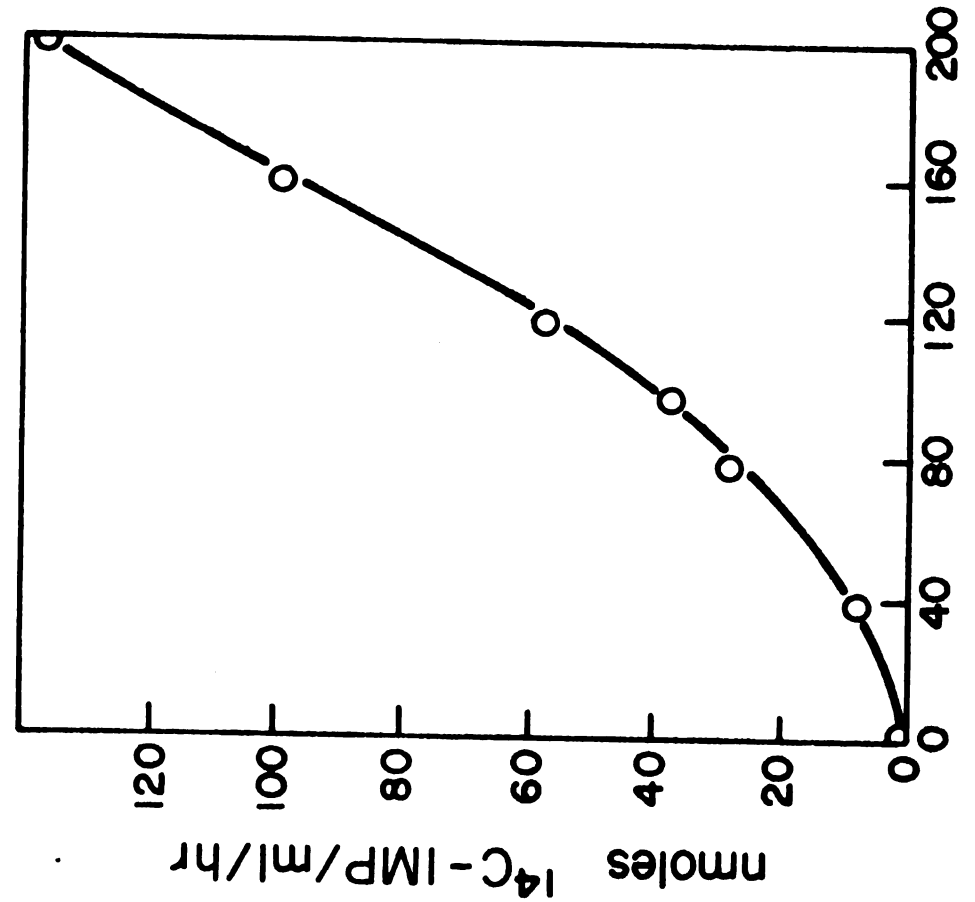


Figure 15

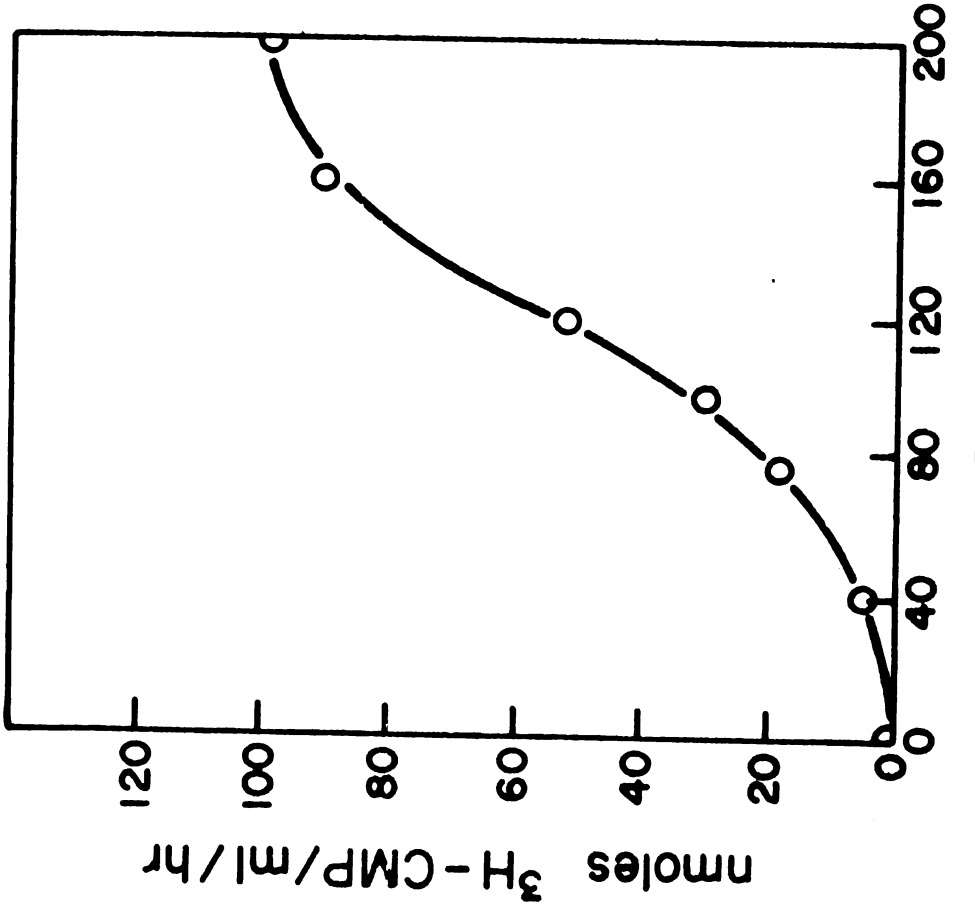


Figure 14

Although the rates for the reaction catalyzed by PC II were greater than those for the reaction catalyzed by PC I at low protein concentrations, the rates appear to converge at higher protein concentrations.

Characteristics of the Interdependent Polymerization of ATP and UTP by PC I RNA Polymerase and PC II RNA Polymerase

PC I RNA Polymerase and PC II RNA Polymerase; Incorporation of ^{3}H-AMP Into TCA Insoluble Material

The catalysis of the interdependent polymerization of ATP and UTP into homopolymers by RNA polymerase (isolated as a single peak by chromatography on phosphocellulose) in the absence of added template has been previously described (9). Since that study, RNA polymerase has been isolated as two fractions, PC I and PC II, both of which were active in the DNA directed synthesis of RNA and in the unprimed interdependent polymerization of ITP and CTP. The capacity of the two components of RNA polymerase to catalyze the unprimed interdependent polymerization of ATP and UTP was investigated. The time course of the incorporation of ^{3}H-AMP into a TCA insoluble form by PC I and PC II is shown in Figure 16. As for the reaction involving ITP and CTP, the length of the lag for the reaction catalyzed by PC II was shorter than that for PC I (20 minutes versus 40 minutes). The steady state

Figure 16.--The time course for the incorporation of ^{3}H-AMP into TCA insoluble material by PC I and PC II in the presence of 0.1 M KCl. The standard reaction mixtures were as described in MATERIALS AND METHODS. The enzyme concentration in both cases was 100 µg/ml. O—O PC I. Δ—Δ PC II.

Figure 17.--The time course for the incorporation of ^{3}H-AMP into TCA insoluble material by PC I and PC II in the absence of KCl. Reaction mixtures and enzyme concentrations were as described for Figure 16, omitting KCl. O—O PC I. Δ—Δ PC II.

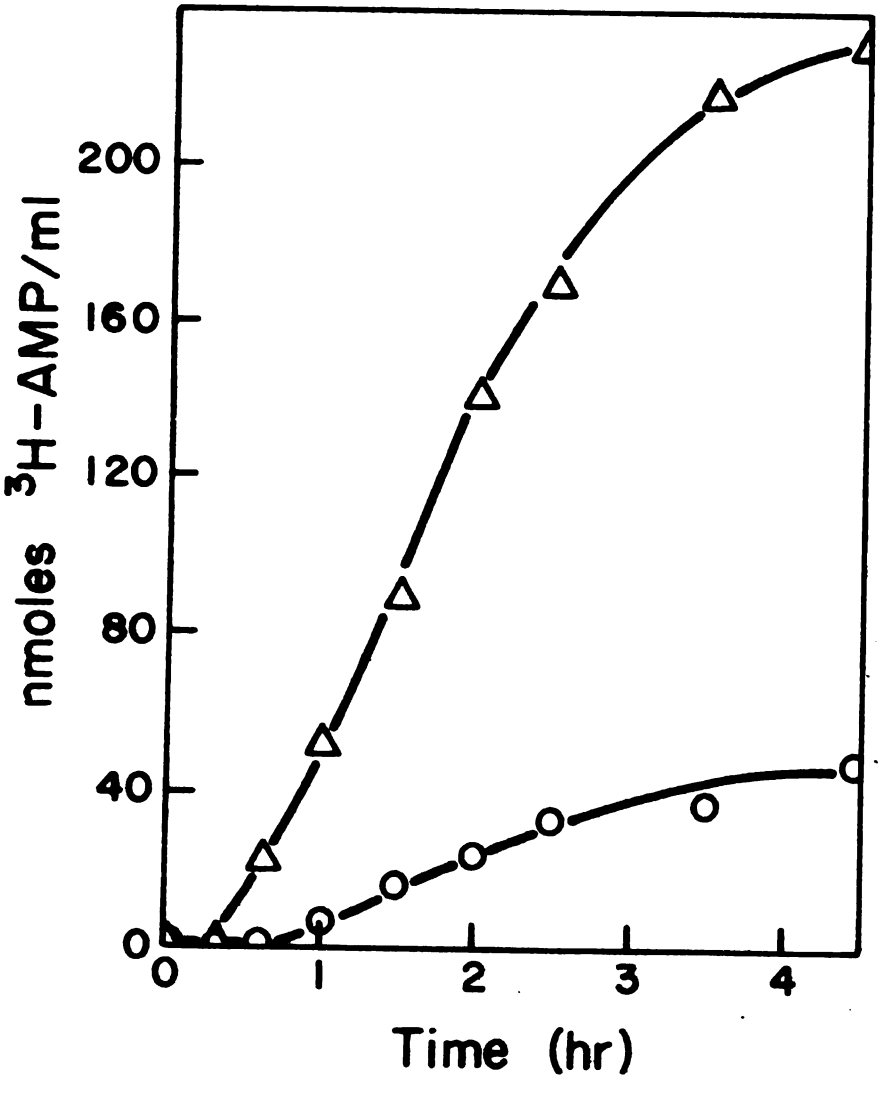


Figure 16

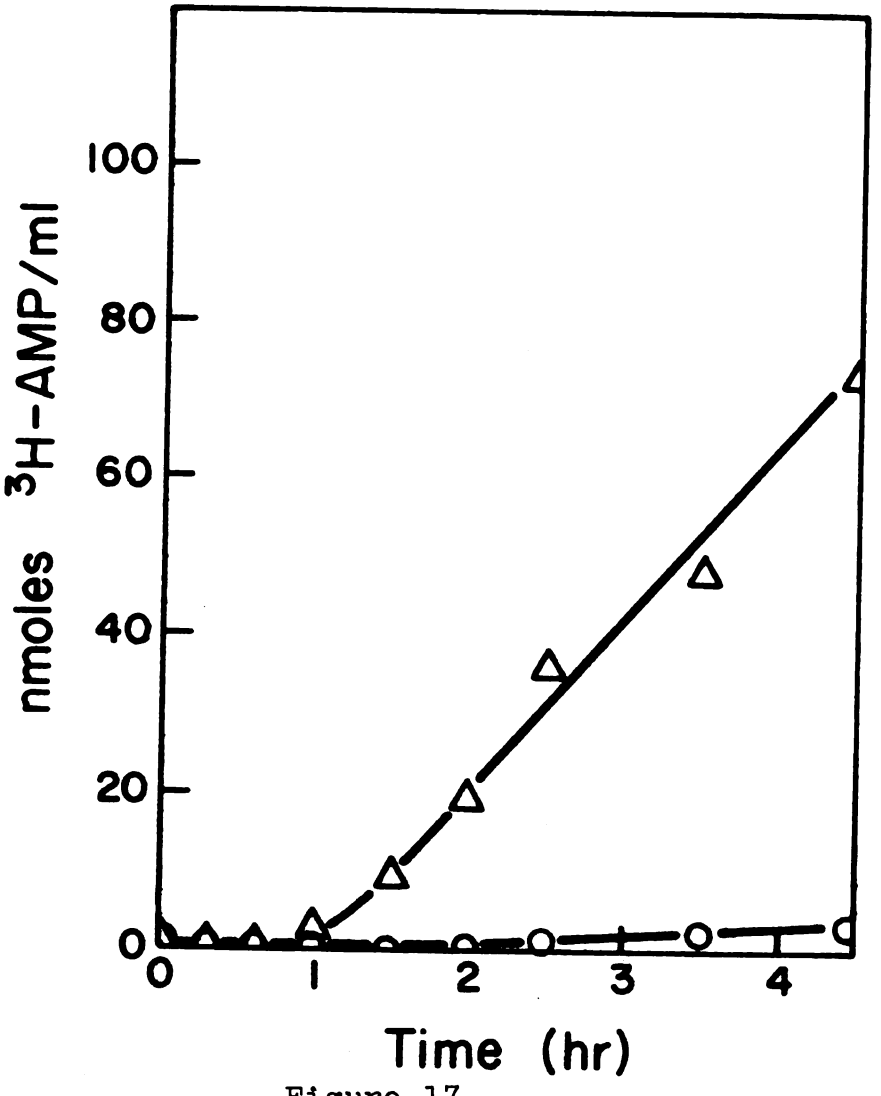


Figure 17

rate for the synthesis of polymers from ATP and UTP by PC II was also greater than for PC I; 89 nmoles ^{3}H-AMP/ml/hr for PC II as compared to 18 nmoles ^{3}H-AMP/ml/hr for PC I. As shown in Figure 17, the same type of incorporation profiles were seen in the absence of KCl, the length of the lag for the reaction catalyzed by PC II being 60 minutes and by PC I, 100 minutes. The rate for the reaction catalyzed by PC II was 22 nmoles ^{3}H-AMP/ml/hr and for the reaction catalyzed by PC I, 2 nmoles ^{3}H-AMP/ml/hr.

Nearest Neighbor Analysis of the Polymer Formed by PC I and PC II Utilizing α-^{32}P-ATP and UTP as Substrates

Nearest neighbor analyses were performed using α-^{32}P-ATP and UTP to determine if the products of the reactions catalyzed by PC I and PC II were homopolymers. The results are presented in Table 6. The ApA sequence, but virtually none of the UpA sequence was detected, indicating the AMP containing product was $r(A)_n$. The UMP containing product must then by $r(U)_n$. Analyses were performed in 0.1 M KCl and in the absence of KCl. No significant differences in the product sequences were noted.

TABLE 6.--Nearest neighbor analysis of the polymer formed from α-^{32}P-ATP and UTP.

	^{32}P-UMP (cpm) Sequence UpA	^{32}P-AMP (cpm) Sequence ApA	% ApA
PC I (0.1 M KCl)	0	4,021	100
PC II (0.1 M KCl)	58	18,189	100
PC I (KCl omitted)	16	2,197	99
PC II (KCl omitted)	52	5,905	99

The reaction mixtures were as described in MATERIALS AND METHODS with the KCl concentration as indicated in parentheses. The enzyme concentration was 100 µg/ml in all cases. Treatment of the product and subsequent analysis were as described in MATERIALS AND METHODS. Cpm were corrected for background radioactivity.

Disc Electrophoresis of RNA Polymerase on Polyacrylamide Gels

Electrophoresis of PC I RNA polymerase and PC II RNA polymerase was performed as described in MATERIALS AND METHODS for the Tris-glycine system. After destaining, several protein bands were evident for both PC I and PC II (Figure 18). In both cases the largest percentage of protein was in a slowly moving band labeled 1. For PC I, 1 was split into two closely migrating bands, 1 and 1', 1' moving slightly faster. For PC II, 1' was absent. However, a band moving more slowly than 1 was observed and is labeled 1". PC I and PC II also had a fast moving component which seemed to be split into two or more bands. These bands are labeled 2. PC I had an additional slowly moving component which migrated only a short distance into the gel.

To test the enzymatic activity of the various bands, electrophoresis of 20 μg samples of PC I and PC II was performed. The resulting gels were incubated in reaction mixtures containing ITP and CTP or ATP and UTP as described in MATERIALS AND METHODS. Duplicate PC I gels were incubated in reaction mixtures from which Mn^{+2} had been omitted. The resulting ribopolymers were stained *in situ* with ethidium bromide. The gels were photographed under ultraviolet light and are pictured in Figure 19.

Figure 18.--Polyacrylamide gel electrophoresis; protein stain. Electrophoresis was performed as described in MATERIALS AND METHODS for the Tris-glycine system. The resulting gels were stained with Coomaasie Brilliant Blue. 20 µg of protein was applied to each gel. On the left is PC I RNA polymerase. PC II is to the right.

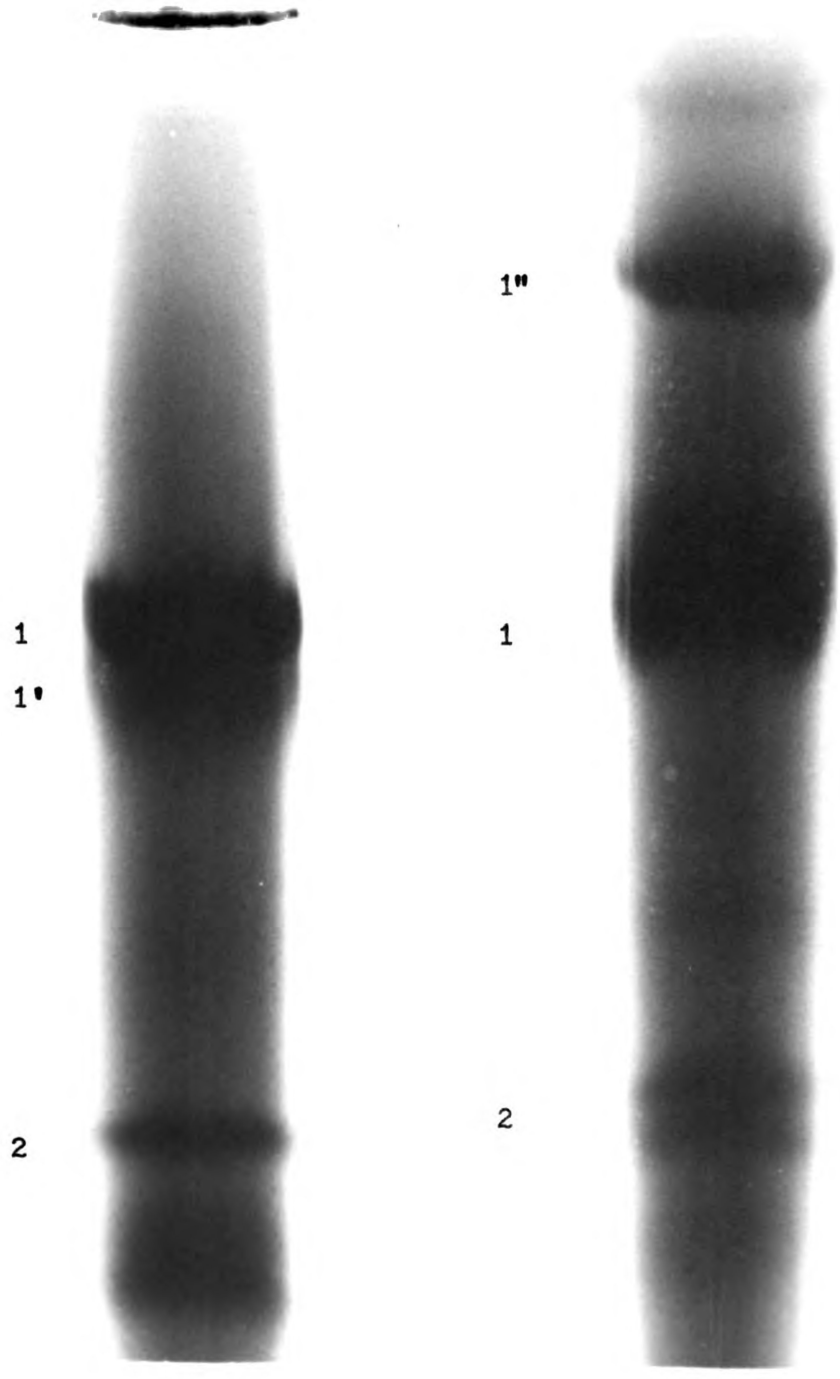


Figure 18

Figure 19.--Polyacrylamide gel electrophoresis; activity stain. Electrophoresis was carried out as for Figure 18, applying 20 µg of protein to each gel. The resulting gels were incubated in reaction mixtures and stained with ethidium bromide as described in MATERIALS AND METHODS. Photographs were taken utilizing ultraviolet light. The set of three gel on the left represent gels which were incubated in reaction mixtures with ATP and UTP as substrates. From the left, PC I, PC II, PC I with $Mn(OAc)_2$ omitted from the reaction mixture. The set of gels on the right were incubated in reaction mixtures containing ITP and CTP. From left to right, PC I, PC II, PC I with $Mn(OAc)_2$ omitted from the reaction mixture.

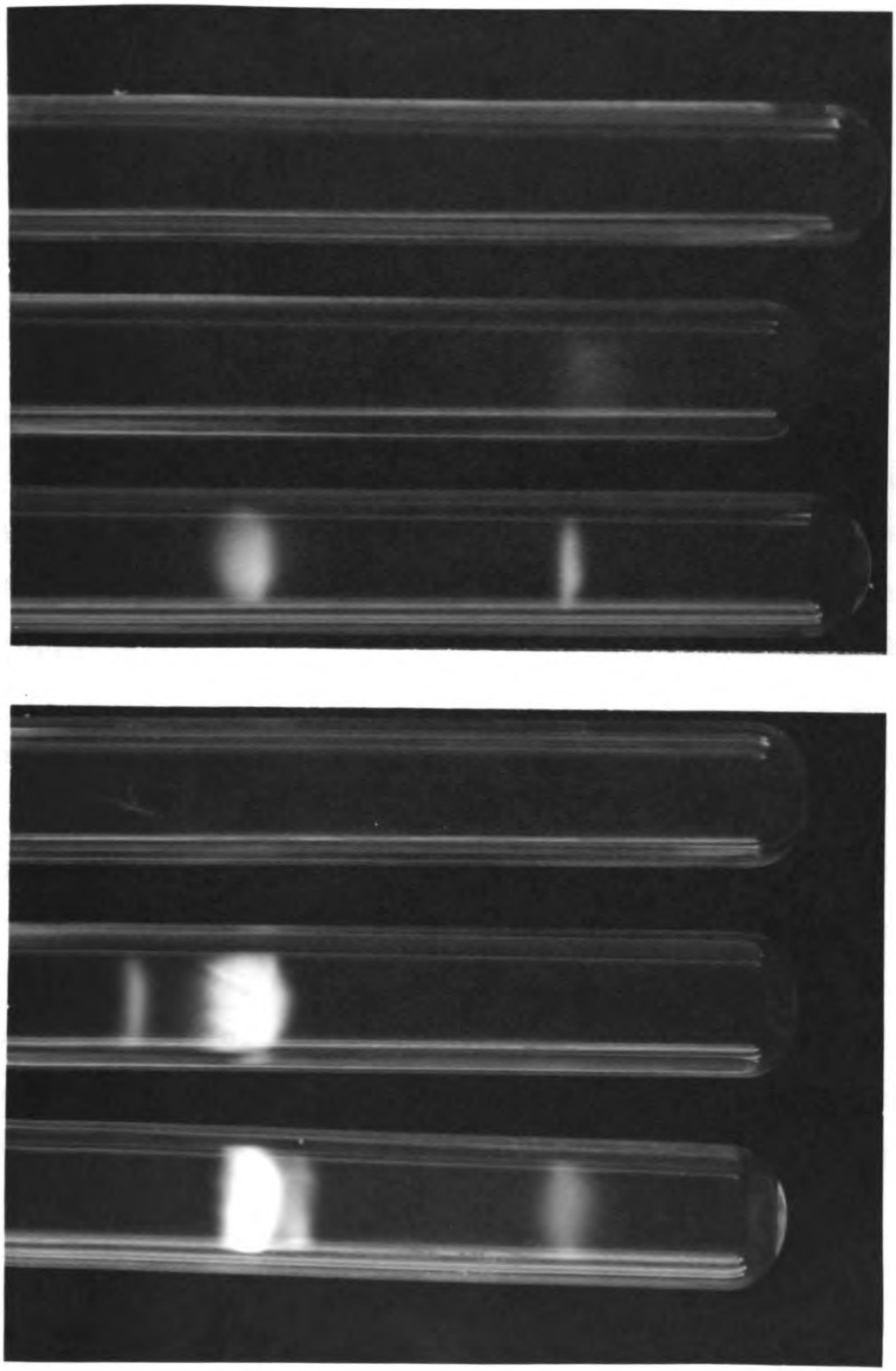


Figure 19

For the synthesis of polymers containing AMP and UMP (the set of gels pictured on the left), bands 1, 1' and at least part of 2 were active for the reaction catalyzed by PC I and 1 and 1' were active in the reaction catalyzed by PC II. Although not evident from the photograph, PC II band 2 synthesized a small amount of polymer from ATP and UTP.

For the synthesis of polymers containing IMP and CMP (the set of gels pictured on the right in Figure 19), PC I, band 1 and part of band 2, were active. In the gel to which PC II had been applied, only band 2 appeared active in the synthesis of IMP, CMP containing polymers (application of more protein, however, resulted in some synthesis in band 1). The results obtained above were reproduced for another preparation of PC I and PC II.

Thus at least part of all bands present in the polyacrylamide gels were capable of catalyzing reactions characteristic of RNA polymerase. The only exception was the very slowly moving, sharply banding protein appearing near the top of the PC I gel. Whether this is a contaminant of the preparation or an inactive high molecular weight aggregate of RNA polymerase is as yet undetermined.

Disc Electrophoresis with Polyacrylamide Gels Using the Sodium Phosphate-SDS System

Proteins with a known subunit molecular weight and PC I RNA polymerase and PC II RNA polymerase were denatured with SDS and β-mercaptoethanol in order to separate the polypeptide chains of the proteins. Electrophoresis was subsequently performed as described in MATERIALS AND METHODS. The resulting gels were stained for protein and photographed. The results are presented in Figure 20. The distance traveled by any subunit is logarithmically related to its molecular weight (15). Thus in Figure 21, the molecular weight of the subunits of the standard proteins are plotted on a logarithmic scale along the ordinate and the distance traveled by that subunit is plotted on the linear abscissa. (The distance was measured in cm from the top of the gel.) As shown in Figure 20, some reaggregation of subunits occurred during electrophoresis to give dimers or trimers of these polypeptide chains. The straight line shown in Figure 21 was used as a standard curve to estimate the molecular weights of the polypeptide chains obtained from similar electrophoresis of PC I and PC II.

PC I had five major polypeptide chains, labeled from the top of the gel down (see Figure 20), A, B, C, D, E with their estimated molecular weights as follows:

A. 150,000-160,000

B. 80,000

Figure 20.--Polyacrylamide gel electrophoresis in SDS-sodium phosphate, pH 7.1. Electrophoresis was performed using the SDS-sodium phosphate system described in MATERIALS AND METHODS. Protein was denatured as described in the text and 20 µg were applied to each gel. From the left, bovine serum albumin, yeast pyruvate kinase, rabbit muscle aldolase, egg white lysozyme, bovine hemoglobin, PC I RNA polymerase and PC II RNA polymerase.

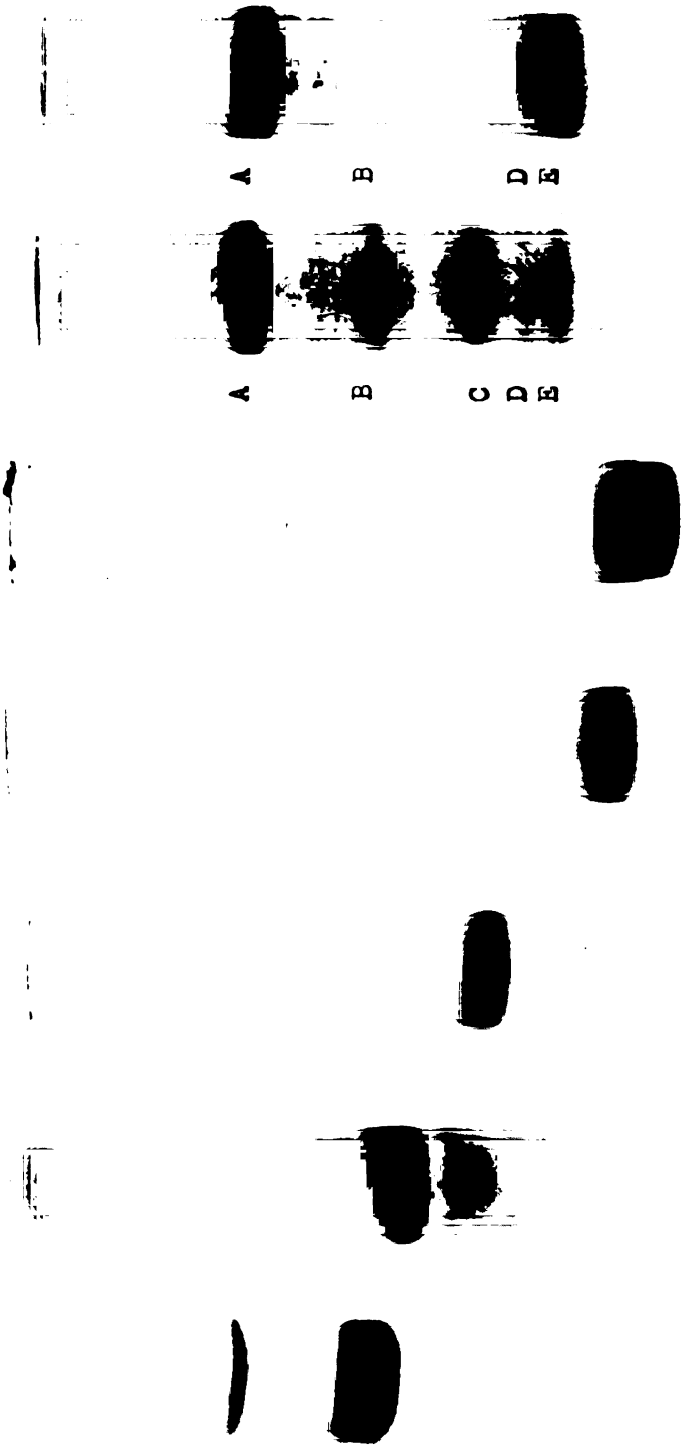


Figure 20

Figure 21.--Standard curve for protein migration in the SDS-sodium phosphate, pH 7.1, polyacrylamide gels. Distances were determined from the gels pictured in Figure 20. The distance migrated is plotted versus the subunit molecular weight of the protein (on log scale). BSA = bovine serum albumin. Hb = hemoglobin. The symbol (x2) or (x3) indicates the subunits were in the form of a dimer or trimer.

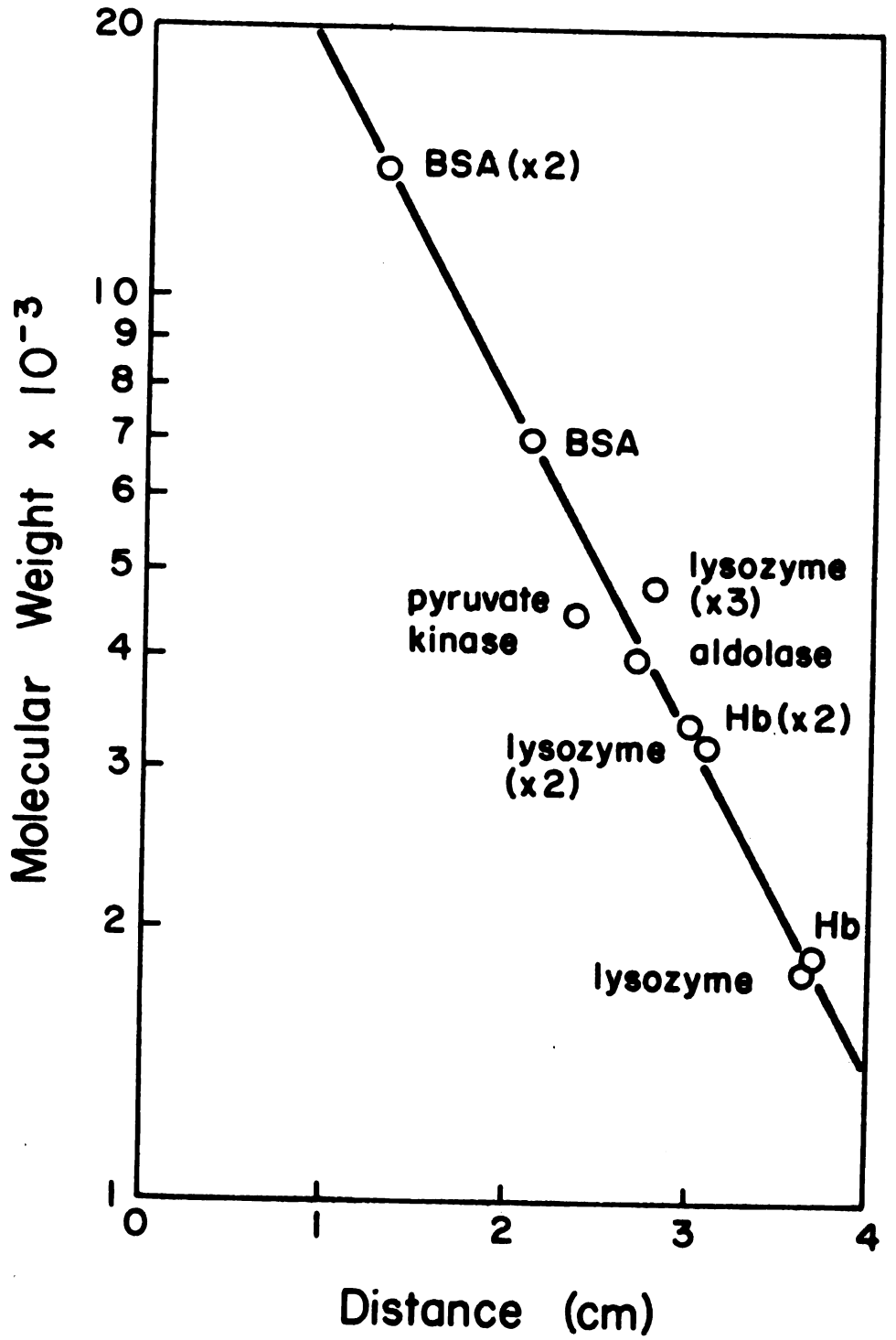

Molecular Weight x 10^{-3}
20
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
Distance (cm)
BSA (x2)
BSA
lysozyme (x3)
pyruvate kinase
aldolase
Hb (x2)
lysozyme (x2)
Hb
lysozyme

Figure 21

C. 50,000

D. 40,000

E. 32,000

PC II contained three major polypeptide chains, A, D and E. Because of the relatively large amounts of D and E present, it is difficult to distinguish them in Figure 20. PC II was missing polypeptide C (50,000) completely and only a trace amount of B (80,000) was detected. Polypeptide C is derived from a protein inactive in RNA synthesis and presumably is a contaminant of the preparation (J. C. Johnson, private communcation).

DISCUSSION

After a lag RNA polymerase from P. putida catalyzes the unprimed interdependent polymerization of ITP and CTP into TCA insoluble material which is primarily homopolymers of inosine and cytidine. In contrast, RNA polymerase from E. coli and from A. vinelandii catalyzes the unprimed synthesis of copolymers from ITP and CTP in which inosine and cytidine are in alternating sequence (10). After the P. putida enzyme is fractionated into two components (PC I and PC II) by chromatography on cellulose phosphate, each component is able to catalyze the unprimed synthesis of homopolymers from CTP and ITP. Although the products of the reactions catalyzed by the two components of RNA polymerase are the same, the length of the lag for the reaction catalyzed by PC I is at least 2.5 times greater than the corresponding lag for the reaction catalyzed by PC II. A possible mechanism for the unprimed interdependent polymerization of ITP and CTP might involve the slow synthesis of either $r(I)_n$ or $r(C)_n$. This polymer might then serve as template for the synthesis of the complementary polymer, which, in turn, could serve as template for the synthesis of its complement. Thus,

with polymers of inosine and cytidine available as templates, rapid synthesis of the homopolymers ensues. RNA polymerase from P. putida catalyzes the unprimed interdependent synthesis of $r(A)_n$ and $r(U)_n$ (9). RNA polymerase from E. coli and from A. vinelandii catalyzes the same reaction, also synthesizing homopolymers from ATP and UTP (6-8). As for the unprimed synthesis of polymers from ITP and CTP, the products of the reactions catalyzed by PC I and PC II from ATP and UTP are homopolymers. Again, however, the length of the lag for the reactions catalyzed by the two components of RNA polymerase is different, with the length of the lag for the reaction catalyzed by PC I being approximately two times longer than the corresponding lag for the reaction catalyzed by PC II.

The rate of the unprimed interdependent synthesis of polymers from ITP and CTP by PC II RNA polymerase is proportional to the square of the enzyme concentration. The rate of synthesis of TCA insoluble material from ITP and CTP by PC I is also related exponentially to the enzyme concentration, with the rate of the reaction approaching proportionality to $[\text{enzyme}]^3$. The rate of synthesis of homopolymers from ATP and UTP by P. putida RNA polymerase is related to the square of the enzyme concentration (9). A mechanism similar to that discussed by Toyama et al. (9) for the unprimed synthesis

of $r(A)_n$ and $r(U)_n$ by P. putida RNA polymerase may also apply to the unprimed polymerization of ITP and CTP. The hypothesis involves a monomer-dimer conversion of the enzyme during the unprimed reaction, with the enzyme existing in the inactive monomeric form at the beginning of the reaction. As the reaction proceeds the generation of polynucleotides facilitates the conversion of monomer to dimer, increasing the concentration of active enzyme.

The protein pattern appearing after electrophoresis of PC I and PC II on polyacrylamide gels (Tris-glycine, pH 9.0, system) shows a major slow moving component and a minor, faster, moving component. The structure of the bands relative to forms of the enzyme is as yet undetermined. Although both bands appear to be composed of several components, at least part of both bands is active in the unprimed reactions catalyzed by RNA polymerase. Relative to the amount of protein which appears to be present in the bands, the faster moving band appears more active in the synthesis of polymers from ITP and CTP than the slower moving band. The synthesis of homopolymers of adenosine and uridine does not show this differential effect. RNA polymerase from A. vinelandii also has two protein bands after electrophoresis on polyacrylamide gels. Both bands of the A. vinelandii enzyme appear active in the unprimed reactions catalyzed by RNA

polymerase (12). The relationship of these bands to those observed with P. putida RNA polymerase is unknown.

After electrophoresis in the SDS-sodium phosphate, pH 7.1, system, the patterns on the polyacrylamide gels indicate PC I has five major polypeptide chains and PC II has three chains. Preliminary data (J. C. Johnson, private communication) indicates one PC I chain (C. M. W. 50,000) is a contaminant. Polypeptide chain B (M. W. 80,000) is then the primary difference between PC I and PC II on the SDS polyacrylamide gels. Depending upon the manner of its purification, E. coli RNA polymerase also gives different polypeptide patterns upon electrophoresis in the SDS polyacrylamide gel system (11). After the E. coli enzyme ($\alpha_2\beta\beta'\sigma$) is passed through a cellulose phosphate column, it loses the ability to synthesize RNA using T_4 DNA as template. However, it can still synthesize RNA using E. coli DNA as template. This difference in catalytic activity is attributed to the loss of the polypeptide chain σ(M. W. 95,000) by chromatography on cellulose phosphate. The similarity of the estimated molecular weights of the polypeptide chains of the enzymes from E. coli and P. putida (β = 165,000, β' = 155,000, A = 150,000-160,000; α = 40,000, D = 40,000, E = 32,000; σ = 95,000, B = 80,000) suggests that, by analogy, RNA polymerase from P. putida may exist as A_2DEB (PC I) and as A_2DE (PC II).

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