Rasmussen, R., and Klein, H. P. (1967), *Biochem. Biophys. Res. Commun. 28*, 415.

Shen, L. C., and Atkinson, D. E. (1970), *J. Biol. Chem. 245*, 5974.

Preparation of Highly Purified Ribonucleic Acid Polymerase; Separation from Polynucleotide Phosphorylase and Polyphosphate Kinase†

D. J. McConnell‡ and J. Bonner*

ABSTRACT: RNA polymerase was prepared from *Escherichia coli*, suitable for studies on the initiation of RNA synthesis. The enzyme appeared >95% pure on sodium dodecyl sulfate polyacrylamide gels. The enzyme preparations were shown to be free of phosphatase and ribonuclease activities. Contamination by deoxyribonuclease was measurable in a highly sensitive assay but was insignificant. Methods are described for the removal or control of trace amounts of polynucleotide phosphorylase. The incorporation of the γ-phosphate from ATP into acid-precipitable form by RNA polymerase preparations in the absence of template did not represent the formation of a phosphoenzyme intermediate, but resulted from the synthesis of polyphosphate, probably by trace amounts of contaminating polyphosphate kinase. Methods are described and assessed for the control of this enzyme. The activities of these contaminants are not usually assayed in studies of this kind but could cause serious misinterpretation of initiation studies if not controlled.

Several methods have been described for the preparation of RNA polymerase from *Escherichia coli* (Chamberlin and Berg, 1962; Furth *et al.*, 1962; Stevens and Henry, 1964; Babinet, 1967; Burgess, 1969); it is now possible to obtain highly purified enzyme in lots of several milligrams. This paper describes the analysis of preparations of such enzyme from *E. coli* D-10 which were intended for use in studies of initiation of transcription. They appeared to be >95% pure on sodium dodecyl sulfate–polyacrylamide gels and were analyzed for a number of potential contaminating activities. No phosphatase activity was detected while the amount of deoxyribonuclease as measured in a sensitive assay was shown to be insignificant.

The preparations used in this study were found to incorporate both adenine and γ-phosphate from ATP into acid-insoluble form in the absence of added template. There were two reasons for studying these reactions more closely. Firstly it was possible that the enzyme was being phosphorylated and adenylated. It has been suggested from kinetic data and from the effects of various inhibitors that RNA polymerase may undergo a number of transitions during initiation (Fuchs *et al.*, 1967; Zillig *et al.*, 1970). There are several well-known cases in which phosphorylated or adenylated enzymes are intermediates in a reaction sequence (Krebs and Fischer, 1962; Little *et al.*, 1967; Shapiro *et al.*, 1967a,b). Goff and Weber (1970) have shown that RNA polymerase does possess sites at which the covalent addition of AMP can occur *in vivo* after T4 infection. The second possible explanation for incorporation of adenine and γ-phosphate was that the enzyme preparations were contaminated by polynucleotide phosphorylase and polyphosphate kinase. It was shown that this was the case.

Methods are described for the control or removal of these activities which can lead to misinterpretation of studies of initiation. In particular any experiments which presume to measure the initiation of RNA synthesis by following the incorporation of the γ-phosphate of ATP into acid-insoluble form (for example, Bautz and Bautz, 1970) must consider the possible artifacts caused by small amounts of contaminating polyphosphate kinase.

Materials and Methods

Cells. Frozen cells of *E. coli* D-10 (one-fourth log phase) were obtained from Grain Processing Corporation, Muscatine, Iowa, and of *E. coli* B ATCC 11303 (midlog phase) from General Biochemicals, Chagrin Falls, Ohio.

RNA Polymerase Assay. The enzyme was assayed in 0.25 ml of 0.05 M Tris (pH 7.9), 0.008 M $MgCl_2$, 0.0001 M Cleland's reagent, and nucleoside triphosphates (0.2 mM each; radioactive ATP, 1 Ci/mole) in the presence of 10–100 μg of DNA, for 10 min at 37°. Acid-precipitable counts were collected on nitrocellulose (25 mm B-6, Schleicher and Schuell) or glass fiber (Whatman GFC) filters. The filters were washed with 5 × 10 ml volumes of 10% trichloroacetic acid, and counted. The GFC filters gave a lower background (about 50% of the nitrocellulose) which could be further reduced by making the trichloroacetic acid solution 0.1 M in sodium pyrophosphate and 0.05 mM in ATP. In some assays 0.8 mM K_2HPO_4 and 0.05 mM ADP were added to inhibit polynucleotide phosphorylase and polyphosphate kinase, respectively. High salt assays contained 0.15 M KCl in addition. One unit of enzyme activity is that which incorporates 1 nmole of ATP/10 min at 37° into acid-insoluble form in the low salt

† From the Division of Biology, California Institute of Technology, Pasadena, California. *Received June 30, 1971.* This is a report of work supported in part by the Lucy Mason Clark Fellowship of the Division of Biology, California Institute of Technology, and in part by U. S. Public Health Service Grant 13762.

‡ Present address: Department of Genetics, Trinity College, Dublin, Ireland.

TABLE I: Yield of Enzyme from *E. coli* D-10.[a]

Stage	Protein (mg)	Total Act. (Units)	Sp Act. (Units/mg)	Estimated Purity (%)
F1		14,000		
F3	35	10,500	300	
Low-salt glycerol gradient	15	9,000	600	
High-salt glycerol gradient	9	9,000	1000	95

[a] Data for 100 g wet weight of cells.

reaction mixture in the presence of excess T7 DNA. The concentration of purified enzyme was estimated from the extinction coefficient, $E_{280}^{1\%} = 6.5$ (Richardson, 1966).

Preparation of RNA Polymerase. Lysis of cells, removal of nucleic acids, and ammonium sulfate fractionation were carried out essentially according to Chamberlin and Berg (1962) to produce the equivalent of their fraction 3. Four methods were used to obtain highly purified material from the F3.

SEDIMENTATION IN LOW-SALT GLYCEROL GRADIENTS. The F3 enzyme was dissolved in 0.01 M Tris (pH 7.9), 0.01 M $MgCl_2$, 0.002 M β-mercaptoethanol, and 0.012 M $(NH_4)_2SO_4$, layered on 60 ml of a 10–30% glycerol gradient in the same buffer, and centrifuged at 25,000 rpm for 36 hr in the Spinco SW 25.2 rotor. Two- to five-milliliter amounts containing up to 100 mg of protein were layered on each gradient.

SEDIMENTATION IN HIGH-SALT GLYCEROL GRADIENTS. The 10–30% glycerol gradients were 0.01 M Tris (pH 7.9), 0.01 M $MgCl_2$, 0.0001 M dithiothreitol, and 1.0 M KCl. Sedimentation was for 45 hr at 25,000 rpm in the SW 25.2 rotor for 10 mg of protein/tube and 60 hr for 20 mg.

DNA CELLULOSE CHROMATOGRAPHY. DNA cellulose was prepared from calf thymus DNA (Sigma Type I) and Whatman CF11 cellulose according to Litman (1968). Eight to ten milligrams of DNA was bound per g of cellulose. F3 (400 mg) in 0.15 M KCl, 0.01 M Tris (pH 7.9), 0.0005 M dithiothreitol, 0.0001 M EDTA, and 25% glycerol was applied to a column of 2 × 40 cm and eluted by 300 ml of a gradient from 0.15 to 1.0 M KCl.

PHOSPHOCELLULOSE CHROMATOGRAPHY was carried out according to Burgess (1969).

Preparation of DNA. T7 virus was purified on CsCl gradients and the DNA extracted with freshly distilled phenol. Seventy per cent of the DNA single strands had an $s_{20,w}$ of 38 S, behaving therefore as complete unknicked molecules (Studier, 1965). *E. coli* DNA and rat ascites tumor DNA were purified by the Marmur (1961) procedure with additional phenol extractions. Final precipitation was with 0.54 volume of isopropyl alcohol in the presence of 0.3 M sodium acetate to select molecules greater than 35 S.

Enzyme Assays. Ribonuclease activity was measured by the reduction in the amount of acid insoluble RNA using *in vitro* generated RNA as substrate.

Deoxyribonuclease was measured by the conversion of unknicked closed circular super-coiled SV-40 DNA (component I) to the knicked form (component II) which sediments more slowly (Bauer and Vinograd, 1968). Routinely 20 μg of SV-40 DNA was incubated at 37° for 15 min with 50 μg of RNA polymerase preparation. EDTA was added to prevent further activity (of DNase I, for example) and the samples were loaded in the synthetic boundary cells and sedimented at 25° through 2.8 M CsCl (pH 7.0) in the Beckman Model E. The assay was most sensitive since the hydrolysis of a single phosphodiester bond changed the *s* value of a molecule.

Polynucleotide phosphorylase generates polynucleotides from ribonucleoside diphosphates (Grunberg-Manago and Ochoa, 1955). Since all samples we have tested of ribonucleoside triphosphates contain about 10% diphosphates the RNA polymerase assay mixture can be used for measuring polynucleotide phosphorylase activity. No template is required and only a single nucleotide. Polyphosphate kinase synthesizes polyphosphate from the γ-phosphate of ATP. The standard assay was 50 μg of protein in 0.25 ml of 0.01 M Tris (pH 7.9), 0.01 M $MgCl_2$, 0.01 M β-mercaptoethanol, and 0.2 mM γ-^{32}P-labeled ATP. Acid-precipitable material was collected and measured.

Phosphatase, adenosine triphosphate phosphatase, and guanosine triphosphate phosphatase were assayed according to Martin and Doty (1949).

Assay for the Presence of σ. The presence of σ was determined from sodium dodecyl sulfate–polyacrylamide gels and by the relative activity of the RNA polymerase on T7 phage DNA and *E. coli* DNA. In the absence of σ the template activities of *E. coli* DNA and T7 DNA are equal while in the presence of σ the T7 DNA was at least eight times more effective than the *E. coli* DNA.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide electrophoresis on 5-cm gels was carried out according to Shapiro *et al.* (1967). After staining with Coomassie Blue the gels were scanned using a Gilford spectrophotometer at 600 mμ. Gels were fractionated for scintillation counting by squeezing through a 20-gauge needle from a 1-ml syringe. Fractions were collected in scintillation vials and 1 ml of Nuclear-Chicago solubilizer was added. They were shaken gently at room temperature for 30 min to extract the gels and then 10 ml of toluene-based scintillation fluid was added and the samples were counted.

Results

Preparation of RNA Polymerase. The results of a single preparation of enzyme from *E. coli* D-10 are summarized in Table I. Cells (600 g) were brought to the F3 stage in 1 day yielding 200 mg of protein with a specific activity of 300 units/mg. This was applied to a single SW 25.2 rotor for low-salt glycerol gradient sedimentation. Ninety per cent of the activity applied was recovered from a distinct peak in the center of the gradient. The fractions were combined, precipitated with 60% saturated $(NH_4)_2SO_4$, dissolved, and applied to a high-salt glycerol gradient where it sedimented with an $s_{20,w}$ of 13 S (Figure 1). The peak fractions were pooled and the enzyme precipitated, was dissolved in storage medium at 5 mg/ml and stored at −20°. This enzyme was used for most of the experiments described in this paper. It had a specific activity of 1000 units/mg and contained only the β', β, σ, α, and ω bands on sodium dodecyl sulfate–polyacrylamide gels as shown in Figure 2. It was estimated to be >95% pure from scans of the stained gels. It was stable for over 1 year. It contained no measurable ribonuclease or phosphatase activity.

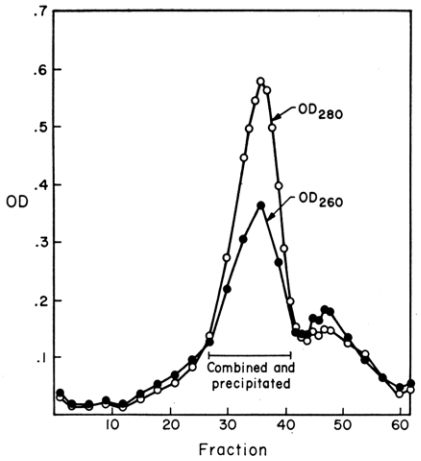

FIGURE 1: High-salt glycerol gradient sedimentation of *E. coli* D-10 RNA polymerase, after previous sedimentation through a low-salt glycerol gradient. RNA polymerase solution (1 ml) in low-salt buffer containing approximately 10 mg of protein was layered on 60 ml of a 10–30% glycerol gradient in high-salt buffer (1 M KCl etc.) and centrifuged at 22,500*g* for 45 hr at 4° in the SW25.2 rotor. Fractions 27–41 were combined and gave an enzyme of specific activity 1000 units/mg on T7 DNA. The enzyme is referred to a G-G-A.

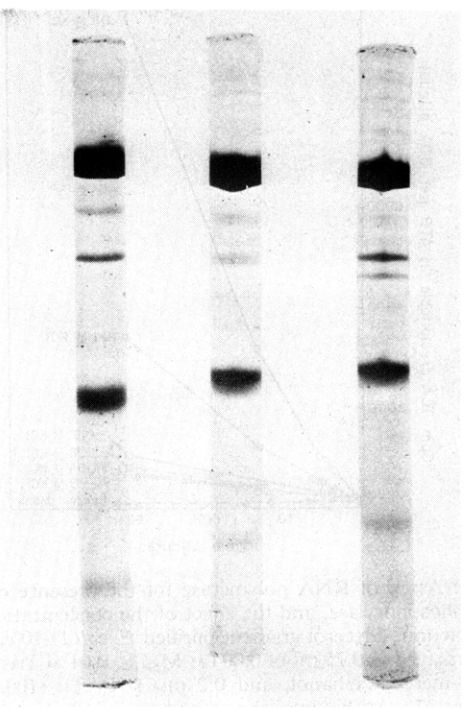

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gels of RNA polymerase preparations (left to right) G-G-A, DC.C., and DC.D. The final steps in purification of these enzymes were high-salt glycerol gradient sedimentation (G-G-A) shown in Figure 1, and DNA cellulose chromatography (DC.C. and DC.D.) shown in Figure 3. The left-hand gel was run at different time from the other two, G-G-A was prepared from *E. coli* D-10 and the others from *E. coli* B.

The method described above has worked well and reproducibly for *E. coli* D-10 one-quarter log-phase cells. With different cells (*E. coli* B) this procedure was not as successful, the major problem being the large amount of protein of low specific activity recovered at the F3 stage. Chromatography on DNA cellulose is effective for dealing with large amounts of low specific activity material. It can also be used for raising the specific activity of relatively pure preparations (Figure 3) as it partly separates high and low specific activity RNA polymerase. Figure 3 shows that the enzyme activity eluting from DNA cellulose was skewed to the trailing edge of the optical density peak. The fractions were combined as shown and sodium dodecyl sulfate gels run on each sample (Figure 2). The specific activities of DC.C and DC.D were respectively 170 and 850 units per mg. DC.D contained approximately three times the amount of σ factor as DC.C, which probably accounts for the difference in specific activities.

Activity of DNase in Polymerase Preparations. Activity was assayed at high concentrations of RNA polymerase (220 μg/ml) and SV-40 DNA component I (100 μg/ml). (Most experiments on the enzyme were conducted at or below this concentration.) In this assay 50% of the SV-40 component I was converted to component II in 20 min at 37°. The total number of knicks produced per molecule of DNA can be calculated from the Poisson distribution $n_x = Ne^{-m}$, where n_x is the number of molecules with x knicks, m is the average number of knicks per molecule, and N is the total number of molecules. For this assay $m = 0.69$.

Contamination by Polynucleotide Phosphorylase. All preparations of RNA polymerase tested incorporated the adenine residues of commercial ATP into acid-precipitable form in the absence of added template and other nucleoside triphosphates (Figure 4). The reaction was strongly stimulated by increasing the salt concentration, and showed an initial lag phase which was especially obvious at high salt. The reaction rate continued to increase up to 0.2 M KCl. The kinetics were unlike those observed for the synthesis of poly(A) by RNA polymerase which reaction shows no lag phase. They were however rather similar to those displayed by polynucleotide phosphorylase of *Azotobacter vinelandii* (Ochoa and Mii, 1961), *M. lysodeikticus* (Singer and Goss, 1962) and *E. coli* (Kimhi and Littauer, 1968) in the absence of oligonucleotide primer.

The following evidence suggests that the reaction was in fact due to contaminating polynucleotide phosphorylase. (1) All samples of commercial ATP which were analyzed

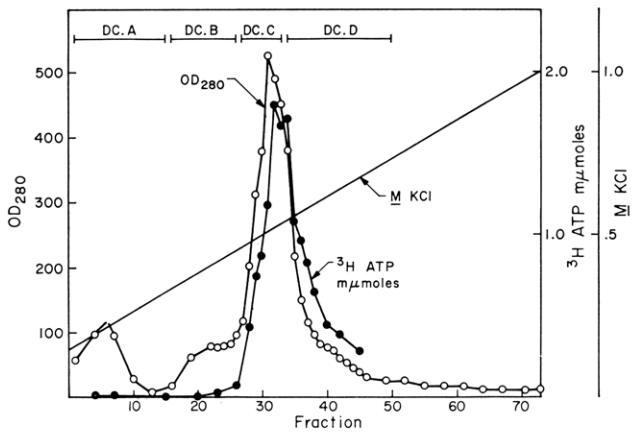

FIGURE 3: DNA cellulose chromatography of a sample of *E. coli* B RNA polymerase which had a specific activity of 350 before this step. Protein (40 mg) was applied in buffer at 0.15 M KCl to a column 1.5 × 30 cm and eluted with a linear gradient (300 ml) from 0.15 to 1.0 M KCl at 15-ml/hr flow rate. Fractions were assayed for RNA polymerase activity (10-μl aliquots) in the presence of 35 μg of ascites tumor DNA in the standard assay 0.15 M KCl + 0.0008 M K_2HPO_4. Fractions were combined as illustrated and the specific activities on T7 DNA for the samples C and D were 170 and 850 units per mg, respectively, after ammonium sulfate precipitation.

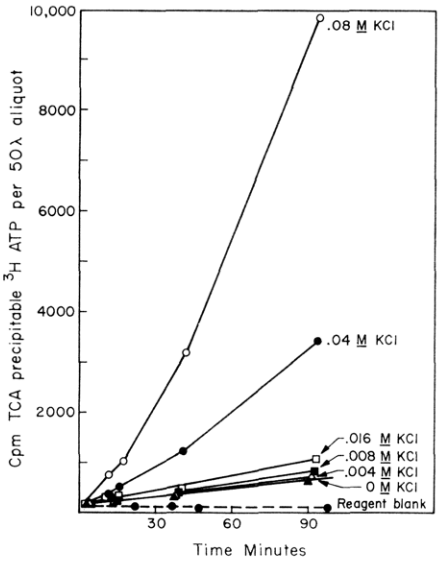

FIGURE 4: Assay of RNA polymerase for the presence of polynucleotide phosphorylase, and the effect of the concentration of KCl on the reaction. Glycerol gradient purified *E. coli* D-10 enzyme (50 μg) was assayed in 0.25 ml of 0.004 M $MgCl_2$, 0.01 M Tris (pH 7.9), 0.01 M β-mercaptoethanol, and 0.2 mM [^{3}H]ATP (100,000 cpm/mmole), at 37°. At the times shown aliquots of 50 μl were removed and precipitated with 10% CCl_3COOH. Samples contained from 0 to 0.08 M KCl. A reagent blank contained no enzyme.

were found to contain 5–10% ADP, a substrate of polynucleotide phosphorylase. (2) The reaction was totally inhibited by 0.8 mM K_2HPO_4 as expected for polynucleotide phosphorylase. (3) The activity separated from the bulk of the RNA polymerase when chromatographed on a phosphocellulose column (Figure 5 and Table II). The run-off contained 100% of the polynucleotide phosphorylase activity. This fraction was the only one which contained a significant band on a sodium dodecyl sulfate–polyacrylamide gel (Figure 6) of molecular weight of approximately 75,000. This is similar to the value of 65,000 reported by Klee (1969) for the subunit of polynucleotide phosphorylase.

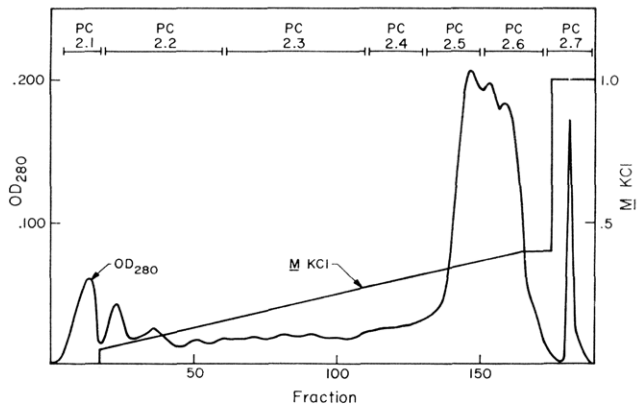

FIGURE 5: Separation of the σ subunit and the core enzyme of RNA polymerase by phosphocellulose chromatography. Protein (10 mg) (glycerol gradient purified enzyme) was applied according to Burgess (1969) to a phosphocellulose column (1 × 7 cm) equilibrated and eluted with 100 ml of gradient from 0.05–0.4 M KCl followed by 1.0 M KCl. Fractions were combined as shown, precipitated with ammonium sulfate. Figure 6 is a photograph of sodium dodecyl sulfate–polyacrylamide gels for these samples PC 2.1–PC 2.7.

TABLE II: RNA Polymerase, Polynucleotide Phosphorylase, and Polyphosphate Kinase Activities of Fractions from the Phosphocellulose Column (Figure 8).

Fraction[a]	RNA Synthesis (nmoles of AMP/10 min per mg)	Polyphosphate Synthesis (nmoles of PO_4/10 min per mg)	Polynucleotide Synthesis[c] (nmoles of AMP/60 min per mg)
PC 2.1	116 (100)[b]	27.5 (100)[b]	8.0 (100)[b]
2.2	38 (33)	0 (0)	(0)
2.3	174 (150)	0 (0)	(0)
2.4	315 (270)	12.5 (45)	(0)
2.5	403 (350)	8.3 (30)	(0)
2.6	360 (310)	4.4 (16)	(0)
2.7	91 (78)	246.0 (900)	(0)

[a] The fractions from the phosphocellulose column (Figure 8) were assayed for RNA synthesis, polyphosphate synthesis, and polynucleotide synthesis by polynucleotide phosphorylase. RNA synthesis was assayed in 0.25 ml of 0.01 M Tris (pH 7.9), 0.01 M $MgCl_2$, 0.01 M β-mercaptoethanol, 0.4 mM CTP, UTP, GTP, and [^{14}C]ATP (5000 cpm/nmole) with 25 μg of *E. coli* DNA as template. The reaction was at 37° for 10 min. Polyphosphate synthesis and the activity of polynucleotide phosphorylase were assayed in the same reaction (0.25 ml) of 0.004 M $MgCl_2$, 0.01 M Tris (pH 7.9), 0.01 M β-mercaptoethanol, and 0.2 mM ATP (90,000 cpm of ^{32}P/nmole of γ-phosphate and 52,000 cpm of ^{3}H/nmole of adenine) incubated at 37°. A time course from 0 to 120 min was plotted after taking four 50-μl aliquots and precipitating with trichloroacetic acid. [b] Values in brackets are normalized to the value of PC 2.1. [c] By polynucleotide phosphorylase.

There was no evidence that the adenine residues were being covalently attached to any of the subunits of RNA polymerase. When reaction mixtures were electrophoresed on sodium dodecyl sulfate–polyacrylamide gels the [^{3}H]adenine residues were not associated with any of the subunits of RNA polymerase.

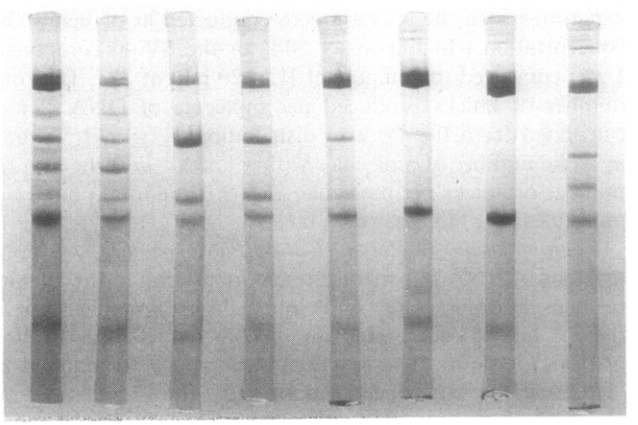

FIGURE 6: Sodium dodecyl sulfate–polyacrylamide gels of the samples from the phosphocellulose column (Figure 5) after staining with Coomassie Blue. Samples were left to right 10 μg of glycerol gradient enzyme (G-G-C), and phosphocellulose enzymes PC 2.1–PC 2.7.

TABLE III: Effect of Antibiotics and Inhibitors on Polyphosphate Synthesis.[a]

Inhibitor	Concn	nmoles of CCl_3COOH-Precipitable PO_4/10 min per mg of Enzyme	% Act.
		1.2	100
Actinomycin D	20 μg/ml	1.2	100
Chloramphenicol	40 μg/ml	1.2	100
Rifampicin	20 μg/ml	1.2	100
Sodium azide	0.08%	0.9	75

[a] A highly purified RNA polymerase preparation, G-G-A (100 μg), was assayed for contaminating polyphosphate kinase in 0.5 ml of 0.01 M Tris (pH 7.9), 0.004 M $MgCl_2$, 0.01 M β-mercaptoethanol, 0.13 mM [2-^{32}P]ATP, and 0.2 mM each of CTP and GTP at 37°. Antibotics were added when shown in the table. Aliquots of 50 μl were precipitated at times between 0 and 30 min with CCl_3COOH, and the precipitates were collected, washed on nitrocellulose membranes, and counted. The incorporation at 10 min was abstracted from the time course and tabulated as a per cent of the incorporation in the absence of antibiotic.

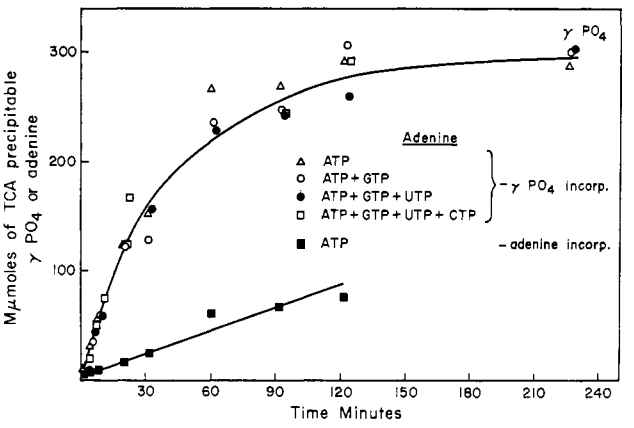

FIGURE 7: Assay of polyphosphate kinase in an RNA polymerase preparation. Glycerol gradient purified D-10 enzyme (200 μg) was assayed in 0.5 ml of 0.1 M Tris (pH7.9), 0.004 M $MgCl_2$, 0.01 M β-mercaptoethanol, and 0.2 mM [^{32}P]- and [^{3}H]ATP, at 37°, in the presence of various nucleoside triphosphates. Samples were (1) ATP alone, (2) ATP + GTP, (3) ATP + GTP + UTP, and (4) ATP + GTP + UTP + CTP. Additional nucleotides were each 0.2 mM. At times shown aliquots of 50 μl were removed and precipitated with 10% CCl_3COOH. Time courses are plotted for γ-PO_4 incorporation for each reaction, and for adenine for reaction 1. (Rates were very similar for 1, 2, 3, and 4.) Specific activities were 8000 and 12,000 cpm per nmole for [γ-^{32}P]- and [^{3}H]adenine, respectively.

Synthesis of Polyphosphate. REACTION. In the absence of DNA, highly purified preparations of RNA polymerase incorporated the γ-phosphate from ATP into acid-precipitable form (Figure 7). The molar incorporation of the γ-phosphate was greatly in excess of the adenine in this experiment where both [γ^{32}P]ATP and [^{3}H]ATP were present as substrates. Hydrolysis of the ATP must therefore have occurred. The incorporation of the ^{3}H label displayed kinetics which were quite different from those of the γ-phosphate, and for the reasons already outlined it was concluded that the former represented the activity of polynucleotide phosphorylase.

The two activities were separated from one another on phosphocellulose (Figure 5 and Table II). The rate of incorporation of phosphate was 3.25 μmoles/10 min per mg of enzyme. It rose to a saturation value between 60 and 120 min of 15 nmoles or one-twelfth of the γ phosphate of ATP in the reaction. Maitra and Hurwitz (1969) observed a similar reaction at a similar rate (4 nmoles/2 hr). It was variable, however, possibly because of differences in the concentration of ADP in the ATP samples. ADP is a powerful inhibitor of the reaction, as shown below.

INHIBITOR STUDIES. The kinetics were rapid, which did not suggest that the reaction was caused by bacterial growth during the incubation. This was confirmed by the fact that chloramphenicol (40 μg/ml), actinomycin D (20 μg/ml), or sodium azide (0.08%) were not inhibitors (Table III). Other nucleoside triphosphates had no effect (Figure 7). Rifampicin, which is a specific inhibitor of *E. coli* polymerase (Hartmann *et al.*, 1967) interacting with the core subunits (di Mauro *et al.*, 1969) and preventing chain initiation (Sippel and Hartmann, 1968), did not inhibit the selective incorporation of γ-phosphate (Table III). *E. coli* DNA (50 μg) caused inhibition, reducing the amount incorporated by 50 μg of enzyme at saturation by a factor of 8 (Figure 8). The DNA also caused the synthesis of poly(A), which depleted the ATP pool. However this was not responsible for all of the inhibition since 30 min after the incorporation of phosphate had ceased, 90% of the ATP still remained. Furthermore, 50 μg of DNA caused 88% inhibition of the incorporation of phosphate at saturation while 37.5 μg of DNA caused 70% inhibition, but both gave the same amount of poly(A) synthesis. It was concluded therefore that the DNA had an inhibitory effect above and beyond the depletion of the ATP pool.

High concentrations of KCl inhibited the reaction completely, while both potassium phosphate and sodium pyro-

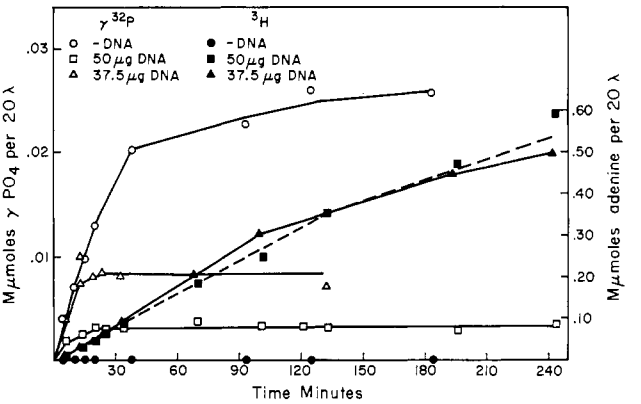

FIGURE 8: The effect of DNA on the activity of polyphosphate kinase in an RNA polymerase preparation. Highly purified D-10 RNA polymerase G-G-A (50 μg) was assayed for polyphosphate kinase in 25 ml of 0.01 M Tris (pH 7.5), 0.004 M $MgCl_2$, 0.01 M β-mercaptoethanol, 0.2 mM GTP, 0.2 mM UTP, and 0.2 mM ATP. The ATP was labeled in the γ-phosphate (260,000 cpm of ^{32}P/nmole) and in the adenine (2150 cpm of ^{14}C/nmole). The samples contained (i) no DNA, (ii) 37.5 μg of DNA, and (iii) 50 μg of DNA. *E. coli* DNA was used. The reaction was carried out at 37°; aliquots of 20 μl were removed at the times shown and precipitated with 10% CCl_3COOH, and the precipitates were collected and washed on nitrocellulose membranes.

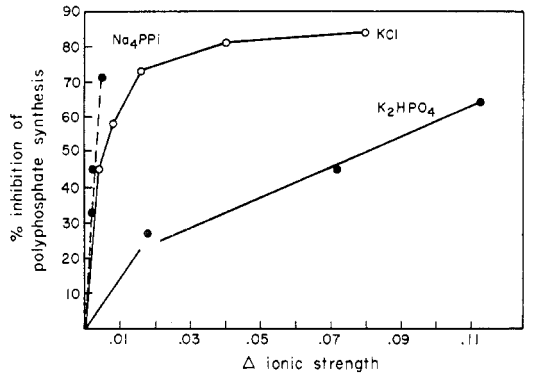

FIGURE 9: The effect of KCl, K_2HPO_4, and sodium pyrophosphate on the activity of polyphosphate kinase in highly purified RNA polymerase preparations. The figure shows the per cent inhibition of the amount of reaction at 10 min *vs.* the change in ionic strength due to the added salt.

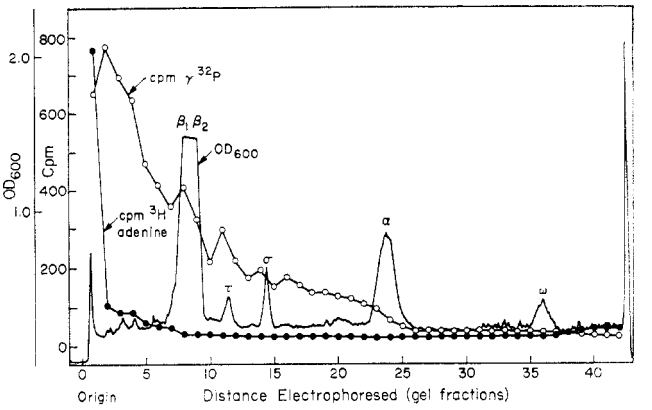

FIGURE 10: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of a fraction eluted from a P-60 column as described in the text. 6000 counts of γ-^{32}P and 1000 counts of ^{3}H were applied to each of three 5-cm gels, and electrophoresed at 45 V for 75 min. Pure enzyme (5 μg) was added to gel 2 as a marker. Gel I was sectioned immediately after electrophoresis (to avoid loss of sample during staining) and the fractions were counted. Gels 2 and 3 were stained, destained, scanned, and then counted. All gels gave the same profile of counts as shown in this figure for gel I, and no loss of counts in fact occurred during staining. 90% of the counts applied were recovered. The OD_{600} profile shows the subunits of the RNA polymerase.

phosphate were also inhibitory. When the per cent inhibition was plotted *vs.* Δ, ionic strength (Figure 9), it was clear that the pyrophosphate was a very effective inhibitor at low molar concentrations (0.2–0.5 mM). ADP stopped the reaction completely at 0.2 mM.

PRODUCT OF THE REACTION. Several enzymes were tested for their ability to degrade the product of the reaction between the RNA polymerase preparation and [γ-^{32}P]ATP in the absence of DNA. RNA polymerase (100 μg) was added to 0.5 ml of reaction mix containing 0.2 mM [γ-^{32}P]ATP under standard conditions. After 90 min, during which 4 $\times$ 50 μl aliquots were taken to measure the progress of the reaction, the tubes were placed in ice, and three further aliquots were removed and added to 50 μl of Pronase (6 mg/ml), ribonuclease (4 mg/ml), or water. These were returned to 37° and incubated for a further 30 min. Pronase caused a 25% reduction in trichloroacetic acid precipitable γ-^{32}P, while the ribonuclease-treated sample showed a 10% increase, neither of which was judged to be very significant in view of the high concentration of enzyme added. If DNase was added at zero time to a reaction, no effect was observed. The product was degraded 100% by alkaline phosphatase or phosphodiesterase.

SIZE OF THE PRODUCT. Three approaches were made to analyze the size of the phosphate compound: glycerol gradient sedimentation, gel filtration, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

For band sedimentation 50 μg of RNA polymerase preparation was added to a standard reaction mix: 0.1 mM [γ-^{32}P]- and [^{3}H]ATP, 0.1 mM GTP, 0.1 mM UTP, 0.01 M Tris (pH 7.9), 0.004 M $MgCl_2$, and 0.01 M β-mercaptoethanol, in 0.25 ml. After 90 min at 37°, the reaction was cooled and dialyzed exhaustively against 0.01 M Tris (pH 7.9), 0.004 M $MgCl_2$, and 0.01 M β-mercaptoethanol. Of the counts remaining after dialysis 20% was precipitable by 10% trichloroacetic acid, and the material was completely stable when stored at 4° for 18 hr. A total of 6000 counts was applied to each of two glycerol gradients, 10–30% glycerol, 0.01 M Tris (pH 7.9), 0.004 M $MgCl_2$, and 0.01 M β-mercaptoethanol, one of which was 0.5 M KCl. A third tube contained a sample of RNA polymerase in the low-salt gradient. Sedimentation was for 10 hr at 50,000*g* at 4°. Seventy per cent of the counts applied was recovered, and for both the high- and low-salt gradients they had scarcely moved from the top, the peak being in fraction 33. The RNA polymerase activity had sedimented to fraction 16. The enzyme was therefore not associated with the γ-phosphate. A similar sample was applied to a P-60 column, 45 $\times$ 0.7 cm, without dialysis, and eluted with 0.01 M Tris (pH 7.5), 0.01 M β-mercaptoethanol, and 0.004 M $MgCl_2$. Sixty-eight per cent of the trichloroacetic acid precipitable γ-phosphate was eluted in the void volume with the peak of OD_{280} of the RNA polymerase. Since from the glycerol gradients, it was known that the enzyme was not associated with the phosphate it was concluded that a high proportion of the phosphate had itself sufficient molecular weight to elute in the void volume.

Aliquots of the peak fraction in the void volumes were electrophoresed on sodium dodecyl sulfate–polyacrylamide gels (5%) for 75 min at 45 V, as described in Materials and Methods section. The gels were stained with Coomassie Blue to locate the subunits of the RNA polymerase, then fractionated and counted. Figure 10 shows the analysis of such a gel. The ^{32}P was distributed heterogeneously and was not associated exclusively with any subunit of the RNA polymerase. The ^{3}H label represented poly(A) synthesized by polynucleotide phosphorylase. It did not run with the phosphate or the enzyme subunits. One-hundred per cent of the counts applied was recovered.

SEPARATION OF THE PHOSPHATE INCORPORATION FROM RNA POLYMERASE ACTIVITY. The activities of phosphate incorporation and RNA synthesis cochromatograph on DEAE-cellulose. They did not separate completely on a high-salt glycerol gradient where the RNA polymerase activity moved a little faster. Chromatography on phosphocellulose (Figure 5) was a partially effective method of separation. Subfractions PC 2.1 to PC 2.7 were assayed for RNA synthesis and phosphate incorporation (Table II), and were analyzed on sodium dodecyl sulfate–polyacrylamide gels (Figure 6). The phosphate incorporation by 2.7 was extremely high, representing a specific activity of 250 nmoles/10 min per mg. This was 30 times the specific activity of the enzyme before phosphocellulose chromatography and more than 20 times the specific activity of any of the other fractions from the column except the run-off. The sodium dodecyl sulfate–polyacrylamide gel of frac-

tion 2.7 revealed two prominent bands between the σ and α subunits of RNA polymerase which did not show up on any of the other gels. The ω subunit was completely absent and σ was almost so. It is not known which of these features was responsible for the phosphate incorporation. The phosphocellulose fractions all contained the β and α subunits and except for 2.6 and 2.7 had significant amounts of σ. None of the fractions showed much RNA polymerase activity, since those with most core had least σ, but it was clear from what was observed and from the gels that specific activity increased from 2.1 to 2.6 and then fell slightly at 2.7. Some fractions showing significant RNA synthesis showed no phosphate incorporation, *e.g.*, 2.2 and 2.3. These were the fractions with the highest proportion of σ.

Discussion

Preparation of RNA Polymerase. Of the methods published recently that reported by Burgess (1969) has been most useful. It differs in two important respects from the procedure described here, firstly in the strain of *E. coli* and secondly in the method of removing DNA.

Burgess has used *E. coli* K-12, grown to three-quarters log phase at 37°. This gave a final yield of enzyme of 27 mg/100 g of cells, a recovery of 56%. This is nearly three times the yield which is obtained from *E. coli* D-10 (recovery 65%), in a procedure which is not very different. Large differences have been observed for the yield and recovery between D-10 and B preparations. It seems possible that K-12 is significantly better than either of these.

After ammonium sulfate fractionation large amounts of F3 were most conveniently handled by a DNA cellulose column. Sixty per cent of the protein which stuck at 0.15 M KCl was RNA polymerase, similar to the observation of Bautz and Dunn (1969). The specific activity after DNA cellulose chromatography of F3 was greater than 500 on T7 DNA. Small amounts of F3 (up to 200 mg) were conveniently purified by low- and high-salt glycerol gradient centrifugation.

No loss of σ occurs on DNA cellulose as on phosphocellulose which has not been used extensively for this reason. After DNA cellulose chromatography, high-salt glycerol gradient sedimentation were effective means of final purification. None of the enzyme preparations contained stoichiometric quantities of σ. The best preparations had molar ratios $\sigma/\beta' = 0.3$. It may be necessary to add σ to such enzymes to inhibit initiation by core enzyme.

Ribonuclease and phosphatase were not observed in any of the highly purified preparations. The DNase activity was very low. Polynucleotide phosphorylase was a measurable contaminant, but several procedures are easily employed to control or remove it. The addition of 0.8 mM phosphate to each assay was used as the most convenient method. The incorporation of γ-phosphate from ATP by RNA polymerase preparations in the absence of template was investigated as possibly representing the formation of a phosphoenzyme intermediate. Since the product of the reaction was heterogeneous, did not cosediment with RNA polymerase in a glycerol gradient, did not electrophorese with any of the subunits in the presence of sodium dodecyl sulfate, and represented an average of eight phosphates incorporated per polymerase molecule, this explanation was unlikely. The activity was almost completely separated from RNA polymerase on phosphocellulose although both activities did cochromatograph on DEAE-cellulose. It was not inhibited by rifampicin nor by deoxyribonuclease. The reaction bears many resemblances to that catalyzed by polyphosphate kinase, being inhibited by ADP and pyrophosphate in a similar manner (Kornberg *et al.*, 1956). The product was digested by alkaline phosphatase and phosphodiesterase but not by ribonuclease. The reduced yield of product after pronase treatment is explained by the fact that polyphosphate is acid precipitable only in the presence of coprecipitant (Kornberg *et al.*, 1956).

It is most important that this reaction should be controlled since it will cause misinterpretation of data concerning initiation, tending to overestimate the number of chains carrying a 5′-ATP residue. GTP does not act as a substrate for polyphosphate kinase. In these preparations approximately 1% of the γ-PO_4 of ATP (0.2 mM) was incorporated into polyphosphate in 15 min (Figure 7). The specific activity of the preparations for polyphosphate synthesis was approximately that of crude lysates reported by Kornberg *et al.* (1956). Where templates such as T7 or T4 are used which probably have only a small number of promoters (Bautz and Bautz, 1970) such that the incorporation into RNA of the γ-phosphate is small anyway, then contamination by polyphosphate kinase will probably cause a very significant error. It is fortunate that the reaction is strongly inhibited by KCl which is usually included in the RNA polymerase assay. This is a further reason for doing so. DNA is also an inhibitor, presumably behaving similarly to polyphosphate and binding to the enzyme. If further control is needed ADP can be added at 0.05 mM.

Acknowledgment

We wish to acknowledge the helpful counsel of our colleagues Stanley Froehner, John Mayfield, Richard Hyman, and Professor Norman Davidson.

References

Babinet, C. (1967), *Biochem. Biophys. Res. Commun. 36*, 925.
Bauer, W., and Vinograd, J. (1968), *J. Mol. Biol. 33*, 141.
Bautz, E. K. F., and Bautz, F. A. (1970), *Nature (London) 226*, 1219.
Bautz, E. K. P., and Dunn, J. J. (1969), *Biochim. Biophys. Acta 34*, 230.
Burgess, R. R. (1969), *J. Biol. Chem. 244*, 6160.
Chamberlin, M., and Berg, P. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 81.
di Mauro, E., Snyder, L., Marino, P., Lamberti, A., Cappo, A., and Tocchini-Valentini, G. P. (1969), *Nature (London) 222*, 533.
Fuchs, E., Millette, R. L., Zillig, W., and Walter, G. (1967), *Eur. J. Biochem. 3*, 183.
Furth, J. J., Hurwitz, J., and Anders, M. (1962), *J. Biol. Chem. 237*, 2611.
Goff, C. G., and Weber, K. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35*, 101.
Grunberg-Manago, M., and Ochoa, S. (1955), *J. Amer. Chem. Soc. 77*, 3165.
Hartmann, G., Honikel, K., Knusel, F., and Neusch, J. (1967), *Biochim. Biophys. Acta 145*, 843.
Kimhi, V., and Littauer, U. Z. (1968), *J. Biol. Chem. 243*, 231.
Klee, C. B. (1969), *J. Biol. Chem. 244*, 2558.
Kornberg, A., Kornberg, S. R., and Simms, E. S. (1956), *Biochim. Biophys. Acta 20*, 215.
Krebs, E. G., and Fischer, E. H. (1962), *Advan. Enzymol. 24*, 263.
Litman, R. M. (1968), *J. Biol. Chem. 43*, 6222.

Little, J. W., Zimmerman, S. B., Oshinsky, C. K., and Gellert, M. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 2004.
Maitra, U., and Hurwitz, J. (1967), *J. Biol. Chem. 242*, 4897.
Marmur, J. (1961), *J. Mol. Biol. 3*, 208.
Martin, J. B., and Doty, P. M. (1949), *Anal. Chem. 21*, 965.
Ochoa, S., and Mii, S. (1961), *J. Biol. Chem. 236*, 3303.
Richardson, J. P. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1616.
Shapiro, B. M., Kingdon, H. S., and Stadtman, E. R. (1967a), *Proc. Nat. Acad. Sci. U. S. 58*, 642.
Shapiro, A. A., Viñuela, E., and Maizel, J. V., Jr. (1967b,) *Biochem. Biophys. Res. Commun. 28*, 815.
Singer, M. F., and Goss, J. K. (1962), *J. Biol. Chem. 237*, 182.
Sippel, A., and Hartmann, G. (1968), *Biochim. Biophys. Acta 157*, 218.
Stevens, A., and Henry, J. (1964), *J. Biol. Chem. 239*, 196.
Studier, F. W. (1965), *J. Mol. Biol. 11*, 373.
Zillig, W., Zachel, K., Rabussay, D., Schachner, M., Sethi, V. S., Palm, P., Heil, A., and Seifert, W. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35*, 47.

Polynucleotides Containing 2′-Chloro-2′-deoxyribose†

John Hobbs, Hans Sternbach, Mathias Sprinzl, and Fritz Eckstein*

ABSTRACT: Polynucleotide phosphorylase from *Micrococcus lysodeikticus* in the presence of Mn^{2+} accepts 2′-chloro-2′-deoxyuridine 5′-diphosphate as well as 2′-chloro-2′-deoxycytidine 5′-diphosphate as substrates. Poly(2′-chloro-2′-deoxyuridylic acid) (poly(Ucl)) and poly(2′-chloro-2′-deoxycytidylic acid) (poly(Ccl)) with *s* values of 7.1 and 10.5 S, respectively, are obtained. Both polymers are stable to alkali and pancreatic ribonuclease. Both are degraded by snake venom phosphodiesterase, spleen phosphodiesterase, DNase I, and micrococcal nuclease at rates considerably slower than for poly(rU) and poly(rC).

The role played by the functional group at the 2′ position of the sugar ring of polynucleotides, in determining structure, function and stability of these compounds, is not well understood (Cross and Crothers, 1971, and references cited therein). Modification of polynucleotides in this position might give some indication as to the factors involved. In addition to polynucleotides containing ribose and deoxyribose, the preparation and properties of homopolynucleotides containing 2′-*O*-methyl-2′-deoxyribose have been described (Bobst *et al.*, 1969; Zmudzka *et al.*, 1969a; Zmudzka and Shugar, 1970). While this manuscript was in preparation the synthesis of two more modified polynucleotides, poly(2′-azido-2′-deoxyuridylic acid) (Torrence *et al.*, 1972) and poly(2′-fluoro-2′-deoxyuridylic acid) (Janik *et al.*, 1972), was reported. The work described here was undertaken to determine the effect of substituting a chlorine atom at the 2′ position. The covalent radius of chloride, r_c^{Cl} = 0.99 Å, should approximate to the spatial bulk of the hydroxyl group (r_c^{O} = 0.74 Å, r_c^{H} = 0.28 Å) and therefore be a good substitute for the hydroxyl group. A recent X-ray study has shown that the crystal structure of 2′-chloro-2′-deoxyuridine (Ucl)[1] is very similar to that of uridine (Suck *et al.*, 1972). A further point of interest concerns the ability of the homopolynucleotide duplex, poly(rI·rC), to induce the formation of interferon and increase resistance to viral attack in cell cultures (Colby, 1971). It has been suggested that this property is a function both of the stability of the duplex (DeClercq and Merigan, 1969), and of its susceptibility to attack by nucleases (DeClercq *et al.*, 1969). A study of the chloro polymers, their stability to enzymic degradation and ability to form stable duplexes, thus provides an opportunity to test this hypothesis.

Materials and Methods

Synthesis of Nucleosides. The synthetic sequence followed for the preparation of Ucl and Ccl was essentially that of Codington *et al.* (1964) and Doerr and Fox (1967). However, 5′-*O*-trityl-O^2,2′-anhydrouridine was conveniently prepared in a single stage from 5′-*O*-trityluridine by treatment with diphenyl carbonate in dimethylformamide at 140° following a procedure by Ruyle and Shen (1967). Ccl was prepared as described below from 2′-chloro-2′-deoxy-4-thiouridine (s^4Ucl).

Synthesis of Nucleotides. Phosphorylation of nucleosides was carried out using $POCl_3$ as described for ribonucleosides by Yoshikawa *et al.* (1967). The method of Hoard and Ott (1965) employing carbonyldiimidazole was used to convert 5′-UclMP to 5′-UclDP; the method of Michelson (1964) employing diphenyl phosphorochloridate was used to prepare 5′-CclDP. The quantity of aUDP and aCDP, respectively, present as impurity was estimated as follows. A sample of the diphosphate (*ca.* 10 A_{260} units) in 100 μl of 0.1 M Tris·HCl (pH 8.9) was digested with alkaline phosphatase (10 μg) for 30 min and the products were applied to a paper chromatogram and developed in system A. The two spots obtained (Ucl, R_F 0.74; aU, R_F 0.65; Ccl, R_F 0.72; aC, R_F 0.67) were

† From the Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, Göttingen, Germany. *Received March 6, 1972.* Part of this work has been published in a preliminary report (Hobbs *et al.*, 1971). This work was supported by the Deutsche Forschungsgemeinschaft. J. H. thanks the Royal Society, London, for a European Programme award.

[1] The following abbreviations are used: Ucl, 2′-chloro-2′-deoxyuridine; Ccl, 2′-chloro-2′-deoxycytidine; s^4Ucl, 2′-chloro-2′-deoxy-4-thiouridine; aU, arabinosyluracil; aC, arabinosylcytosine; poly(Um), poly(2′-*O*-methyluridylic acid); poly(Cm), poly(2′-*O*-methylcytidylic acid); poly(Ucl), poly(2′-chloro-2′-deoxyuridylic acid); poly(Ccl), poly(2′-chloro-2′-deoxycytidylic acid); poly(Uz), poly(2′-azido-2′-deoxyuridylic acid); poly(Uf), poly(2′-fluoro-2′-deoxyuridylic acid); poly(Ua), poly(2′-amino-2′-deoxyuridylic acid).