INVESTIGATIONS ON (i) CHROMOSOMAL RIBONUCLEIC ACID OF ASCITES TUMOUR, (ii) RNA POLYMERASE OF E. COLI

Thesis by

David John McConnell

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy.

California Institute of Technology

Pasadena, California

1971

(Submitted 9th October, 1970)

To Janet with childe.

ACKNOWLEDGEMENTS

When Janet and I arrived in Pasadena carrying our suitcases we went straight to the laboratory, where I met Dr. Bonner whose first question was "Well, when can you start work?" I replied, with the deferment which a student must show to his Professor in Europe, "Whenever is convenient?" That was a mistake for his response was "How about right now?" So, at the end of a 6000 mile journey I was promptly dispatched in the capable care of Mike Dahmus who was instructed to find out if I could use a pipette, while Janet was whisked off to discover the Caltech Housing Office. I worked with Mike for the first year, and the first chapter of this thesis is a study started by him. During that time I learnt from him a great number of laboratory techniques, but more importantly, the standards he set for the work and the cautious judgements which he made were important influences in my subsequent research. My induction into the mysteries of molecular biology was mainly his doing, and the occasional tennis game, folk session and Sierra trip or the more common TGIF set the scene for the next four years. In the words of the local slang, in the gospel according to O'Casey, "no better man."

Apart from Mike and Grace too, I have enjoyed the company of many others who have been on their way through the group. In particular John Smart, John Mayfield, Sally

Elgin and Rick Firtel have always been ready to waste an afternoon in discussing female chauvinism, moonlighting, the Middle East, or the joys of a mathematician who has discovered biology, each his own topic but not in that respective order. In less serious moments all of these friends have never failed to offer perceptive and constructive criticism of the latest unexplicible experiment. My special thanks to Sally for instruction in and use of the electrophoresis apparatus. It has been helpful recently to have had another member of the group, Stan Froehner, trying to psyche out RNA polymerase. Apart from the empathy which we shared I have been able to charge some expensive substrates to his name, which saved me from occasional flurries, ruffles, eddies or what have you, raised by our local chartered accountant, Ingelore. She is indispensible and with incredible energy keeps the laboratory supplied and the machines running, and the measure of how much our research depends on her is what happens when she is away. Without Ludia and Sue life would have been duller.

Among many others I will not forget Yuriy Sivolap, Cornell Phillips or Anil Sadgopol who seemed to me to be special people whom I was lucky and privileged to meet and know, and with a little luck we will meet again.

Outside our group, it was a novel experience to take Bill Dreyer's seminar on "Anything but Advanced Biochemistry"- a sort of think-tank set up to design experiments for the

reasonably distant future. Caltech is peculiar maybe because it allows faculty and students to work together informally and enjoyably and this seminar was one of the best examples of that characteristic. Dr. Davidson has in this last year been invaluable as a guide and critic of my work, which to a large extent was derived from the earlier studies in his group by Richard Hyman. Richard was quite amazing and will stand in my memory as the uncommon man of the incisive wit. Recently, Louise Chou has carried out some studies on the T7 DNA-RNA polymerase complex which have helped to direct my own research on the same topic. Mike Raftery has always been willing to listen to a problem and to suggest an answer. I owe much to all of these people.

Above all I owe a great debt to Dr. Bonner who firstly as an author and later as my professor has fascinated and stimulated my interest in Biology. I am especially grateful to him for the huge freedom given to me in my research, which has sometimes by necessity deviated from the central interests of the group. This freedom has been beautiful, and has added to the fun and enjoyment and happiness which everyone experiences when here. His examples of living life fully, always reaching outwards for new ideas and new schemes, perceptively, constructively, and positively, these things for me are the "take-home lessons."

This thesis would never have been completed but for a little reason in my life, called Janet, who has had to put

up with a totally abnormal schedule, consistent grumblings about experiments which never seemed to work, and enough crazy ideas about what to do in the future to satisfy a domestic fiction writer. The first line of the song suffices- "Love is pleasin'."

Finally I want to say a secret thank-you to another group "the Committee for the Conservation of David", Judy, Sandy, Sally, Della, Betsy , and Karen who will understand what I mean.

ABSTRACT

PART I: When chromatin isolated from rat ascites cells is dissociated in the presence of high salt and the chromosomal proteins separated from the DNA by buoyant density centrifugation, a portion of the RNA contained in the chromatin remains associated with the chromosomal proteins. This RNA (chromosomal RNA) is characterized by its small size, $s_{20,w}$ = 3.3S, its high content of dihydroribothymidine and its ability to form hybrid with about 4% of the nuclear DNA. It appears to have no sequences in common with ascites transfer, ribosomal, or messenger RNA.

A class of RNA (cytoplasmic 3S RNA) with similar properties but associated with the cytoplasmic proteins has also been isolated. This RNA hybridizes to about 2% of the nuclear DNA and contains very few, if any, sequences not also contained in chromosomal RNA. This fraction of RNA is however unable to compete with about 50% of the sequence present in chromosomal RNA indicating that a large portion of chromosomal RNA is confined to the chromatin. A further class of RNA associated with the nuclear sap proteins appears to be identical to the RNA associated with cytoplasmic proteins.

A further class of RNA (nuclear 3S RNA) with hybridization properties similar to the cytoplasmic 3S RNA has been isolated from the nuclear sap. It hybridises to a lower extent than chromosomal RNA and is strongly competed by cytoplasmic 3S RNA.

PART II. RNA polymerase, more than 95% pure, has been prepared from E. coli D-10 and B. It is free from ribonuclease and phosphatase. It carries out poly A synthesis on E. coli or ascites tumour DNA but not on T7 DNA. Phosphate incorporation (TCA precipitable) from the γ phosphate of ATP in the absence of primer may be caused by a slight contamination with polyphosphate kinase. This can be controlled. Initiation of synthesis on T7, E. coli and ascites tumour DNA differ in the response to the σ sub-unit. RNA synthesized on T7 DNA can initiate with A or G. A salt- or rifampicin-stable complex between T7 DNA can be formed when a single nucleoside triphosphate (commercial reagent) is present, but requires a small amount of propagation. After purification of the nucleotides the efficiency of complex formation is greatly reduced.

TABLE OF CONTENTS

PART I.

INVESTIGATIONS ON CHROMOSOMAL RIBONUCLEIC ACID OF ASCITES CELLS.

Chapter I. "Chromosomal Ribonucleic Acid of Rat Ascites Cells." Publication: Dahmus, M.E., and McConnell, D.J., (1969). Biochemistry 8,1524.

Appendix. (i) Base Analysis of the Ascites Chromosomal Ribonucleic Acid in the RNA/DNA hybrid.

(ii) Extraction of Ascites 3 S Ribonucleic Acid from the Nuclear Sap.

Part I.

Chapter I.

CHROMOSOMAL RIBONUCLEIC ACID OF RAT ASCITES CELLS.

(Publication: Dahmus, M.E., and McConnell, D.J., (1969). Biochemistry 8, 1524.)

Chromosomal Ribonucleic Acid of Rat Ascites Cells

It was shown earlier that the chromosomal proteins dissociated by salt from pea bud chromatin contain associated RNA of a special class (Huang and Bonner, 1965). This RNA has been assigned the name chromosomal RNA and can be distinguished from previously reported classes of RNA by its small size and high content of dihydrouridine. It has been suggested that chromosomal RNA may play a key role in gene regulation by conferring specificity on the DNA–chromosomal protein interaction (Bonner and Huang, 1966; Bonner and Widholm, 1967; Bekhor *et al.*, 1969; Huang and Huang, 1969).

The following work shows that such an RNA is also associated with the chromosomal proteins of rat ascites cells.

Methods

Growth and Labeling of Cells. The Novikoff ascites tumor line used in the following investigation was maintained by serial transplantation in male albino Sprague–Dawley rats purchased from Berkeley Pacific Laboratories. The transfer was carried out on days 6 or 7 of the cycle. Cells for experimental use were harvested on days 6 and 7.

Uniformly ^{32}P-labeled chromosomal RNA was prepared from cells which had been grown for 24–48 hr in the presence of ^{32}P. Rats, infected 4–5 days prior with tumor, were given an intraperitoneal injection of carrier-free [^{32}P]orthophosphate (Volk Radiochemical Co.) in 0.5 ml of physiological saline (0.15 M NaCl). The standard injection of ^{32}P was 3 mCi/rat; dosages as low as 500 μCi and as high as 5 mCi/rat have been used.

For the preparation of ^{3}H-labeled DNA, 4-day-old tumor-infected rats were each injected with 1 mCi of [^{3}H]thymidine (14,600 μCi/μmole; Nuclear-Chicago) and the cells were harvested 48 hr later.

The tumor cells were purified from the ascites fluid by preferential lysis of the blood cells followed by differential centrifugation. The ascites fluid was diluted with an equal volume of TNKM (0.05 M Tris buffer (pH 6.7)–0.13 M NaCl–0.025 M KCl–0.0025 M $MgCl_2$) and centrifuged for 6 min at 700*g* in the International refrigerated centrifuge. The cells were then suspended in three volumes of deionized water and immediately centrifuged at 700*g*. The supernatant was then removed and the pellet was washed by repeated centrifugation until no contaminating erythrocytes were apparent. The purified cells were then used immediately or frozen in ethanol–Dry Ice and stored at −80°.

Preparation of Chromatin. The initial investigations were carried out on chromatin isolated in the presence of EDTA following the procedure of M. Nicolson (personal communication). The second procedure is a direct modification of the method of Marushige and Bonner (1966) and was developed to study the effect of the absence of EDTA on the isolation of chromosomal RNA. The two methods of extraction resulted in purified chromatin of the same chemical composition and a final recovery of about 70–80% of the DNA. The amount of chromosomal RNA associated with each was identical. Because of its simplicity, the following procedure was routinely used. All extraction procedures were carried out at 0–4°.

Isolation of chromatin in the absence of EDTA. The cells were first suspended by hand with the aid of a Teflon homogenizer in 15 volumes of cold deionized water. Nuclei and unlysed cells were then pelleted by

centrifugation at 1500*g* for 15 min. The pellet was then examined to determine the extent of cell lysis. If lysis was incomplete (*i.e.*, less than 70% of the cells lysed) the water wash was repeated. The crude nuclear pellet was then homogenized by hand with the aid of a Teflon homogenizer in 0.01 M Tris buffer (pH 8.0), stirred slowly for 30 min on a magentic stirrer, and centrifuged at 10,000*g* for 15 min. The chromatin was then washed three to four times by repeated suspension in 0.01 M Tris buffer (pH 8.0) and sedimentation at 10,000*g*. The chromatin at this stage is referred to as crude chromatin. If purified chromatin was to be prepared, the chromatin was suspended in an equal volume of 0.01 M Tris buffer (pH 8.0). Aliquots (5 ml) were then layered onto 25 ml of 1.7 M sucrose containing 0.01 M Tris buffer (pH 8.0) and the top two-thirds of the tube was stirred to form a two-step gradient and centrifuged for 2 hr at 22,000 rpm in the Spinco SW25 rotor. The purified chromatin was recovered as a clear gelatinous pellet.

Chemical Composition. DNA was determined by the diphenylamine reaction as described by Burton (1956) using rat liver DNA as a standard. RNA was determined by the orcinol reaction following the method of Dische and Schwarz (1937) using purified yeast RNA as a standard. Histone was extracted from chromatin with 0.2 N H_2SO_4 at 4° and precipitated with 20% trichloroacetic acid. The amount of protein was determined following the method of Lowry *et al.* (1951) using rat liver histone as a standard. The nonhistone protein content of the acid-insoluble, alkali-soluble material was determined by the same procedure, using bovine serum albumin fraction IV as a standard.

Preparation of Chromosomal RNA. Chromosomal RNA was prepared from both purified and crude chromatin. The purified RNAs isolated from the two sources are identical in size, base composition, and hybridization properties. Crude chromatin was, therefore, routinely used as a starting material for the preparation of chromosomal RNA. The following procedure was carried out at 0–4° with the exception of the steps indicated.

The chromatin pellets were suspended in an equal volume of 0.01 M Tris buffer (pH 8.0) and diluted with two volumes of 6 M CsCl in 0.01 M Tris (Industrial Grade CsCl, American Potash and Chemical Corp.). The resulting solution was extremely viscous and was homogenized for 30 sec at 20 V in a Waring blender to facilitate solution of the chromatin. The solution was then centrifuged for 15 hr at 36,000 rpm in the Spinco 40 rotor. Under these conditions the DNA pellets, while the chromosomal proteins, being buoyant, form a skin at the top of the tube. The skins were removed with a spatula and washed three times with 70% ethanol. The chromosomal proteins were then digested by treatment with 2–4 mg ml of pronase (pronase, Grade B from Calbiochem, was preincubated for 90 min at 37°) in 0.01 M Tris buffer (pH 8.0) for 4–6 hr at 37°. Sodium dodecyl sulfate was added to a final concentration of 1% followed by the addition of an equal volume of water-saturated phenol containing 0.1% 8-hydroxyquinoline (Kirby, 1962). After shaking for 30 min the phases were separated by centrifugation and the phenol phase was extracted with one-half volume of water. The combined aqueous phase was then reextracted twice with one-half volume of phenol. The nucleic acids were precipitated by the addition of one-tenth volume of 20% potassium acetate and two volumes of 95% ethanol. After 2 hr at −20° the precipitate was recovered by centrifugation, washed once with 70% ethanol, and dissolved in several ml of 0.2 M NaCl–7 M urea 0.01 M Tris buffer (pH 8.0).

The nucleic acids accompanying the chromosomal proteins include some DNA in addition to chromosomal RNA. This DNA was separated from the chromosomal RNA by chromatography on A-25 DEAE-Sephadex. Fractionation was routinely carried out on a 9 mm × 25 cm column, eluted with a linear gradient of NaCl ranging from 0.2 to 1.0 M in the presence of 7 M urea and 0.01 M Tris buffer (pH 8.0). Chromosomal RNA was recovered by precipitation with two volumes of 95% ethanol in the presence of 2% potassium acetate at −20° for 2 hr. The purified RNA was dissolved in and dialyzed against two-times standard saline citrate (0.3 M NaCl and 0.03 M sodium citrate).

Preparation of Extranuclear Protein-Bound RNA. To avoid the possibility of contamination by chromosomal RNA, RNA was isolated from the supernatant from the first water lysis of the cells. This fraction contains no visible nuclei nor does it contain detectable DNA as measured by the diphenylamine method of Burton (1956). It is therefore unlikely that this supernatant is contaminated with a significant amount of chromatin. The supernatant was first precipitated with two volumes of 95% ethanol in the presence of 2% potassium acetate. The precipitate was then dissolved in 0.01 M Tris buffer (pH 8.0) and diluted with two volumes of 6 M CsCl–0.01 M Tris. Under these conditions the majority of ribosomal proteins are dissociated and the RNA pellets in the following centrifugation. Centrifugation, pronase treatment, phenol extraction, and DEAE-Sephadex chromatography were carried out exactly as in the preparation of chromosomal RNA.

The RNA associated with the cytoplasmic proteins and purified by the above procedure elutes from DEAE-Sephadex as a single sharp peak at a NaCl concentration of 0.55 M. It has a sedimentation constant of 3.2–3.5 S and will be referred to as 3S cytoplasmic RNA.

Preparation of t- and rRNA. Total cytoplasmic RNA was isolated from the cytoplasmic fraction by cold phenol extraction following the method of Attardi *et al.* (1966). The RNA was first fractionated on a Sephadex G-100 column (1.5 × 200 cm equilibrated and eluted with two-times standard saline citrate). The r- and tRNA fractions were diluted to a final salt concentration of less than 0.1 M NaCl in 0.05 M sodium phosphate buffer (pH 6.7) and separately chromatographed on methylated albumin kieselguhr as described by Mandell and Hershey (1960). The RNA was eluted with a linear gradient of NaCl, concentrated by pressure dialysis followed by ethanol precipitation, and rechromatographed on Sephadex

G-100. The finally purified RNA was precipitated with ethanol and dialyzed against two-times standard saline citrate.

Preparation of Pulse-Labeled RNA. Pulse-labeled RNA was prepared from *in vitro* labeled cells. The cells were pelleted directly from the ascites fluid, washed once with Eagle's Medium (Eagle, 1959) deficient in phosphate, containing 5% dialyzed calf serum, and suspended in ten volumes of the same medium preheated to 37°. After 5 min neutralized ^{32}P was added (4 mCi of ^{32}P was added for each rat sacrificed) and the incubation was continued at 37° for 10 min. The cells were then diluted with cold TNKM and washed free of contaminating erythrocytes by differential centrifugation at 4°. A portion of the cells was extracted with phenol at 66° according to the method of Scherrer and Darnell (1962). Nucleic acids associated with the chromosomal proteins were extracted from the remainder of the cells as previously described in this section. The final nucleic acid extracts were exhaustively dialyzed against 0.05 M sodium phosphate buffer (pH 6.7) and chromatographed on methylated albumin kieselguhr as described by Mandell and Hershey (1960). The radioactivity of each fraction was determined by evaporation of 0.2-ml aliquot on a planchet and counted in a Nuclear-Chicago D-47 gas-flow counting system.

In Vivo Methylation and Extraction of Labeled RNA. Each rat, infected 6 days previously with tumor, was given an intraperitoneal injection of 50 μCi of [^{14}C]-methylmethionine (12 μCi/μmole purchased from New England Nuclear Corp.). After 5 hr the rats were sacrificed and the tumor was harvested and washed as previously described. Chromosomal, 3S cytoplasmic, and tRNA were prepared from the same cells. Chromosomal and 3S cytoplasmic RNA were prepared as previously described in this section. tRNA was prepared from a portion of the cytoplasmic fraction used in the preparation of 3S cytoplasmic RNA by phenol extraction according to the method of Attardi *et al.* (1966). All nucleic acid fractions were purified by chromatography on A-25 DEAE-Sephadex and developed with a linear gradient of NaCl from 0.2 to 1.0 M containing 0.01 M Tris (pH 8.0) and 7 M urea. tRNA elutes at a salt concentration of 0.53 M NaCl, rRNA is irreversibly bound to the column. Each fraction (1-ml aliquots) was precipitated with cold 10% trichloroacetic acid in the presence of 1 mg of carrier yeast RNA and acid-insoluble material collected by filtration on trichloroacetic acid presoaked membrane filters (Schleicher & Schnell B-6). The filters were then washed with 10 ml of cold 10% trichloroacetic acid, dried, and counted in the Beckman liquid scintillation spectrometer.

Preparation of in Vitro Synthesized RNA. RNA was synthesized *in vitro* by *Escherichia coli* RNA polymerase (f_1 of Chamberlin and Berg, 1962) using ascites-purified chromatin or ascites DNA as template. The complete incubation mixture for RNA synthesis contained in a final volume of 10 ml: 400 μmoles of Tris buffer (pH 8.0); 40 μmoles of $MgCl_2$; 10 μmoles of $MnCl_2$; 120 μmoles of β-mercaptoethanol; 8 μmoles each of GTP, UTP, and CTP; 8 μmoles of ATP-8-^{14}C (2 μCi/μmole); ascites DNA (100 μg) or chromatin (an amount containing 200 μg of DNA); and 1 mg of f_1. After 2 hr incubation at 30°, sodium dodecyl sulfate was added to a final concentration of 1% and the sample was extracted at 4° with an equal volume of water-saturated phenol containing 0.1% 8-hydroxyquinoline. Nucleic acids were precipitated from the aqueous phase with two volumes of ethanol in the presence of 2% potassium acetate at −20° for 2 hr. The precipitate was dissolved in 4 ml of TKM (0.05 M Tris buffer (pH 7.4)-0.025 M KCl-0.0025 M $MgCl_2$) and treated with 30 μg/ml of electrophoretically purified DNase (Worthington Biochemical Corp.) at 25° for 1 hr. The sample was again phenol extracted, the nucleic acid was precipitated, and dissolved in and dialyzed against two-times standard saline citrate.

Preparation of DNA. Rat ascites DNA was prepared from crude chromatin by the procedure of Marmur (1961) followed by an additional step including pronase digestion and phenol extraction. After RNase treatment (20 μg/ml) for 2 hr at 37° (RNase had been previously heated at 80° for 15 min), the DNA was incubated for 2 hr at 37° with 40 μg/ml of pronase (pronase, Grade B from Calbiochem, previously autodigested for 90 min at 37°). After digestion, the solution was made 1% in sodium dodecyl sulfate and extracted with an equal volume of water-saturated phenol. The extraction was repeated twice in the absence of sodium dodecyl sulfate with 0.5 volume of phenol. The DNA was then spooled from the aqueous phase with the addition of two volumes of 95% ethanol, dissolved in $\frac{1}{100}$ standard saline citrate, and reprecipitated with 0.54 volume isopropyl alcohol in the presence of 0.3 M sodium acetate plus 0.1 mM Na_2EDTA (pH 7.0) (Marmur, 1961). The purified DNA was then dissolved in and dialyzed against $\frac{1}{100}$ standard saline citrate. RNase activity was monitored by incubation of the DNA with ^{32}P-labeled RNA in two-times standard saline citrate at 37° for 20 hr. DNA preparations which resulted in any loss of trichloroacetic acid precipitable counts were subjected to additional phenol extractions until no detectable RNase activity remained.

DNA-RNA Hybridization. Denatured DNA was immobilized on nitrocellulose filters (Schleicher & Schuell B-6, 25 mm) as described by Gillespie and Spiegelman (1965). DNA filters were prepared in the presence of six-times standard saline citrate and contained 40 μg of denatured DNA. Trace amounts of ^{3}H-labeled ascites DNA were added to allow easy monitoring of the DNA in subsequent steps.

Hybridization was carried out at 66° or at 25° in the presence of 30 vol % formamide (Bonner *et al.*, 1967). The specificity of hybrid formation is identical under both conditions as measured by the specificity for ascites DNA and the total amount of hybrid formed. Before the addition of the filters the hybridization solution containing the RNA was heated at 95° for 10 min and cooled to 0 4°. Each vial contained two DNA filters and one blank filter in a volume of 1 ml and a final salt concentration of two-times standard saline citrate. At the end of the incubation the filters

TABLE I: Chemical Composition of Rat Ascites Chromatin.

Component	Mass Ratio	
	Crude Chromatin	Purified Chromatin
DNA	1.00	1.00
RNA		
Chromosomal	0.04	0.02
"Free"	0.17	0.11
Total	0.21	0.13
Histone		1.16
Nonhistone		1.00

TABLE II: Base Hydrolysis and RNase Digestion of Fractions I and II.[a]

Treatment	Fraction	Input (cpm)	Acid Soluble (cpm)	% Acid Soluble
0.3 N KOH, 18 hr, 37°	I	1748	1742	99.7
	II	1981	56	2.8
RNase,[b] 18 hr, 37°	I	1648	1608	97.6
	II	9499	780	8.2

[a] All samples were made 5% in $HClO_4$ at the end of the incubation and the radioactivity of the supernatant was determined. [b] RNase digestion was carried out in two-times standard saline citrate in the presence of 20 μg/ml of pancreatic RNase.

were removed, rinsed with two-times standard saline citrate in a large beaker, and washed on each side by filtration with 50 ml of two-times standard saline citrate. RNase digestion was carried out at 25° in a large volume of two-times standard saline citrate containing 20 μg ml of preheated pancreatic RNase (about 24 filters 100 ml). After 1-hr incubation, the filters were rinsed with two-times standard saline citrate and again washed on each side with 50 ml of two-times standard saline citrate. The filters were dried in a vacuum oven and counted in a Beckman liquid scintillation spectrometer.

Results

General Properties of Ascites Chromosomal RNA. Purified chromatin of Novikoff ascites tumor cells is composed of DNA, chromosomal proteins, both histone and nonhistone, and RNA in the mass ratios shown in Table I. When chromatin is fractionated by separation of the DNA from the chromosomal proteins by salt dissociation and buoyant density centrifugation in CsCl, a portion of the nucleic acid remains associated with the chromosomal proteins. Purification of this nucleic acid as described under Methods and chromatography on DEAE-Sephadex results in the two peak elution profiles shown in Figure 1. Both peaks exhibit a characteristic nucleic acid ultraviolet absorption spectra with absorption maxima at 257 mμ. As shown in Table II the first peak which elutes at a NaCl concentration of 0.55 M, is totally base labile, and is sensitive to RNase; the second peak, which elutes at 0.65 M NaCl, is not hydrolyzed by base and is resistant to RNase. The RNA contained in the first peak is referred to as chromosomal RNA and represents only a fraction of the RNA contained in the chromatin. The ultraviolet absorption spectrum of chromosomal RNA is shown in Figure 2.

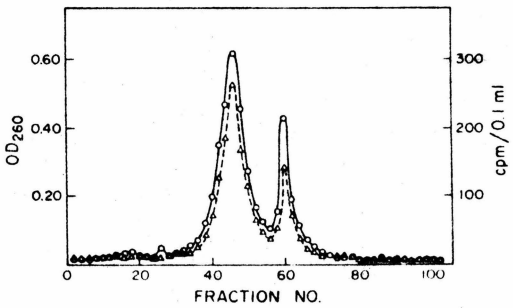


FIGURE 1: Elution profile of uniformly ^{32}P-labeled chromosomal RNA and DNA from DEAE-Sephadex. Nucleic acid eluted with a linear gradient of NaCl from 0.2 to 0.1 M in the presence of 7 M urea and 0.01 M Tris buffer (pH 8.0). ○—○, OD_{260}; △---△, ^{32}P counts per minute.

The remainder of the RNA associated with chromatin pellets with the DNA and is referred to as "free" RNA. The nature of this "free" RNA is not known.

TABLE III: Nucleotide Composition of Different RNA Fractions of Rat Ascites Cells.[a]

Species of RNA	Mole Per Cent					
	A	C	Dihydro-ribothymidine	U	G	G + C
Chromosomal	17.4 ± 1.1	24.2 ± 0.8	8.1[b]	19.4 ± 1.8	30.9 ± 1.9	55.1
Ribosomal	18.1 ± 0.4	28.4 ± 0.6		18.2 ± 0.7	35.4 ± 0.5	63.8
Transfer	17.5	26.0		23.4	33.0	59.0

[a] The base composition of the various RNA fractions was determined by alkaline hydrolysis of the RNA in 0.3 N KOH at 37° for 18 hr and separation of the nucleotides on a column of Dowex 1-X8, formate form (Cohn and Volkin, 1953). [b] Taken from Jacobson and Bonner (1968).

TABLE IV: Degree of Methylation of Various RNA Fractions.

Species of Nucleic Acid	Methylation[a] (cpm/mg of RNA × 10^{-2})	% Methylation Rel to tRNA
tRNA	387.0	100
Chromosomal RNA	77.0	20
3S RNA	65.0	17
Fraction II (DNA)	6.5	1.7

[a] Average specific activities of the peak fractions. In the case of tRNA the value reported is the average of the fractions at the leading edge of the peak.

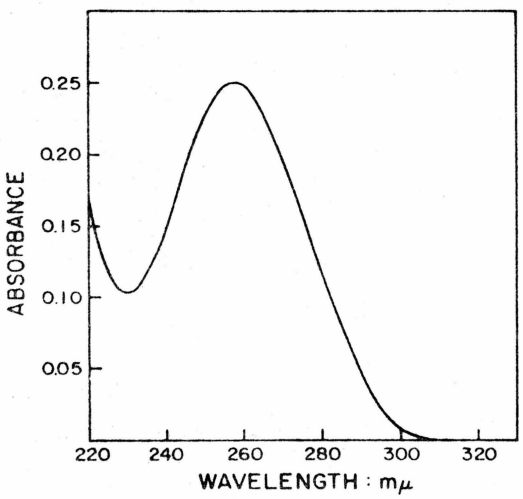


FIGURE 2: Absorption spectrum of purified rat ascites chromosomal RNA in 0.01 M Tris buffer (pH 8.0).

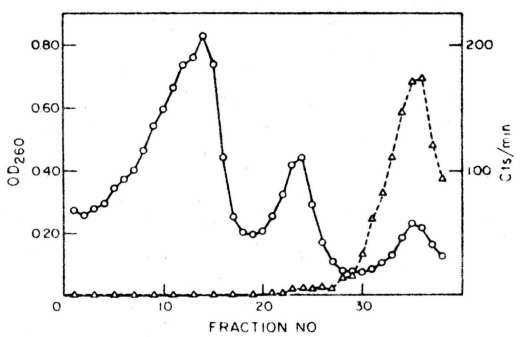


FIGURE 3: Sedimentation pattern of ^{32}P-labeled ascites chromosomal RNA in the presence of total ascites RNA. Sucrose (5–20%) gradient in the presence of 0.01 M sodium acetate buffer (pH 5.1) 0.1 M NaCl. Centrifugation at 39,000 rpm at 4° for 4.5 hr in the Spinco SW39 rotor. ○—○, OD_{260}; △--△, ^{32}P counts per minute.

The fact that chromosomal RNA elutes from DEAE-Sephadex as a single sharp peak indicates that it is composed of molecules of a relatively homogeneous chain length (Hall *et al.*, 1965). The pattern obtained from sucrose density gradient centrifugation of labeled chromosomal RNA in the presence of total ascites cytoplasmic RNA is shown in Figure 3. Chromosomal RNA moves as a sharp band and under these conditions is not distinguishable from tRNA. Analytical band velocity sedimentation gives an $s_{20,w}$ of 3.3 S. Equilibrium sedimentation in the Spinco Model E, according to the method of Van Holde and Baldwin (1958), yields a molecular weight of 10,140 daltons (D. Brutlag, personal communication).

The chromosomal RNA from pea cotyledon and

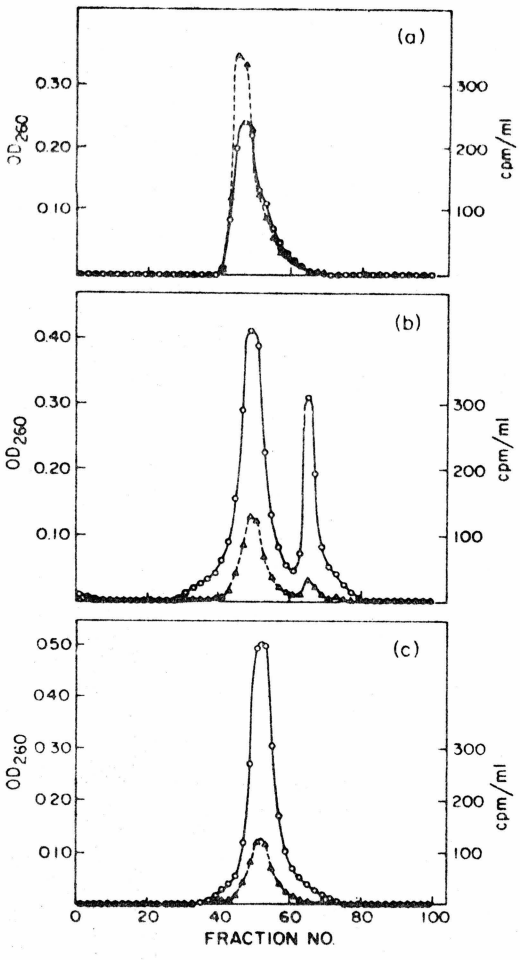


FIGURE 4: Elution profile of *in vivo* ^{14}C-methylated ascites tRNA (a), chromosomal RNA (b), and 3S cytoplasmic RNA (c) from DEAE-Sephadex. ○—○, OD_{260}; △--△, ^{14}C counts per minute.

chick embryo is characterized by a relatively high content of dihydropyrimidine; 8.5 mole % for pea cotyledon (Jacobson and Bonner, 1968) and 9.6 mole % for chick embryo (Huang, 1969). Dihydrouridine has been identified in pea bud and dihydroribothymidine in rat ascites cells. The base composition of ascites chromosomal RNA is presented in Table III along with the compositions of ascites t- and rRNA.

The different classes of RNA can be distinguished by their different content of methylated bases. The mole per cent of methylated bases contained in the various classes of HeLa cell RNA is as follows: tRNA = 8.3, 18S rRNA = 2.1, and 28S rRNA = 1.4 (Brown and Attardi, 1965). The extent of methylation of chromosomal RNA was measured by exposing the cells to [^{14}C]methylmethionine and measuring the extent of incorporation of the label into RNA. Incorporation into chromosomal RNA was compared with that into t- and 3S cytoplasmic RNA. The various fractions of RNA were prepared as described under Methods and purified by chromatography on DEAE-Sephadex. The elution profiles of transfer, chromosomal, and 3S cytoplasmic RNA are shown in Figure 4. In the case of chromosomal and 3S cytoplasmic RNA (4b,c) the specific activity throughout the peak is constant. In the case of tRNA (4a), however, the leading edge of the peak clearly has a higher specific activity than the remainder. This may be due to the presence of small amounts of 3S cytoplasmic RNA which elute slightly behind tRNA. The specific activities of the various fractions of RNA are listed in Table IV. The leading edge of the tRNA peak contains five times the [^{14}C]methyl group activity of the chromosomal or 3S cytoplasmic RNA fractions. The degree of methylation of 3S cytoplasmic RNA is probably not significantly different from that of chromosomal RNA. Chromosomal RNA is therefore methylated to a degree comparable with rRNA but clearly much less than tRNA.

These results make it unlikely that chromosomal RNA is a degradation product of tRNA, for if this were the case, we would expect the former to be methylated to the same degree as the latter.

Nascent RNA may be closely associated with the chromosomal proteins and may be isolated by the procedure used for the preparation of chromosomal RNA. The possible relationship between these classes of RNA and chromosomal RNA was investigated by comparing the amount of incorporation of ^{32}P into various classes of RNA following a short pulse of label. Ascites cells were therefore subjected to a 10-min pulse of ^{32}P and chromosomal and total RNA were extracted as described under Methods. The RNA fractions were chromatographed on methylated albumin kieselguhr and the specific activities of the different RNA species was determined. (Independent experiments in which chromosomal RNA was cochromatographed with total nucleic acid extracts have shown chromosomal RNA to elute in the region of tRNA.) The elution profiles are shown in Figure 5. In Figure 5a, total RNA, the first ultraviolet-absorbing peak is tRNA, the second rRNA. Rapidly labeled mRNA elutes at the tail end of the ribosomal peak as is apparent from the high specific activity in that region. Comparing this profile with that of chromosomal RNA (Figure 5b), it is clear that chromosomal RNA is not rapidly labeled as is mRNA but is synthesized at a rate comparable with that of tRNA. (The presence of DNA incompletely resolved from chromosomal RNA accounts for the decreased specific activity in fractions 70–100.)

Hybridization of Ascites Chromosomal RNA to Nuclear DNA. The data contained in Table V show that the hybridization of ascites chromosomal RNA is specific for rat ascites DNA. About $\frac{1}{6}$ the amount of hybrid is formed with calf thymus DNA and between $\frac{1}{10}$ and $\frac{1}{20}$ the amount with pea DNA.

The per cent of DNA hybridized in the presence of increasing amounts of chromosomal RNA is shown in

TABLE V: Hybridization of Rat Ascites Chromosomal RNA to Various DNAs.

		Ascites Chromosomal RNA		
Expt	DNA Source	Input (μg)	Hybridized (μg of RNA/filter)[a]	μg of RNA Hybridized/μg of DNA × 10^2
1	Ascites	60	0.521	1.28
	Pea embryo	60	0.026	0.06
	Sea urchin	60	0.011	0.03
2	Ascites	27	0.254	0.72
	Calf thymus	27	0.052	0.13
	Pea embryo	27	0.025	0.08

[a] Heterologous DNA filters of expt 1 contained 2 μg of ^{3}H-labeled ascites DNA. The amount of RNA expected to be hybridized to this amount of DNA has been subtracted (0.08 μg). The DNA filters of expt 2 contained no labeled DNA and no correction has been made. Incubation at 66° for 10 hr.

Figure 6a. The double-reciprocal plot presented in Figure 6b shows that, at an infinite RNA concentration, 3.8% of the DNA would be expected to be hybridized. The percentage of DNA hybridized at saturation, by different preparations of chromosomal RNA, ranged from 3 to 4%. The kinetics of hybrid formation and the final saturation level were not changed when the hybridization was performed at 25° in the presence of 30% formamide. Additional purification of chromosomal RNA by chromatography on methylated albumin kieselguhr or Sephadex G-50 did not alter its level of hybridization.

The level of hybridization obtained with ascites t- and rRNA is shown by the data contained in Table VI. For comparison, the table also includes data on the hybridization of chromosomal RNA. Since concentrations of t- and rRNA up to 600 μg/ml do not completely saturate the DNA, it seems unlikely that this interaction represents the specific hybridization of t- or rRNA. (The hybridization may be due to the presence of trace amounts of mRNA.) The specific activities of the t- and rRNA (1000–2000 cpm/μg) are not sufficiently high to allow an accurate measure of such low levels of hybridization. It is clear however that chromosomal RNA has very different hybridization properties than either t- or rRNA.

Competition between Labeled Chromosomal and Unlabeled t-, r-, and in Vitro Generated mRNA. Neither transfer nor rRNA compete with chromosomal RNA in the hybridization to ascites DNA (Table VII). This result is not surprising since both t- and rRNA are capable of forming hybrids with less than 0.06% of the DNA. rRNA, however, when present in large amounts relative to chromosomal RNA, does intefere with the hybridization of chromosomal RNA. This appears to be due to interaction between ribosomal and chromosomal RNA.

The interference of rRNA in the hybridization of chromosomal RNA made it impossible to carry out competition experiments with pulse-labeled RNA. It has been shown by Paul and Gilmour (1966) and by

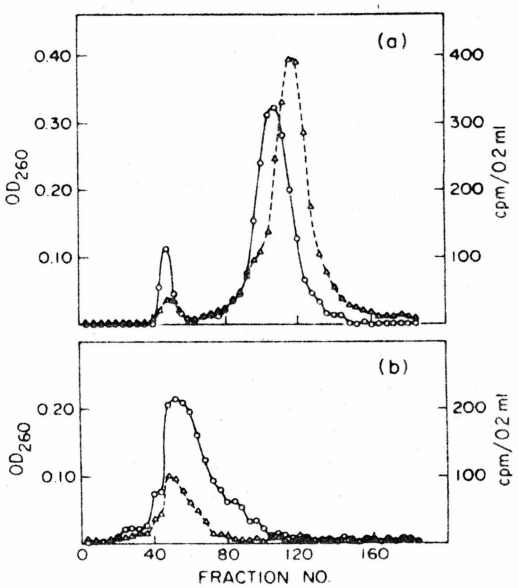


FIGURE 5: Elution profile of ^{32}P pulse-labeled total ascites RNA (a) and chromosomal RNA (b) from methylated albumin kieselguhr. ○—○, OD $_{260}$; △--△, ^{32}P cpm/0.2 ml.

Smith *et al.* (1969) that the RNA, generated by chromatin *in vitro*, possesses complete sequence homology with the mRNA of the tissue from which the chromatin was isolated. We have thus compared chromosomal RNA with the RNA generated by *E. coli* RNA polymerase with ascites purified chromatin as template. The RNAs generated from pea embryo chromatin and ascites DNA were also assayed for their ability to compete with chromosomal RNA in hybridization to denatured DNA. The results of such an experiment are presented in Table VIII. The addition of ten times the amount of RNA generated from either ascites or

TABLE VI: Hybridization of Rat Ascites Chromosomal, t- and rRNA to Ascites Nuclear DNA.[a]

Species of Ascites RNA	Sp Act. (cpm/μg)	Input RNA (μg)	cpm Hybridized/Filter	Hybridized RNA (μg/filter)	μg of RNA Hybridized/μg of of DNA × 10^2
Chromosomal	1007	53	367	0.365	0.91
	1615	225	1246	0.740	2.16
Transfer	1727	307	40	0.024	0.058
Ribosomal	1409	246	34	0.024	0.058

[a] Incubation at 25° for 10 hr in the presence of 30% formamide.

TABLE VII: Hybridization Competition between Labeled Chromosomal RNA and Unlabeled Ascites t- and rRNA.[a]

μg of ^{32}P Ascites Chromosomal RNA	Competing RNA Species	Competing RNA Amount (μg)	Total RNA/^{32}P Chromosomal RNA	μg of ^{32}P RNA Hybridized/Filter	% Labeled Hybrid Remaining
390			1	0.767	100
390	Transfer	1108	3.8	0.795	103
390	Ribosomal	1108	3.8	0.792	103
390	Chromosomal	593	2.5	0.580	76
390	Chromosomal	1108	3.8	0.382	50

[a] Incubation at 66° for 10 hr.

TABLE VIII: Hybridization Competition between Labeled Chromosomal RNA and *in Vitro* Generated RNA.[a]

μg of ^{32}P Ascites Chromosomal RNA	Competing RNA Species (Template)	Competing RNA Amt (μg)	Total RNA/^{32}P Chromosomal RNA	μg of ^{32}P RNA Hybridized/Filter	% Labeled Hybrid Remaining
60				0.384	100
60	*In vitro* RNA (ascites chromatin)	540	10	0.380	98.9
60	*In vitro* RNA (pea cotyledon chromatin)	540	10	0.396	103.0
60	*In vitro* RNA (ascites DNA)	540	10	0.348	91.0
60	Ascites chromosomal RNA	540	10	0.104	27

[a] Incubation at 25° for 10 hr in the presence of 30% formamide.

pea embryo chromatin had no effect on the level of hybridization of chromosomal RNA while the same amount of added homologous chromosomal RNA caused a 73% reduction in the amount of hybrid formed. RNA generated from ascites DNA slightly reduced the level of hybrid formation. From these data it is clear that ascites chromosomal RNA and ascites messenger RNA, synthesized *in vitro*, have no sequences in common. The low level of competition observed with RNA transcribed from DNA is probably due to the synthesis of small amounts of chromosomal RNA.

Sequence Homology between Chromosomal RNA and 3S RNA. The data of Figure 7 show that 3S cytoplasmic RNA hybridizes at saturation with about 1.8% of denatured ascites nuclear DNA.

The competition curve obtained when ^{32}P-labeled chromosomal RNA is hybridized in the presence of increasing amounts of unlabeled homologous or 3S cytoplasmic RNA is shown in Figure 8. The data of Figure 8 show that unlabeled 3S cytoplasmic RNA is an effective competitor for about 50% of the chromosomal RNA binding sites. This result is in agreement with the hybridization saturation curve obtained for 3S cytoplasmic RNA in which it saturated about one-half the amount of DNA as did chromosomal RNA. That this competition is in fact specific site competition, and

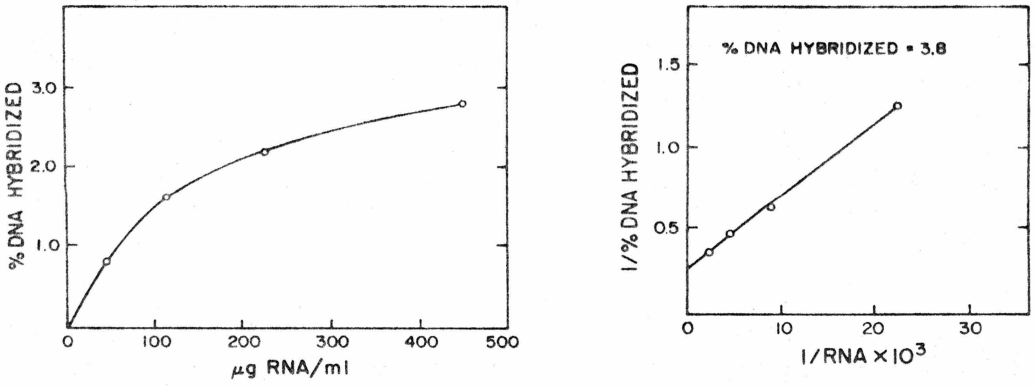


FIGURE 6: Hybridization of ascites chromosomal RNA to ascites nuclear DNA. Incubation at 66° for 10 hr. (a) Hybridization as a function of input RNA concentration. (b) Double-reciprocal plot of DNA saturation curve.

TABLE IX: Nucleotide Composition of Chromosomal RNA from Various Sources.

Source of RNA	Mole Per Cent						
	A	C	Dihydro-pyrimidine	U	G	% G + C	Pu/Pyr
Rat ascites[a]	17.4	24.2	8.1	19.4	30.9	53.9	0.93
Pea bud[b]	39.8	13.1	8.5	19.2	19.3	32.4	1.45
Chick embryo[c]	27.6	25.6	9.6	12.8	24.6	50.2	1.09

[a] Taken from Jacobson and Bonner (1968). [b] Huang and Bonner (1965) report a slightly different base composition. The present values are based on the more accurate analytical methods now available. [c] Taken from Huang (1969).

not interference of the type observed with rRNA, was shown by sequential hybridization experiments in which the DNA was first hybridized with 3S cytoplasmic or chromosomal RNA followed by hybridization with labeled chromosomal RNA.

The data for Figure 9 show the competition observed when ^{32}P-labeled 3S cytoplasmic RNA is hybridized in the presence of increasing amounts of unlabeled 3S cytoplasmic or chromosomal RNA. It is apparent that chromosomal RNA is a more effective competitor than 3S cytoplasmic RNA and is capable of competing for at least 90% of the 3S RNA binding sites. That is to say there are very few, if any, sequences contained in 3S cytoplasmic RNA that are not also contained in chromosomal RNA. The fact that a given concentration of chromosomal RNA produces more competition than the same concentration of 3S cytoplasmic RNA indicates that the 3S cytoplasmic RNA fraction contains less of the high hybridizing component than does chromosomal RNA and therefore is not a pure fraction.

RNA has also been prepared from the nuclear lysate, after pelleting of the chromatin, by the same procedure used for the preparation of 3S cytoplasmic RNA. This fraction of RNA has hybridization properties identical with those of 3S cytoplasmic RNA. It is capable of competing for only 50% of the chromosomal RNA binding sites and contains no sequences not also contained in chromosomal RNA. It competes on an equal basis with 3S cytoplasmic RNA in hybridization competition experiments with ^{32}P-labeled 3S cytoplasmic RNA. A portion of the chromosomal RNA, about 50% of the sequences present, therefore, appears to be confined to the chromatin. The remainder of the chromosomal RNA is homologous to a fraction of RNA present in the nuclear sap and cytoplasm.

Discussion

Chromosomal RNA isolated from rat ascites cells has many properties like pea bud and chick embryo chromosomal RNA (Huang and Bonner, 1965; Bonner and Huang, 1966; Bonner and Widholm, 1967; Huang and Huang, 1969; Huang, 1969). The amount of chromosomal RNA (relative to DNA) contained in purified chromatin from rat ascites, pea bud, and chick embryo is between 2 and 4% even though the "free" RNA content of these different chromatins varies from 4 to 17%. Commerford and Dehlias (1966) have reported extremely small amounts of RNA associated with the chromosomal proteins of mouse liver and intestine. This result may be the consequence of their method of nucleohistone preparation. The chromosomal RNA from rat liver reported by Benjamin *et al.* (1966) was

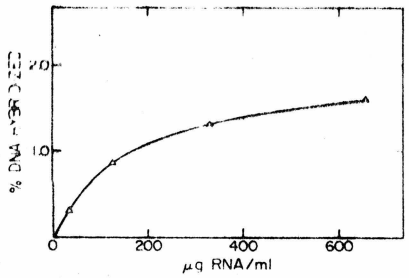


FIGURE 7: Hybridization of 3S cytoplasmic RNA to ascites nuclear DNA in the presence of increasing amounts of RNA. Hybridization at 66° for 10 hr.

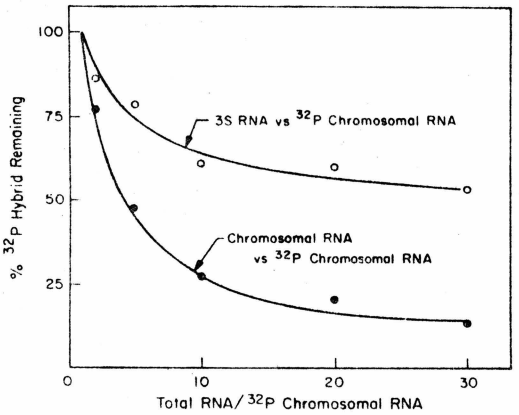


FIGURE 8: Ability of ascites 3S cytoplasmic or chromosomal RNA to compete with ^{32}P-labeled chromosomal RNA in hybridization to ascites DNA. All hybridization at 66° for 10 hr with 60 μg of labeled ascites chromosomal RNA throughout.

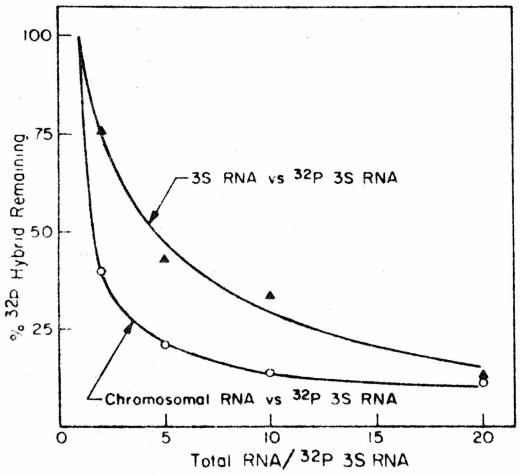


FIGURE 9: Ability of ascites chromosomal or 3S cytoplasmic RNA to compete with ^{32}P-labeled 3S cytoplasmic RNA in hybridization to ascites DNA; 60 μg of labeled ascites 3S cytoplasmic RNA throughout. Hybridization for 10 hr at 25° in the presence of 27% formamide.

followed by radioactivity only; we thus have no indication of the actual amount of RNA present. An additional complication of their work results from the short labeling time which they used; a time during which we would expect very little incorporation into chromosomal RNA of the nonreplicating liver chromosomes, but a large amount into nascent messenger. This is indicated by the fact that a 10-min pulse of ^{32}P given to rat ascites cells results in only slight incorporation into chromosomal RNA. In fact the base analysis and sedimentation profile reported by Benjamin *et al.* (1966) are most representative of mRNA. A more detailed investigation is required to determine if this RNA is really analogous to the chromosomal RNA reported in other systems.

Pea bud, chick embryo, and ascites chromosomal RNA are all small and size homogeneous. This is indicated by the fact that all three species of chromosomal RNA elute from DEAE-Sephadex as a sharp peak at the same molarity of NaCl. The sedimentation constant of 3.3 S for ascites chromosomal RNA is in good agreement with the sedimentation constants of pea (3.2 S) and chick embryo (3.8 S).

The base compositions of ascites, pea bud, and chick embryo chromosomal RNA are presented in Table IX. The base compositions are similar only in the sense that each contains about 9 mole % saturated pyrimidine. The significance of these differences is unclear at the present time.

The hybridization properties of rat ascites chromosomal RNA are in good agreement with those reported for pea chromosomal RNA by Bonner and Widholm (1967). Pea chromosomal RNA hybridizes to about 5% of pea nuclear DNA and has no sites in common with either t- or rRNA. Bonner and Widholm (1967) presented no evidence concerning a partially homologous cytoplasmic fraction. However, no competition was observed in the hybridization of pea chromosomal RNA in the presence of 32 times the amount of total cytoplasmic RNA. The chromosomal RNA of chick embryo is also complementary to a large portion of chick nuclear DNA, hybridizing at saturation to about 4% of the DNA (R. C. Huang, 1967, personal communication). It has no sites in common with chick t- or rRNA.

The hybridization properties of chromosomal and 3S cytoplasmic RNA are very similar to those reported by Shearer and McCarthy (1967) for pulse-labeled nuclear and cytoplasmic RNA from mouse L cells. They have reported the presence of a nuclear RNA that hybridizes to about 4.4% of the nuclear DNA. An additional fraction isolated from the cytoplasm hybridizes to about 1% of the nuclear DNA and contains no sequences not contained in the nuclear RNA. Thus, a fraction of the RNA isolated from the nucleus is confined to the nucleus. An important distinction between this work and that of Shearer and McCarthy (1967) is that their RNA is rapidly labeled while chromosomal RNA does not appear to be. In addition, ascites chromosomal RNA appears to possess no sequence homology to ascites mRNA.

Chromosomal RNA constitutes about 1-2 % of the

total cellular RNA. From the present experiments it is not possible to estimate the amount of 3S cytoplasmic and 3S nuclear RNA. We can however estimate the total amount of nonchromatin-bound 3S RNA to be about 5% of the total cellular RNA. The fact that these RNAs have not been observed previously may be due to their association with proteins which have prevented their extraction with phenol.

In establishing chromosomal RNA as a new class of RNA it is necessary to very carefully consider the possibility that it has arisen from the degradation of other classes of RNA. The experiments reported here, although they do not prove the point, strongly suggest that it does indeed represent a new class of RNA. The hybridization properties of ascites chromosomal RNA distinguish it from the various other RNA species studied. It is immediately distinguished from t- and rRNA by its high level of hybridization, being complementary to about 4% of ascites nuclear DNA. Neither t- nor rRNA are effective competitors for chromosomal RNA binding sites and in unreported data we have found that degradation of rRNA into 3S fragments does not increase its efficiency as a competitor. The lack of competition observed with *in vivo* generated mRNA suggests that chromosomal RNA is also distinct from mRNA. This is further supported by the fact that it is not pulse labeled. Because of the low degree of methylation of chromosomal RNA it is unlikely the result of degradation of tRNA. These results indicate that chromosomal RNA is not a degradation product of any one of these RNA fractions. The high content of dihydroribothymidine (8 mole %) is evidence that it is not a general composite degradation product.

Bekhor *et al.* (1969) and Huang and Huang (1969) have demonstrated that chromatin, completely dissociated in the presence of 2 M NaCl and then reconstituted under the proper conditions, supports the synthesis of the same spectrum of RNA sequences as does native chromatin. However, chromosomal RNA appears to be required for this sequence-specific reconstitution, *i.e.*, destruction of the chromosomal RNA by RNase or $Zn(NO_3)_2$ before reconstitution prevents sequence-specific reconstitution. In ascites cells only a fraction of the chromosomal RNA is confined to the chromatin. RNA homologous to the remaining fraction is found both in the nuclear sap and cytoplasm. What can be the role of this class of RNA? The close homology between 3S RNA and chromosomal RNA suggests that it also may be involved in gene regulation.

The properties of ascites chromosomal RNA clearly show that it is homologous to the chromosomal RNA isolated from pea bud and chick embryo. The presence of chromosomal RNA in rat, pea, and chick suggests that chromosomal RNA may be of general occurrence in higher organisms.

References

Attardi, G., Parnas, H., Hwang, M. I. H., and Attardi, B. (1966), *J. Mol. Biol. 20*, 145.

Bekhor, I., Kung, G., and Bonner, J. (1969), *J. Mol. Biol. 39*, 351.

Benjamin, W., Levander, O. A., Gellhorn, A., and DeBellis, R. H. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 858.

Bonner, J., and Huang, R. C. (1966), *in* Ciba Foundation Study Group No. 24, de Reuck, A. V. S. and Knight, J., Ed., Boston, Little, Brown, p 18.

Bonner, J., Kung, G. M., and Bekhor, I. (1967), *Biochemistry 6*, 3650.

Bonner, J., and Widholm, J. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1379.

Brown, G. M., and Attardi, G. (1965), *Biochem. Biophys. Res. Commun. 20*, 298.

Burton, K. (1956), *Biochem. J. 62*, 315.

Chamberlin, M., and Berg, P. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 81.

Cohn, W., and Volkin, E. (1953), *J. Biol. Chem. 203*, 319.

Commerford, S. L., and Dehlias, N. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1759.

Dische, Z., and Schwarz, K. (1937), *Mikrochim. Acta 2*, 13.

Eagle, H. (1959), *Science 130*, 432.

Gillespie, D., and Spiegelman, S. (1965), *J. Mol. Biol. 12*, 829.

Hall, J., Sedat, J., Adiga, P., Uenura, I., and Winnick, T. (1965), *J. Mol. Biol. 12*, 162.

Huang, R. C. (1969), *Biophys. J. Soc. Abstracts 9*, A86.

Huang, R. C., and Bonner, J. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 960.

Huang, R. C., and Huang, P. C. (1969), *J. Mol. Biol. 39*, 365.

Jacobson, R. A., and Bonner, J. (1968), *Biochem. Biophys. Res. Commun. 33*, 716.

Kirby, K. S. (1962), *Biochim. Biophys. Acta 55*, 545.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Mandell, J. D., and Hershey, A. D. (1960), *Anal. Biochem. 1*, 66.

Marmur, J. (1961), *J. Mol. Biol. 3*, 208.

Marushige, K., and Bonner, J. (1966), *J. Mol. Biol. 15*, 160.

Paul, J., and Gilmour, R. (1966), *Nature 210*, 992.

Scherrer, K., and Darnell, J. E. (1962), *Biochem. Biophys. Res. Commun. 7*, 486.

Shearer, R. W., and McCarthy, B. J. (1967), *Biochemistry 6*, 283.

Smith, K., Church, R., and McCarthy, B. (1969), *J. Mol. Biol.* (in press).

Van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.

Part I.

Appendix.

(i) BASE ANALYSIS OF ASCITES cRNA IN THE RNA/DNA HYBRID

(ii) EXTRACTION OF ASCITES 3 S RNA FROM THE NUCLEAR SAP.

INTRODUCTION.

Two questions were raised in the work reported in Part I Chapter I (Dahmus and McConnell, (1969). 1. Is the hybrid RNA representative of the input cRNA sequences, or is it a sub-class of the cRNA preparation? 2. What is the role of 3 S RNA in the control of gene expression? Is it present in the nuclear sap? Base analysis of the input RNA and the hybrid RNA eluted from the nitrocellulose membrane show that that they have the same base composition. We have been able to prepare 3 S RNA from the nuclear sap, and its hybridization properties are identical to those of 3 S cytoplasmic RNA (Part I Chapter I).

MATERIALS AND METHODS

(i) General.

Methods for the growth and labelling of ascites tumour cells, preparation of chromatin, cRNA and DNA were carried out as described in Part I Chapter I.

(ii) DNA - RNA hybridization and base analysis of the hybridized RNA

Hybrids were formed between DNA (40-50 ug) immobilized on nitrocellulose membranes (Schleicher and Schuell B-6, 25 mm) (Gillespie and Spiegelman, 1965) and RNA in 2 x SSC at 66°C for 10 hrs or in 2 x SSC 30% formamide at 25°C for 10 hours. To assay the true hybrid formed the filters were then washed, ribonucleased, washed again and counted as described in Part I Chapter I.

For some analyses of the base composition of the hybrid, the filters were either not treated with ribonuclease or treated with pancreatic ribonuclease (20 ug/ml) which cleaves between the 3' phosphate of a pyrimidine and the 5' position of its neighbour, or T1 ribonuclease (50 units/ml) which hydrolyses the bond between the 3' phosphate of a guanylate residue and its neighbour (Egami et al., 1964).

To obtain enough hybridized RNA to carry out a base analysis 24 DNA filters and 3 blanks were incubated in 3 mls of 2 x SSC 30% fomamide at 25°C for 10 hrs. The filters were then divided into 3 lots of 8 DNA filters and 1 blank

each, and either (a) washed with 100 mls 2 x SSC (b) washed with 100 mls 2 x SSC, treated with 20 ug/ml pancreatic ribonuclease (1 hr at room temperature) and washed with a further 100 mls 2 x SSC or (c) as in (b) except treated with T1 ribonuclease at 50 units/ml. 1 DNA filter and 1 blank from each lot were then counted.

The RNA associated with the remaining filters was extracted by 4 x 3 ml volumes of deionized water at 100°C for 15 minutes. Carrier (2 mg.) was added and the RNA was precipitated with 2 volumes of 95% ethanol in the presence of 2% potassium acetate at -20°C overnight. The precipitate was collected and dissolved in 2 ml .3N KOH and hydrolyzed at 37°C for 18 hr. The solution was made .5N perchloric acid (PCA), and after 30 minutes on ice the insoluble potassium perchlorate and any DNA which might have eluted from the filters were removed by centrifugation. The precipitate was washed with .5N PCA and the washings added to the supernatant. The solution was neutralized to pH 8 to 8.5 with .3N KOH, and kept at -20°C for 2 hrs to remove as much potassium perchlorate as possible. The supernatant was diluted to approximately 100 mls with deionized water and applied to a Dowex-1 x 8 column for base analysis according to Cohn and Volkin, (1953).

(iii) Preparation of 3 S RNA from cytoplasmic sap and nuclear sap

The procedure was exactly the same as that used to

prepare 3S cytoplasmic RNA from the supernatant of the first water lysis during the preparation of chromatin (Part I Chapter I), but was applied to all supernatants from both water and tris lyses. The supernatants were treated with potassium acetate - ethanol, the precipitates dissolved in 4 M CsCl .01M tris pH 8.0 and centrifuged to equilibrium. The proteinaceous skins were pronased, and phenol extracted and the nucleic acids chromatographed on DEAE Sephadex A-25 as described in Part I Chapter I.

Suspension of ascites cells in H_2O lyses the cellular membrane. The subsequent lyses are carried out in .01 M tris pH 8.0 when the nuclei are lysed.

RESULTS.

(i) Base analyses of RNA from cRNA - DNA hybrids.

The effect of ribonuclease treatment on the amount of RNA associated with the filters is summarized in Table 1. Neither ribonuclease significantly reduced the cpm hybridized by more than 15%, and the blank filters had always less than 3% of the counts on the DNA filter. We conclude that the hybrid formed under these conditions does not have extended regions of mismatch. Analysis of the RNA which is precipitated by ethanol after extraction from the membranes revealed that more than 90% is recovered in the pellet in the case of no treatment or pancreatic ribonuclease treatment, while only 40% is precipitable after T1 ribonuclease. The RNA which remains in the supernatant after incubation with ribonuclease T1 is 40% G. The most likely explanation for these observations is that ribonuclease T1 is not specific for single-stranded RNA.

The results of the base analyses are given in Table II, with the average of seven determinations for chromosomal RNA and the range of error. In the absence of RNAase treatment the hybridized RNA has a base composition which is not significantly different from that of the input RNA, suggesting that the hybrid contains a representative spectrum of the sequences of the input.

After treatment with pancreatic RNAase or T1 RNAase,

Table I: Effect of ribonuclease treatment on the RNA associated with the filters.

	cpm/filter No ribonuclease	cpm/filter Pancreatic ribonuclease	cpm/filter T1 ribonuclease
*DNA filter	8145	6841	7942
Blank filter	228	175	239

*The DNA filter carried 43 µg of denatured DNA. Hybridization was at 25°C, 2 x SSC, 30% formamide for 10 hr, 24 DNA filters and 3 blanks in 3 mls. total volume at 393 µg/ml of ^{32}P chromosomal RNA. Nuclease concentrations as in Materials and Methods.

Table II: Base composition of RNA attached to the filter in the RNA - DNA hybrid.

	INPUT RNA	HYBRIDIZED RNA						
		No RNAase		Pancreatic RNAase		T1 RNAase		
		exp.1.	exp.2.	exp.1.	exp.2.	exp.1.	exp.2.	exp.3.*
C	18.9 ± 1.1	20.1	20.4	16.0	16.4	27.6	29.7	20.6
A	26.3 ± .8	26.5	25.3	28.4	24.2	27.4	27.6	25.4
U	21.1 ± 1.8	20.8	19.3	16.2	18.2	26.6	24.3	22.2
G	33.6 ± 1.9	32.7	35.1	41.4	41.2	18.4	18.3	31.6
cpm analyzed		6619	5468	3040	3114	1159	814	2444

* Total RNA extracted from the filters analyzed, since 60% of the RNA after T1 RNAase treatment is not ethanol precipitable. Hybridization conditions as in Table I.

the ethanol precipitable RNA is appropriately reduced in the % C + U and % G respectively. The RNA which remains bound to the filter after T1 RNAase treatment but which is not ethanol precipitable has a high % G. If the % base composition of both the ethanol precipitable and ethanol soluble RNA is calculated it becomes very similar to that of the RNA isolated after no ribonuclease (Table 2).

(ii) Preparation of nuclear sap 3 S RNA

Every supernatant during the preparation of chromatin yielded a peak of 3 S RNA which chromatographed on DEAE Sephadex exactly as chromosomal RNA. Equal quantities of this RNA were isolated from the first two water lyses, and are referred to as W1 and W2 3S RNA. The pellet after the second water lysis contains nuclei but no whole cells when examined in the microscope. The nuclei are very difficult to lyse and usually five homogenizations in .01 M tris pH 8.0 by a Teflon homogenizer are required to break them open. All of the tris supernatants produce significant but decreasing amounts of 3S RNA referred to as T1-T5 3S RNA. Because the cytoplasmic membrane is delicate and easily ruptured, while the nuclear membrane is resilient, it seems most likely that at least some of this 3S RNA is nuclear in origin. If it was all cytoplasmic it should appear mostly in the W1 and W2 preparations whereas the yield of T1+T2+T3+T4+T5 equalled that of W1+W2. The W1+W2 3S RNA could however be derived

from nuclei ruptured by homogenization in H_2O. The pellets after water lyses contain no whole cells but some of the nuclei are irregularly shaped as if they have been punctured. It is a reasonable interpretation therefore that all 3S RNA may be of nuclear origin.

(iii) Hybridization of 3S RNA

A saturation curve for T1 3S RNA is compared with one for chromosomal RNA in Figure 1. As for cytoplasmic W1 3S RNA (Part I Chapter I) the chromosomal RNA is about twice as effective in the formation of hybrid as the 3S RNA. Figure 2 shows the results of a competition hybridization experiment between ^{32}P chromosomal RNA and cold W1 3S RNA, W2 3S RNA, T1 3S RNA and T2 3S RNA. All of the 3S RNAs behave identically and the results are similar to those of Figure 8 (Chapter I), in that only a small proportion of the ^{32}P chromosomal RNA hybrids are competed by the 3S RNA. To show whether or not the same sequences were being competed out by the various 3S RNA preparations a competition experiment was carried out between ^{32}P T1 3S RNA and cold W1 3S RNA, W2 3S RNA, and T2 3S RNA (Figure 3). The results show that each of these RNAs behaves the same, so it is concluded the RNAs isolated from the water and tris lyses supernatants are the same.

Figure 1. % hybridization of chromosomal RNA and 3S nuclear sap RNA (T1) to denatured ascites tumour DNA (43 µg) immobilized on nitrocellulose membranes (Schleicher and Schuell 25 m.m. B6) in 2 x SSC (1 ml.) at 66°C for 10 hrs. Specific activities were 1463 cpm/µg (cRNA) and 1717 cpm/µg (T1 3S RNA). Values are for duplicate DNA filters from which a blank filter background was subtracted.

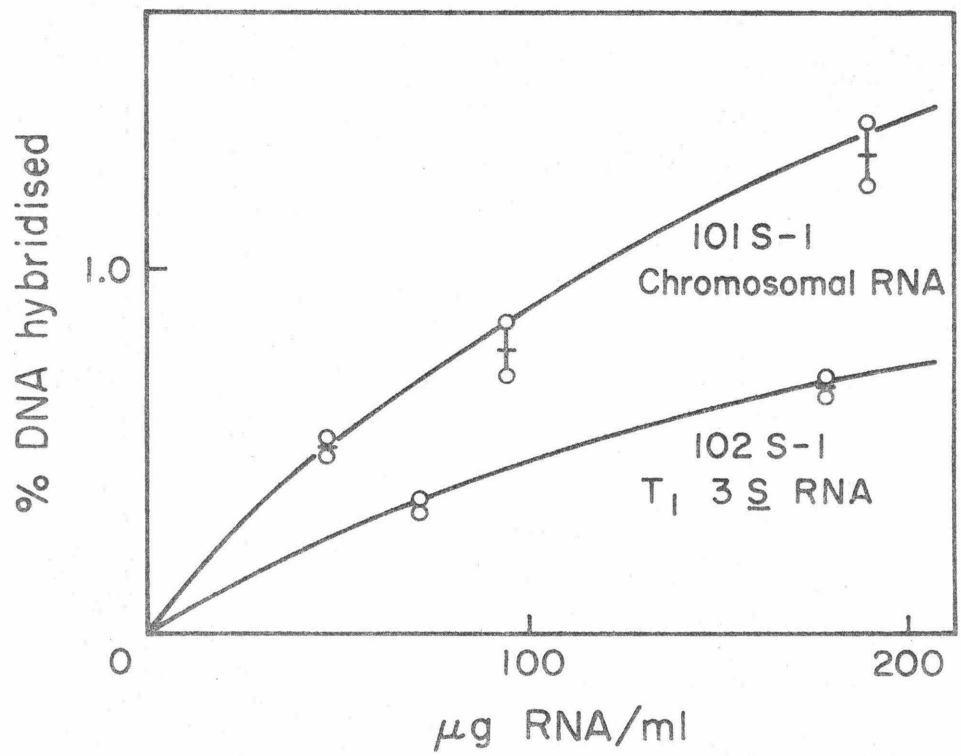

% DNA hybridised
1.0
0
100
200
μg RNA/ml
101 S-1
Chromosomal RNA
102 S-1
T1 3S RNA

Figure 2. Competition hybridization between ^{32}P chromosomal RNA and cold W1, W2, T1 and T2 3S RNA. Reactions contained 30 µg of ^{32}P chromosomal RNA and increasing quantities of each cold RNA. Conditions otherwise as in Figure 1. Specific activity of the chromosomal RNA was 1215 cpm/µg.

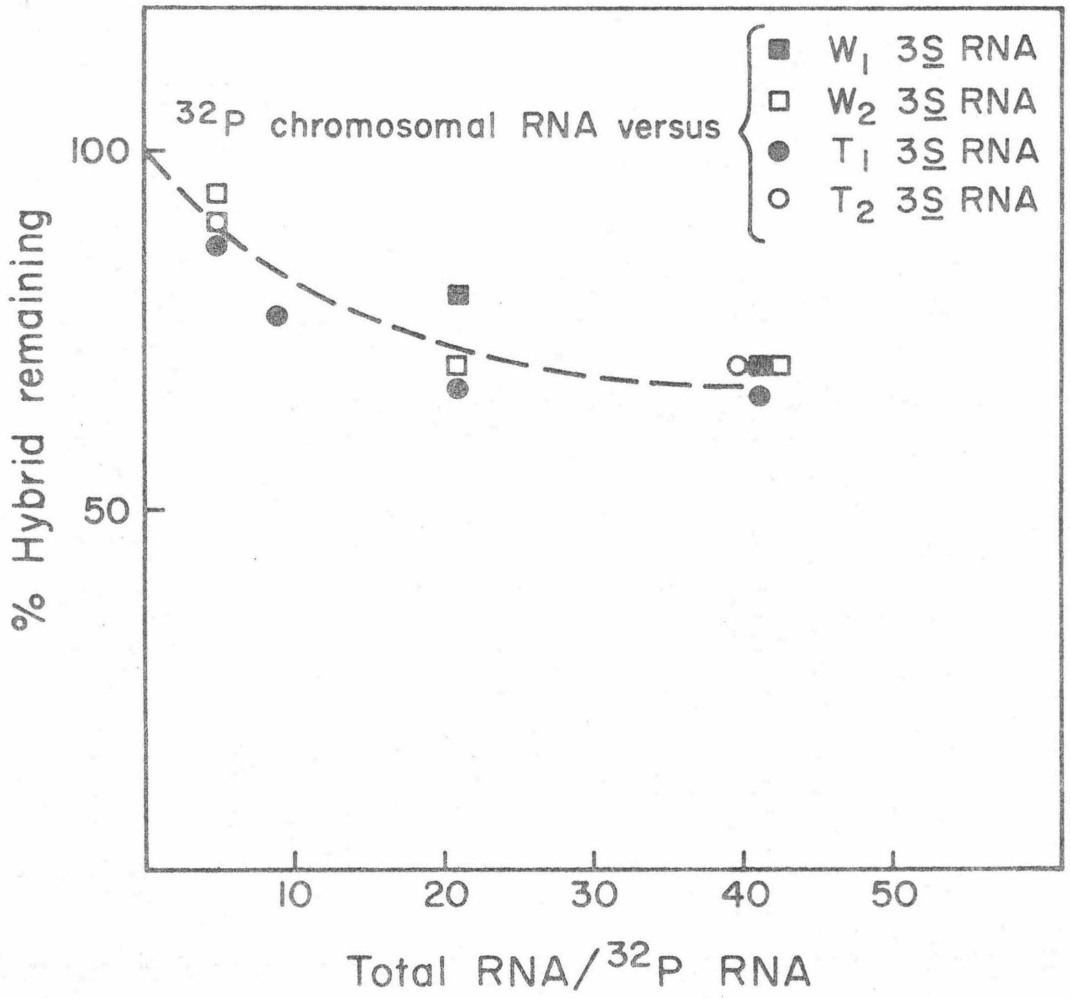
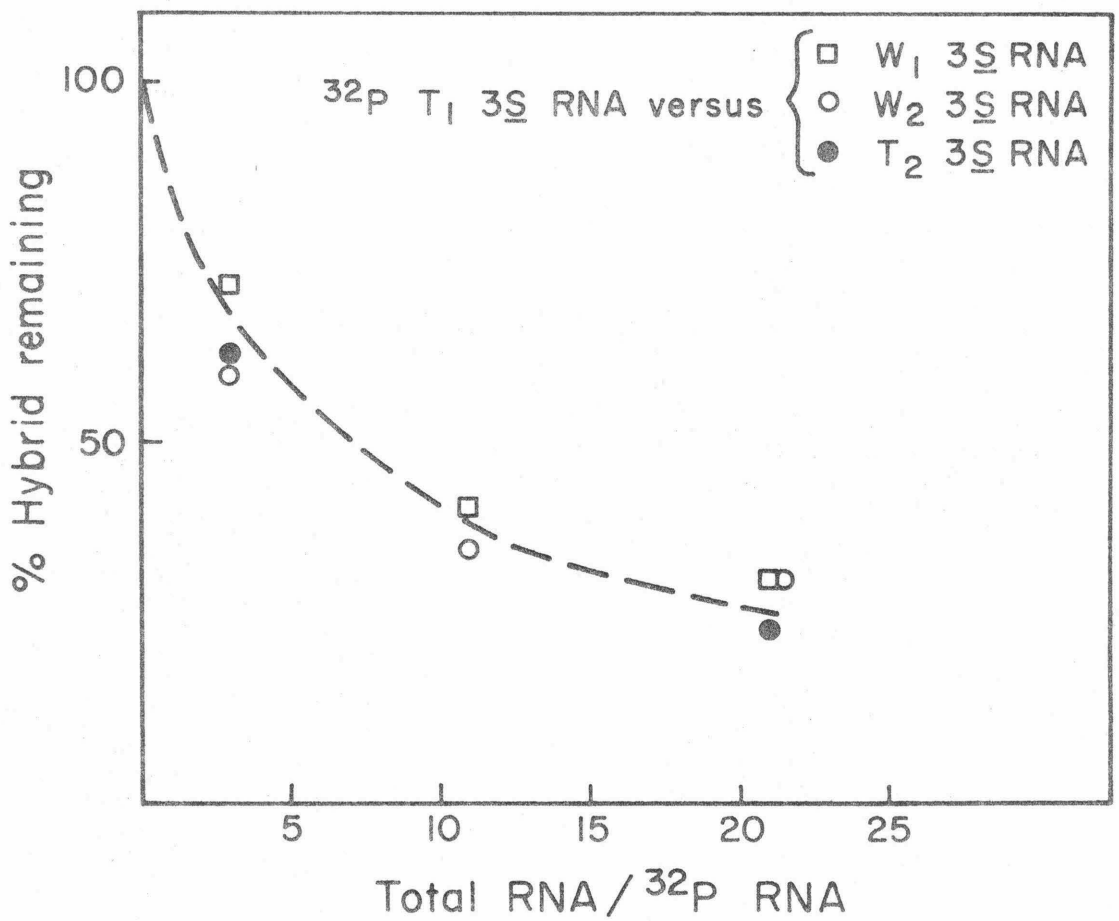

^{32}P chromosomal RNA versus
W_1 3S RNA
W_2 3S RNA
T_1 3S RNA
T_2 3S RNA
% Hybrid remaining
100
50
10
20
30
40
50
Total RNA/^{32}P RNA

Figure 3. Competition hybridization between ^{32}P T1 3S RNA and cold W1, W2 and T2 3S RNA. Reactions had 60 ug of ^{32}P T1 3S RNA and increasing quantities of cold RNA. Specific activity of ^{32}P T1 3S RNA was 1465 cpm/ug.


100
50
% Hybrid remaining
^{32}P T_1 3S RNA versus
W_1 3S RNA
W_2 3S RNA
T_2 3S RNA
5
10
15
20
25
Total RNA / ^{32}P RNA

DISCUSSION

The results of the base analyses provide further support for the proposal that chromosomal RNA is a distinct species of RNA associated with chromatin and not a general degradation product. If cRNA contained breakdown products of DNA-like RNA which hybridized extensively to the DNA and of rRNA and tRNA which reduced its effective specific activity it might have been expected that the base composition of the hybrid RNA would differ from the input total RNA. This has not been observed. The negative results however do not rule out the possibility that the hybridizing RNA although different has the same base composition as the total chromosomal RNA. However, the other properties of the chromosomal RNA such as its unusually high proportion of dihydropyrimidine make the general breakdown produce hypothesis unlikely (Jacobson and Bonner, 1968).

3S RNA with nybridization properties very similar to 3S cytoplasmic RNA (Chapter I) (Dahmus and McConnell, 1968) has been extracted from the nuclear supernatant. Both cytoplasmic and nuclear 3S RNA are clearly related to chromosomal RNA which has been implicated in genetic control mechanisms (Bekhor et al., 1969;Huang and Huang,1969). The fact that sequences all of which are present in chromosomal RNA are present in the cytoplasm was hard to reconcile with such a role. These results suggest that the presence of 3S RNA in the cytoplasm

may be an artifact caused by the rupture of some nuclei by the first water lysis which then leak some of their 3S nuclear sap RNA into the so-called cytoplasmic supernatant. If 3S RNA were localized in the nucleus it would be easier to incorporate it into models of genetic control, for example as a precursor of chromatin bound chromosomal RNA, or as a fraction of the chromosomal RNA population free to respond to changes in the concentration of effector molecules in the nuclear sap. The interesting point remains that more than 50% of the sequences in chromosomal RNA are not represented in the sequences of 3S RNA (Dahmus and McConnell1, 1968).

REFERENCES.

Bekhor, I., Kung, G., and Bonner, J., (1969). J. Mol. Biol., 39, 351.

Cohn, W., and Volkin, E., (1953). J. Biol. Chem., 203, 319.

Dahmus, M.E., and McConnell, D.J., (1969). Biochemistry, 8, 1524.

Gillespie, D., and Spiegelman, S., (1965). J. Mol. Biol., 12, 162.

Huang, R.C., and Huang, P.C., (1969). J. Mol. Biol., 39, 365.

Jacobson, R.A., and Bonner, J., (1968). Biochem. Biophys. Res. Comm. 33, 716.

Egami, F., Takahashi, K., and Uchida, T., (1964). Prog. in Nucleic Acid Research, 3, 59.

PART II.

STUDIES ON RNA POLYMERASE OF E. coli

Part II

Chapter I.

THE PREPARATION OF RNA POLYMERASE

INTRODUCTION.

Since the first isolation procedure for E. coli RNA polymerase was reported (Chamberlin and Berg, 1962) many variations on it, as well as several new methods, have been devloped (Furth et al., 1962; Stevens and Henry, 1964; Fuchs et al., 1967; Babinet, 1967; Burgess, 1969). It is now possible to obtain the enzyme in large quantities more than 95% pure. However, differences in the strain of E. coli, the medium on which the cells are grown, and the stage in the growth cycle at which they are harvested may lead to differences in the efficiency with which a given method can be applied. Great variation in the protein and nucleic acid content is observed for example, and can create serious problems. Some cells contain so much nucleic acid that it is almost impossible to prepare enzyme completely dependent for activity on added template. Two strains, E. coli D-10 and E. coli B have been used in most of this work. E. coli D-10 was much superior. After a description of the various methods, the processes of purifying the enzyme from these two strains will be compared.

Since these enzyme preparations have been intended for studies on the mechanism of binding and initiation special attention has been paid to contaminating enzyme activities such as ribonuclease, deoxyribonuclease, phosphatase and polynucleotide phosphorylase which might affect the results

of experiments into these problems. Ribonuclease and phosphatase have not been detected in assays on purified enzymes, but the others have, DNAase at a very low level in a very sensitive assay, and polynucleotide phosphorylase.

It is well established that RNA polymerase catalyses the synthesis of homopolymer in an incomplete mixture of nucleoside triphosphates (Chamberlin and Berg, 1962; Ibid, 1964; Stevens, 1964). It is not likely, but it has not been shown conclusively, that the reaction does not occur when all four nucleotides are present. If it is an artifact it may cause serious misinterpretation of experiments in which RNA polymerase and DNA are reacted in the presence of one, two, or three nucleotides to form a putative initiation complex. Such preincubation has been shown to stabilize the initiation complex against high salt [.4M $(NH_4)SO_4$] and rifampicin (di Mauro et al., 1969; Anthony et al., 1966; Hyman and Davidson, 1970), though the role of homopolymer which is certainly made under the conditions of some of these experiments, has not been evaluated. If homopolymer is synthesized the complex under study may really be a mixture of enzyme-homopolymer, enzyme-DNA, and enzyme-homopolymer-DNA and deductions concerning the interaction of the enzyme with its real initiation site may not be valid.

The possible error is greatest in the case of ATP, for poly A is synthesized at a much greater rate than the other homopolymers (Chamberlin and Berg, 1962; Stevens, 1964).

Effects of GTP due to formation of homopolymer can be ruled out since the synthesis of poly G on a natural template has not been observed (Ibid).

The synthesis of poly A can be reduced by a variety of methods (Chamberlin and Berg, 1962; Stevens, 1964; Niyogi and Stevens, 1965; So et al., 1967). These have been applied to develop a readily interpretable system.

Another activity associated with RNA polymerase in all preparations examined has been the ability to make polyphosphate. Initially the incorporation of the gamma phosphate from ATP into acid precipitable form by highly purified enzyme in the absence of DNA was thought to represent the formation of a phosphoenzyme. This has been ruled out. Attempts to dissociate the activity from RNA polymerase have not been completely successful and it is suggested that this may be another activity of RNA polymerase. Alternatively it may represent tenacious contamination by polyphosphate kinase (Kornberg, et al., 1956).

MATERIALS AND METHODS.

(i) Assay for RNA synthesis.

The reaction mixture for RNA synthesis is : .05 M tris buffer pH 7.9, .008 M $MgCl_2$, .0001 M Cleland's Reagent, (or .01 M β mercaptoethanol). Sometimes .8 X 10^{-3} M K_2HPO_4 and .05 X 10^{-3} M ADP are added as inhibitors of polynucleotide phosphorylase, and polyphosphate kinase, respectively. The final volume is .25 ml. The high salt assay contains .15 M KCl. Some assays have varied from the one above and they are described in detail in appropriate places. Assays contain between 10 and 100 µg of DNA. Incubation is at 37°C in a water bath, usually for 10 minutes. Reactions are carried out in hydrophobic plastic 17 mm X 100 mm 12 ml tubes (Falcon Plastics, Bioquest). These have the advantage that visible fibrous precipitates formed when high concentrations of DNA and protein are used, i.e. >50 µg/assay, do not adsorb so readily to the sides of the tubes. Such precipitates stick strongly to glass and represent a serious source of error in recovery. The plastic tubes are superior in that the walls are thin, and reach the temperature of incubation more quickly than the heavier glass ones. The reactions are stopped by the addition of 1 ml of ice cold 5 M NaCl, followed quickly by 5 ml of 10% (W/V) trichloroacetic acid (TCA). The solution is mixed in a Vortex, and a further 5 ml of 10% TCA added. The NaCl solution is squirted into the tube with an automatic pipette, so mixing

and diluting the reaction. This stops the reaction effectively and reduces the chance of formation of fibrous precipitates. If the combined amounts of DNA and protein in the reaction are small, i.e. <10 µg, the NaCl should contain 50-100 µg/ml fraction V bovine serum albumin as coprecipitant. After the addition of TCA the tubes are stood on ice for at least 30 minutes. The precipitates are collected by filtration through nitrocellulose membranes (25 mm B-6 Schleicher and Schuell), or glass fibre filters (Whatman GFC), and washed five times with 10 ml volumes of 10% TCA. The extensive washing is carried out routinely to reduce the background, and is expecially necessary when high specific activity nucleotides are used. GFC filters have a lower background, approximately 50% that of nitrocellulose membranes. The background can be further reduced by soaking and washing the filters with 10% TCA containing .1 M sodium pyrophosphate and .05 µm/ml cold ATP. Since the rate of reaction is measurable at 4°C, it is not convenient to handle more than 12 assays at once. Filters are dried at room temperature, or under a desk lamp for fifteen minutes, and then at 80°C in vacuo for 10 minutes, to ensure complete evaporation of the TCA, which is a strong quenching agent. If nitrocellulose membranes are not dried at room temperature before being placed in the vacuum oven they become singed, another source of quenching.

(ii) Isotopes

8-^{14}C ATP or ^{3}H ATP were obtained from Schwarz Bio-Research or New England Nuclear. If crystalline the ATP was used directly, if in 50% ethanol it was lyophilized before use. For standard assays ^{14}C-ATP was diluted to approximately 1 millicurie per millimole, and ^{3}H-ATP to approximately 5 millicuries per millimole. Concentrations of nucleoside triphosphates were calculated using the following absorption coefficients for solutions in tris buffer pH 7.5. (Ref. Handbook of Biochemistry).

	ε at λ Max.	λ Max.	250/260	280/260
ATP	15.4×10^3	259 Mμ	.78	.16
CTP	9.0×10^3	271 Mμ	.84	.97
UTP	10.0×10^3	262 Mμ	.75	.38
GTP	13.7×10^3	253 Mμ	1.17	.66

Samples of triphosphates were sometimes applied to the Picker Nuclear Nucleic Acid Analyser. The column was developed with a gradient of .04 M-1.5 M ammonium formate pH 4.35 at 71°C. Mono-, di- and triphosphates were easily separated in that order. If radioactive ATP is kept in concentrated solution at high specific activity there is significant degradation from auto-irradiation even at low temperature. After 2 months only 85% of the ^{3}H of a sample of ^{3}H ATP chromatographed as ATP, the remainder recovered in the diphosphate

and monophosphate regions. The same is true of solutions of ^{32}P ATP.

(iii) Scintillation Counting

Two cocktails were used routinely

(a) Dioxane

Per 3 kg of dioxane, 360 g of naphthalene, 12 g of PPO, .15 g of POPOP

(PPO ≡ 2,5 diphenyloxazole)
(POPOP ≡ p bis (2-(5-phenyloxazolyl)) benzene

(b) Toluene

Per 3.79 l of toluene, 26.6 g of PPO and .75 g of POPOP

Depending on the salts up to 2 ml aqueous samples were counted in 10 ml of Dioxane with very little quenching of ^{14}C or ^{32}P. If an aqueous sample was less than 100 microlitres it was usually dried on a filter and counted in toluene. Precipitates collected on filters were counted in Toluene. Specific activities of radioactive nucleotides, DNA and protein were always calculated from controls counted under identical conditions to the sample. All filters counted in toluene were dried in air at room temperature for 10 minutes, and then in vacuo at 80°C for 10 minutes. Counting aqueous

samples of ^{3}H in dioxane is subject to a major source of error. The efficiency of counting falls off rapidly with time over a period of 4 days. This is also observed with almost every method tried, but never at the same rate, to the same extent, or over such a long period as in the case of dioxane and aqueous solutions. It is therefore extremely important to make standards for determining specific activities at the same time as the samples, and this is done routinely. The efficiencies of counting ^{3}H under four conditions is tabulated below. For the filters the sample was applied and dried thoroughly. Values are those when the rate of change of efficiency is less than 2% per day, and are normalized to the highest value.

Toluene and NCS	100
Toluene and filter	40
Dioxane and water	30
Dioxane and filter	10

NCS, an organic alkaline solubilizer (Nuclear Chicago) was sometimes used to allow the counting of ^{3}H solutions in toluene without drying. It is particularly convenient for counting fractions from glycerol gradients. Up to .5 ml of aqueous glycerol solution containing ^{3}H can be counted in the presence of .5 ml NCS in 10 ml of toluene. The concentration of glycerol in the solution has a small effect. .5 ml of 10% glycerol gives approximately 10% greater

efficiency than .5 ml of 30% glycerol. As the volume of sample increases from .4 ml to .5 ml the efficiency increases by 20%. Larger volumes were not tested. NCS was also used to permit the counting of fractions of SDS-polyacrylamide gels in toluene. Fractions are obtained most successfully by squeezing the gels through a 20 gauge needle from a 1 ml syringe. The plunger is driven by a screw so that the size of the fractions can be controlled. They are collected directly in scintillation vials, 1 ml of NCS is added, and they are shaken gently at room temperature for 30 minutes to facilitate the extraction of the gels. The polyacrylamide is not soluble in NCS but the gel is fragmented by the passage through the needle and the protein is easily extracted, even after staining. If the protein has been stained by Coomassie Blue it is simple to identify by eye which fractions contain which bands, and secondly as the extraction with NCS proceeds the dye becomes soluble and provides a measure of the degree of solution of the protein. 10 ml of toluene are added per fraction. Up to 80 fractions can be conveniently obtained from a 6 cm gel. Under these conditions chemiluminescence due to NCS is not observed. NCS cannot be used for nitrocellulose membranes, due to excessive chemiluminescence in the range of energy of ^{3}H disintegrations.

(iv) Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis

The method used is essentially that described by

Shapiro et al., (1967).

Stock solutions are:

A. Temed [NNN'N' tetramethylene diamine.].

.4 ml per 100 ml H_2O. Stored in the dark in a refrigerator.

B. Acrylamide, NN-bismethylene acrylamide, phosphate and SDS. 20 g acrylamide plus .52 g bisacrylamide plus 2 ml of 20% sodium dodecyl sulphate plus 40 ml sodium phosphate buffer pH 7.1 plus H_2O to a final volume of 100 ml, stored at room temperature. It is usually necessary to warm slightly before use, to dissolve the acrylamide.

C. Ammonium persulphate.

.12 g ammonium persulphate per 100 ml H_2O. This solution must be made directly before use.

7.5 ml of solution C are added to 1.5 ml of A and mixed thoroughly. 3 ml of solution B are added and rapidly mixed. Glass tubes 7.5 cm by 5 mm placed in a Perspex holder are filled to a line .5 cm from the top. A layer of H_2O is carefully placed on top of the acrylamide solution and the gels are allowed to form by standing for 2 hours at room temperature.

The final concentrations in the gel are:

5% acrylamide
.1% sodium dodecyl sulphate
.13% bisacrylamide
.05% NNN'N' tetramethylene diamine
.1M sodium phosphate pH 7.1
.075% ammonium persulphate

The tray buffer in the electrophoresis apparatus is .1 M sodium phosphate pH 7.1, .1% SDS. Samples are prepared for application to the gels by making them .1% SDS, .01 M sodium phosphate pH 7.1, .1% β mercaptoethanol, 10% glycerol. 100 microlitres of samples can be applied per gel. If the salt concentration of the sample is low ($<$.2 M) and provided the concentration of potassium is less than .05 M, 50 microlitres of sample may be added directly to an equal volume of .2% SDS, .02 M sodium phosphate pH 7.1, 2% β mercaptoethanol and 20% glycerol. Potassium dodecyl sulphate is insoluble, while high salt concentration may reduce the "stacking". Initially samples were heated at 60°C for 30 minutes before applying to the gel but no advantages were observed, and the procedure was dropped.

Electrophoresis of RNA polymerase is carried out routinely for 75 minutes at 45 Volts at constant voltage. Gels are then removed from the tubes and stained in a solution of 25% Coomassie Brilliant Blue R-250 in methanol - acetic acid - water (5:1:5) for 90 minutes. Destaining is carried out in 10% acetic acid 5% methanol in a horizontal field of 90 Volts for one hour. Gels are stored in 10% acetic acid 5% methanol in the dark. Under these conditions gels retain their stain for over a year. The volume of sample applied does not affect the resolution of different bands up to 100 microlitres. Larger samples have not been tried. For highly purified RNA polymerase ($>$ 90% pure) a

sample of 5 micrograms gives excellent results. Above 10 micrograms the rate of electrophoresis of the bands slows markedly, but large amounts are sometimes used to show up minor bands. The characteristic β_1 β_2 σ ω band pattern of RNA polymerase is striking and easy to recognize even in a highly contaminated sample. Controls of purified polymerase are always run to make certain of the identification of bands. Gels were scanned by a Gilford Spectrophotometer at 600 mμ at a slit width of .05 mm.

(v) Sedimentation coefficients.

Analyses of sedimentation coefficients of DNA preparations at neutral pH were carried out according to Studier, (1965). For T7 DNA runs were made under alkaline conditions to estimate the percentage of single strands which were unknicked.

(vi) Chemical and spectrophotometric assays.

DNA is determined by the diphenylamine reaction described by Burton (1956), RNA by the orcinol method of Dische and Schwarz (1937). The concentration of RNA polymerase is estimated from the extinction coefficient

$$E_{280}^{1\%} = 6.5 \text{ (Richardson, 1966).}$$

(vii) Preparation of E. coli DNA from frozen cells of strain B.

Frozen cells of E. coli B were obtained from General Biochemicals, Chagrin Falls, Ohio, and stored at -80°C.

20 g of cells were suspended in 75 mls .01 M tris .01 M EDTA pH 7.5 at 37°C. The presence of EDTA renders the cell wall susceptible to egg white lysozyme which is added at a concentration of 1 mg/ml. The suspension is stirred at 37°C for 15 minutes becoming extremely viscous. It may be necessary to dilute it further to obtain a manageable solution. DNA is extracted by the procedure of Marmur (1961). Two initial extractions are made with chloroform/isoamyl alcohol in the presence of 1% SDS and 2 M NaCl. 2 volumes of 95% ethanol are added slowly and the DNA spooled out on a glass rod. The RNA which is a significant contaminant at this stage, forms a flocculent precipitate, but most of this is not spooled out with the DNA. The DNA is dissolved in 200 ml 15 M NaCl .015 M Na Citrate [Standard Saline Citrate, (SSC)], and then incubated at 37°C for 2 hours in the presence of ribonuclease A (40 µg/ml), followed by 2 hours at 37°C in the presence of pronase (40 µg/ml). The RNAase A is heated at 80°C for 15 minutes before use, to destroy any contaminating DNAase. For the same reason Pronase (Grade B, Cal Biochem.) is autodigested at 37°C for 90 minutes. The solution is then made to 1% SDS, 2M NaCl and extracted with an equal volume of re-distilled phenol containing .1% 8 hydroxyquinoline (Kirby 1962) to facilitate phase separation. The extraction must be repeated at least three times to ensure complete removal of ribonuclease. The DNA is precipitated with ethanol, dissolved in 1/100 SSC, and dialyzed

to remove trace amounts of phenol. It is re-precipitated with .54 volume isopropyl alcohol in the presence of .3 M sodium acetate, .1 M EDTA pH 7.0 (Marmur 1961). This procedure leaves small DNA and RNA in the supernatant. The precipitate is dissolved in 1/100 SSC, extensively dialysed, and finally stored at 4°C over chloroform. It is then assayed for RNAase activity as outlined in paragraph (xiii). If RNAase activity is measurable, further extraction with phenol is effective in removing it. The sedimentation pattern of DNA prepared in this way has an S20, W of 35. Total yield is 25 mg of DNA per 20 g of cells. The OD 280/OD 260 = .42 and the OD 230/OD 260 = .51.

(viii) Growth of Novikoff Ascites Tumour Cells.

The method is described by Dahmus and McConnell (1969).

(ix) Preparation of Ascites Tumour DNA.

The procedure is identical to that described in Dahmus and McConnell (1969). DNA had an S20,W > 20, a hyperchromicity of 40%, and did not contain ribonuclease activity. Its spectrum showed an OD 280/OD 260 of .54 and an OD 230/OD 260 of.51.

(x) Preparation of RNA polymerase.

1. Buffers

Buffer A .01 M tris pH 7.9

.01 M Magnesium chloride

.01 M β mercaptoethanol

	5% glycerol
Buffer LSB (low salt buffer)	.01 M tris pH 7.9
	.01 M magnesium chloride
	.002 M β mercaptoethanol
	.012 M ammonium sulphate
Buffer HSB (high salt buffer)	.01 M tris pH 7.9
	.01 M magnesium chloride
	.0001 M Cleland's reagent
	1.0 M KCl
Storage medium (SMB) (Burgess, 1969)	.01 M tris pH 7.9
	.01 M magnesium chloride
	.01 M potassium chloride
	.0001 M EDTA
	.0001 M Cleland's reagent
	50% glycerol (Matheson, Coleman and Bell, Spectral Grade)
Buffer D (DEAE cellulose)	.01 M magnesium chloride
	.0001 M EDTA
	.0001 M Cleland's reagent
	.002 M potassium phosphate pH 7.5
	5% glycerol
Buffer P (Phosphocellulose)	.0001 M EDTA
	.0001 M Cleland's reagent

.02 M tris pH 7.9

5% glycerol

Buffer DC (DNA cellulose)

.01 M tris pH 7.9

.0005 M Cleland's reagent

.0001 M EDTA

25% glycerol

2. Lysis of cells

Method A.

60 g (wet weight) of cells (E. coli B ATCC 11303, mid-log phase, General Biochemicals, Chagrin Falls, Ohio,) or E. coli D-10,(1/4 log phase, Grain Processing Corp., Muscatine, Iowa) are homogenized at 100 volts for 20 minutes in a Waring blender with 130 mls of buffer A and 180 g of glass beads (Superbrite 100-500). The temperature is maintained at less than 4°C by occasionally cooling the container in an ethanol/dry ice bath. Larger batches are always homogenized in 60 g lots to maximize contact with the blades.

Method B.

60 g of cells are suspended in 130 ml .01 M tris base .01 M EDTA pH 7.5 and incubated in the presence of lysozyme, 1 mg/ml at 37°C for 15 minutes. The solution becomes exceedingly viscous and sometimes unmanageable due to the release of DNA. Deoxyribonuclease II (Worthington, HDAC.) 10 µg/ml, can be added to reduce the viscosity.

It is inactive in the presence of Mg++, and at alkaline pH so that in the event that a small amount contaminates the final enzyme preparation its effect will be reduced. However it should be used with caution where the integrity of the template is vital. To complete the lysis the solution is made 5% glycerol, .03 M $MgCl_2$ .01 M β mercaptoethanol, cooled to less than 4°C and homogenized with glass beads as in Method A. The use of lysozyme is a convenient method for increasing the efficiency of lysis and was used routinely. DNAase treatment was not used in most preparations.

3. Low and high speed centrifugation of crude extract

The crude extract is first centrifuged at 30,000 g for 10 minutes to remove unlysed cells and glass beads. The supernatant is then run at 100,000 g for 3 hours to pellet ribosome, membranes, cell wall material etc. The final supernatant is F1.

4. Fractionation by precipitation with Streptomycin and Protamine

Method A.

RNA polymerase and DNA are precipitated by different concentrations of streptomycin sulphate and protamine (Chamberlin and Berg, 1962). For the first step, the amount of 10% streptomycin, which precipitates the maximum fraction of DNA with little or no loss of enzyme, is determined by adding increasing amounts from 0 to .13 ml to 1 ml aliquots of F1 while stirring vigorously. The samples are stood on

ice for 15 minutes, centrifuged, and 50 or 100 microlitre aliquots of each supernatant are assayed for RNA synthesis in the presence and absence of added calf thymus DNA (100 µg). The results of such an assay are shown in Figure 1 (a). The rise inactivity of the supernatant at low streptomycin concentrations is characteristic. The addition of .07 ml of 10% streptomycin precipitates 80% of the DNA but none of the RNA polymerase. This amount is added per ml of F1, while stirring, the solution is stood on ice for 15 minutes and the supernatant collected after centrifuging. Precipitation by protamine proceeds very similarly, in this case the amount of 1% protamine required to precipitate 90% of the enzyme is determined in a preliminary assay (Figure 1 (b)).

Method B.

A two step protamine precipitation avoids the use of streptomycin and only requires a single assay. Increasing amounts of 1% protamine from 0 to .35 ml are added to 1 ml aliquots of F1. After standing on ice for 15 minutes the samples are centrifuged, and the supernatants assayed for RNA polymerase activity as before. The results of such an assay are shown in Figure 1(c).It is possible to add .15 mls of 1% protamine per ml of extract, precipitating 60% of the DNA but none of the enzyme. This precipitate is discarded and a further .15 mls of 1% protamine are added per ml of supernatant to precipitate the RNA polymerase. The

Figure 1 (a). The effect of streptomycin sulphate on the RNA polymerase activity of a crude extract of E. coli D-10. Activity measures the cpm of ^{14}C AMP which is acid precipitable after incubation of a 100 λ aliquot of a streptomycin supernatant with or without added calf thymus DNA (10 ug) for 10 minutes at 37°C in a standard RNA polymerase low salt assay. .07 mls of streptomycin per ml of extract gives optimum yield of enzyme and high dependency on added DNA.

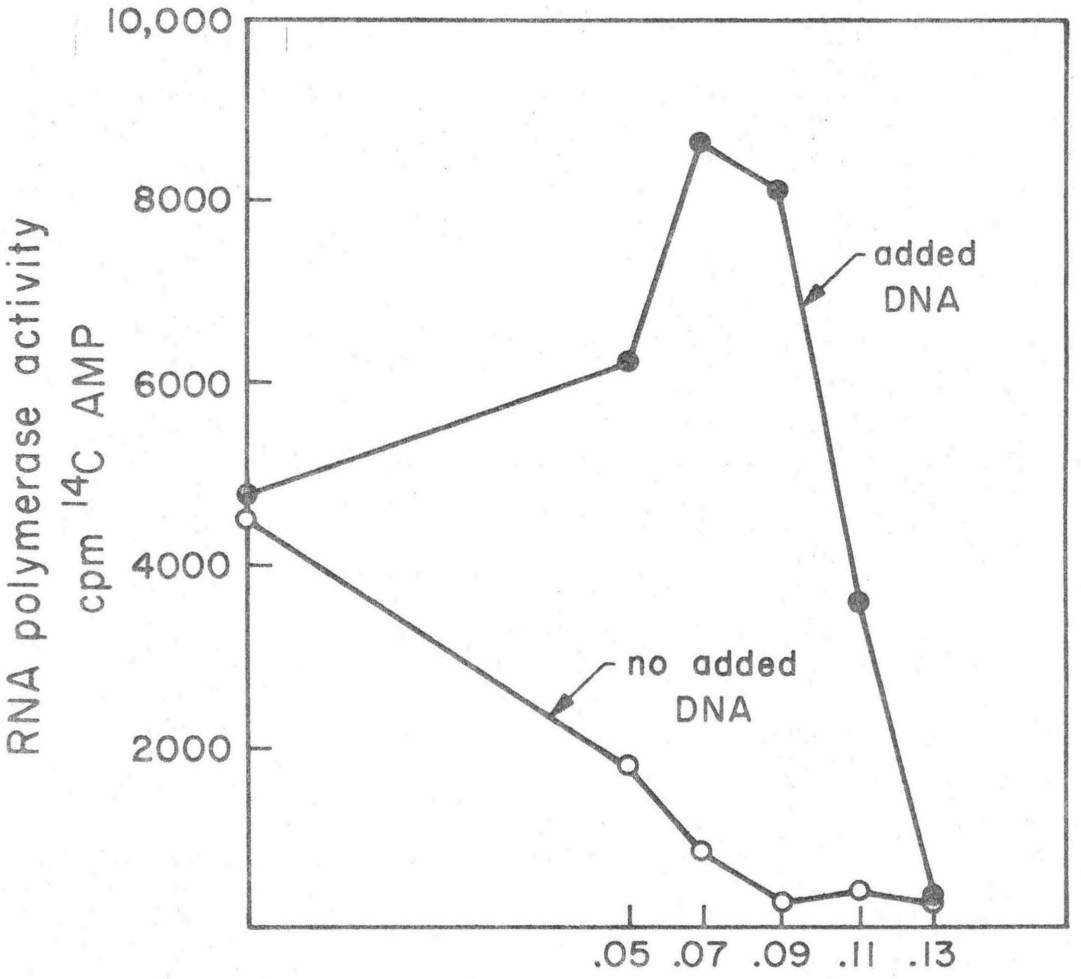

10,000
8000
6000
4000
2000
RNA polymerase activity
cpm ^{14}C AMP
added
DNA
no added
DNA
.05 .07 .09 .11 .13
Vol. 10% streptomycin added per ml of
crude extract

Figure 1 (b). The effect of protamine sulphate on the RNA polymerase activity of the extract of E coli D-10 after the streptomycin (.07 10%/ml of extract) precipitation of most of the nucleic acid. Assay exactly as in Figure 1 (a). .07 ml of 1% protamine precipitates 100% of the activity.

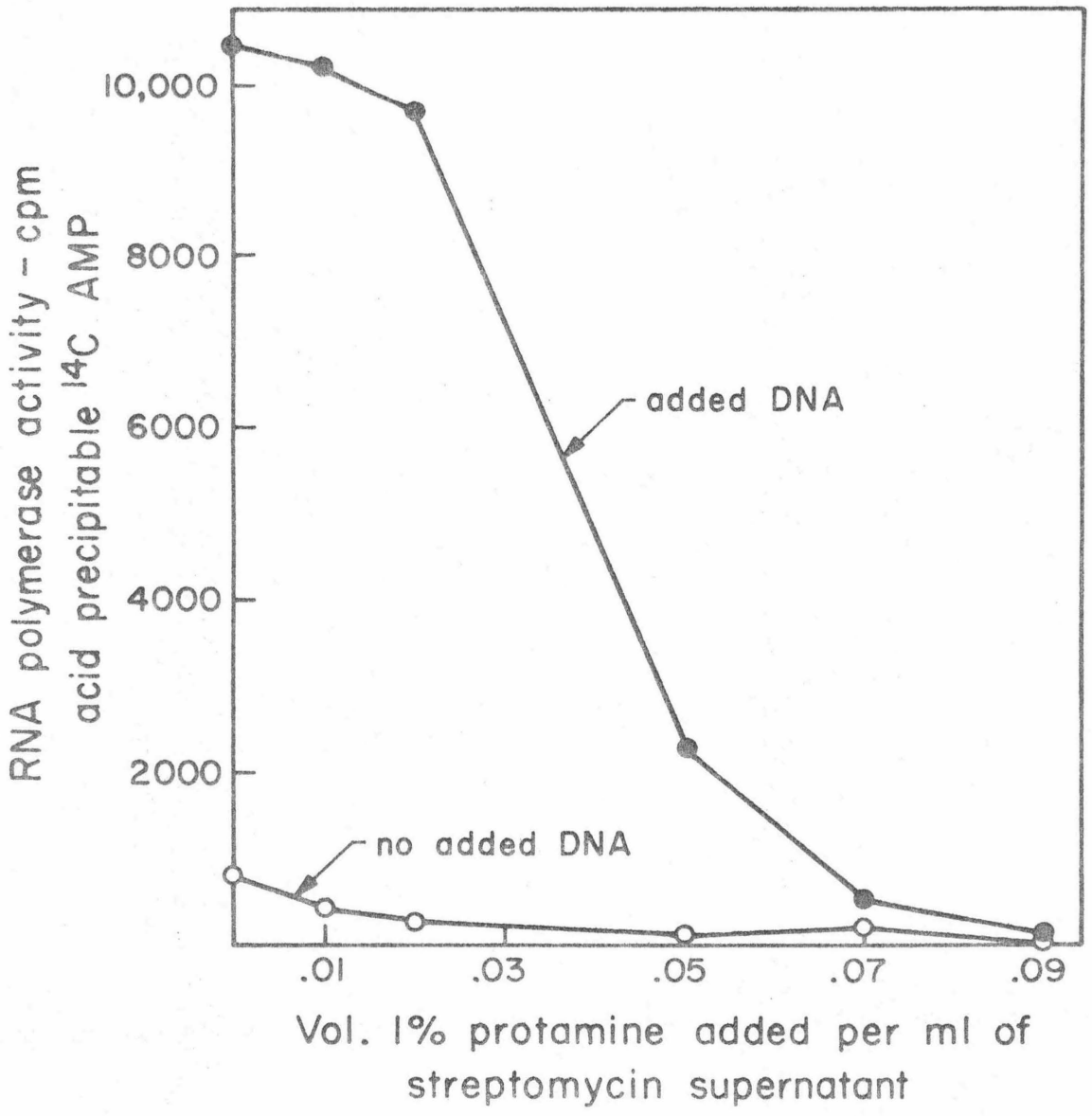
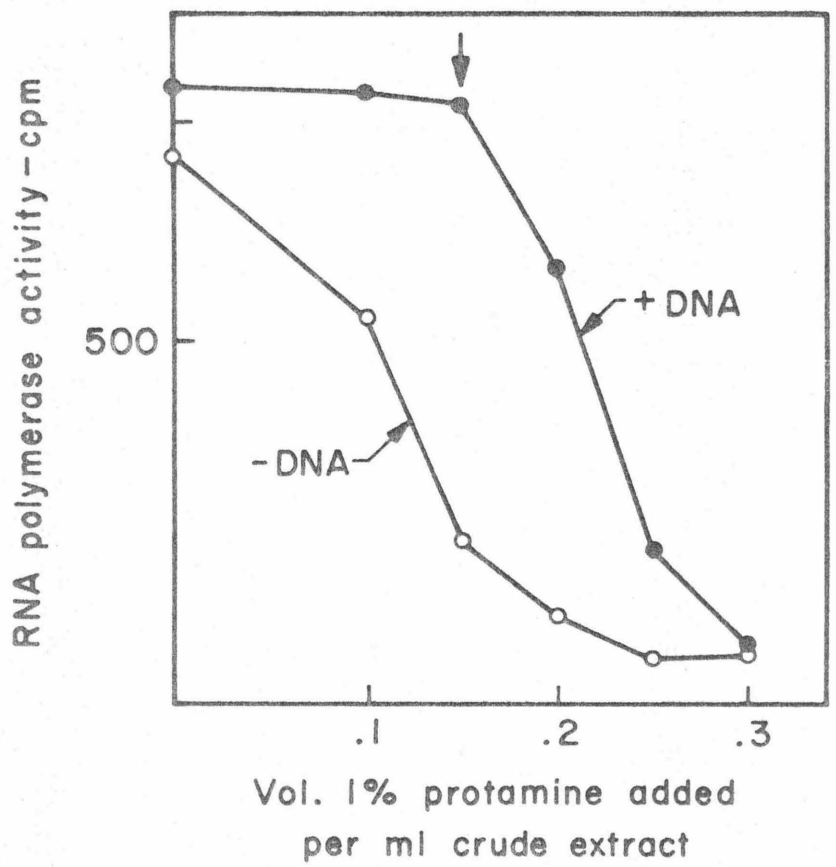

RNA polymerase activity - cpm acid precipitable ^{14}C AMP
10,000
8000
6000
4000
2000
added DNA
no added DNA
.01
.03
.05
.07
.09
Vol. 1% protamine added per ml of streptomycin supernatant

Figure 1 (c). The effect of protamine sulphate on the RNA polymerase activity of a crude extract of E.coli B— no previous precipitation with streptomycin. Activity in 50 λ of the supernatant after precipitaion with the designated amount of protamine, 50 ug of E. coli DNA being added to one of duplicate assays. .15 ml 1% protamine per ml of extract is added to remove most of the DNA, followed by another .15 ml to precipitate the enzyme activity.


RNA polymerase activity - cpm
500
+ DNA
- DNA
.1
.2
.3
Vol. 1% protamine added
per ml crude extract

precipitate is F2. This method was used routinely as being equally effective, more convenient and more reproducible.

5. Extraction with magnesium acetate and ammonium sulphate fractionation

The protamine precipitate is suspended gently in .1 M magnesium acetate buffer A^+ with a teflon homogeniser. Approximately 20 ml are required per 70 g of cells. The suspension is centrifuged at 30,000 g for 10 minutes, and the supernatant which has a high OD 260, discarded. The pellet is dissolved in .1 M ammonium sulphate buffer A^+ (20 mls per 70 g cells) by gentle homogenisation*. Insoluble material is removed by centrifuging, and re-extracted with ammonium sulphate. The supernatants are combined , a 33% saturated ammonium sulphate precipitate is discarded and the enzyme precipitated by 66% ammonium sulphate. The final precipitate may be washed with 58% ammonium sulphate which does not dissolve the polymerase but does remove some other proteins (Burgess, 1969). This is F3.

6. Low salt glycerol gradient sedimentation

The precipitate F3 is dissolved in LSB and dialysed extensively to reduce the concentration of ammonium sulphate. 2 to 5 ml amounts containing up to 100 mg of protein are then layered on 60 ml linear glycerol gradients (10-30%) in LSB, covered by a thin film of paraffin oil to reduce surface denaturation and spillage, and centrifuged at 22.5K for

36 hours at 4°C in the Beckman SW 25.2 rotor. 1 ml fractions are collected after puncturing the bottoms of the tubes, and representative samples are assayed. Results for such a gradient are shown in Figure 2. Fractions containing polymerase are combined and the enzyme is precipitated by 60% saturated ammonium sulphate. This sample was then applied to the high salt glycerol gradient of Figure 3, which shows the enzyme to have been more than 60% pure after the low salt sedimentation.

7. High salt glycerol gradient sedimentation

2-5 ml samples are applied to 60 ml linear glycerol gradients (10-30%) in buffer HSB as above, and centrifuged at 25 K for 45 hours. Fractions are collected and assayed as usual. Figure 3 shows the result of such an experiment. The enzyme from this run had a specific activity of 1000 on T7 DNA. The dependency of the sedimentation velocity on protein concentration is high, and the time of centrifugation should be adjusted accordingly. The above condition is appropriate for 10 mg of protein per tube. For 20 mg the conditions are 25 K for 60 hrs. An SDS gel is shown in Fig. 7.

8. DEAE cellulose chromatography

DEAE cellulose #70 .89 meq. per g is obtained from Schleicher and Schuell. 10 g of resin is sufficient for a column bed volume of 100 mls. It is treated as follows to remove extraneous material.

Figure 2. Low salt glycerol gradient sedimentation of an E. coli D-10 fraction 3 (F3) RNA polymerase preparation. 2 ml of LSB containing 100 mg of protein were layered on 60 ml of 10%-30% glycerol in LSB and centrifuged at 22.5 K for 36 hr at 4°C in the SW 25.2 rotor. Fractions were collected by dripping, OD 280 was read and selected fractions were assayed for RNA polymerase activity in a low salt assay with 10 µg of calf thymus DNA as template. Fractions 11-30 inclusive were combined, precipitated with ammonium sulphate and applied to the high salt glycerol gradient shown in Figure 3. after dialysis against LSB.

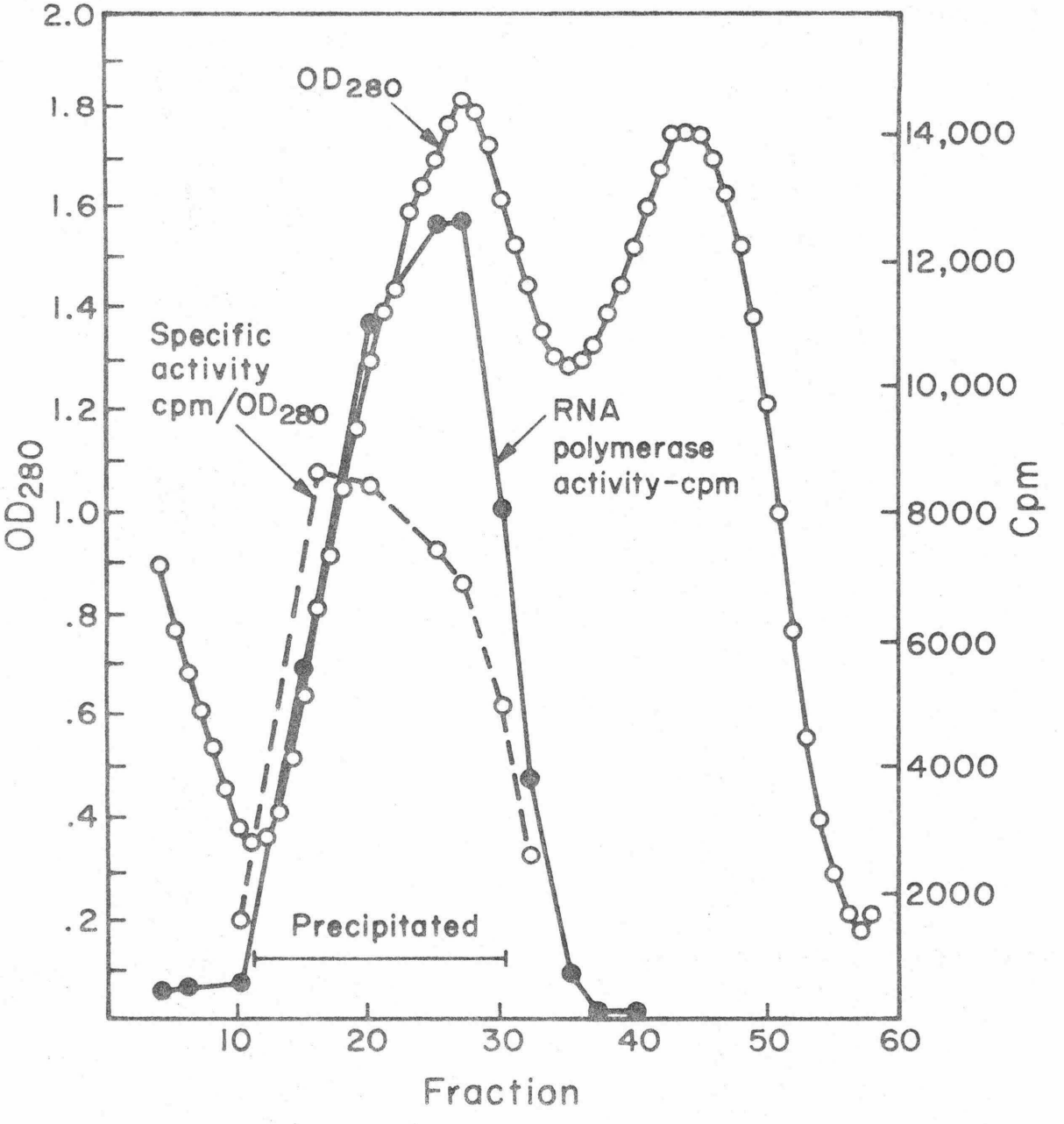
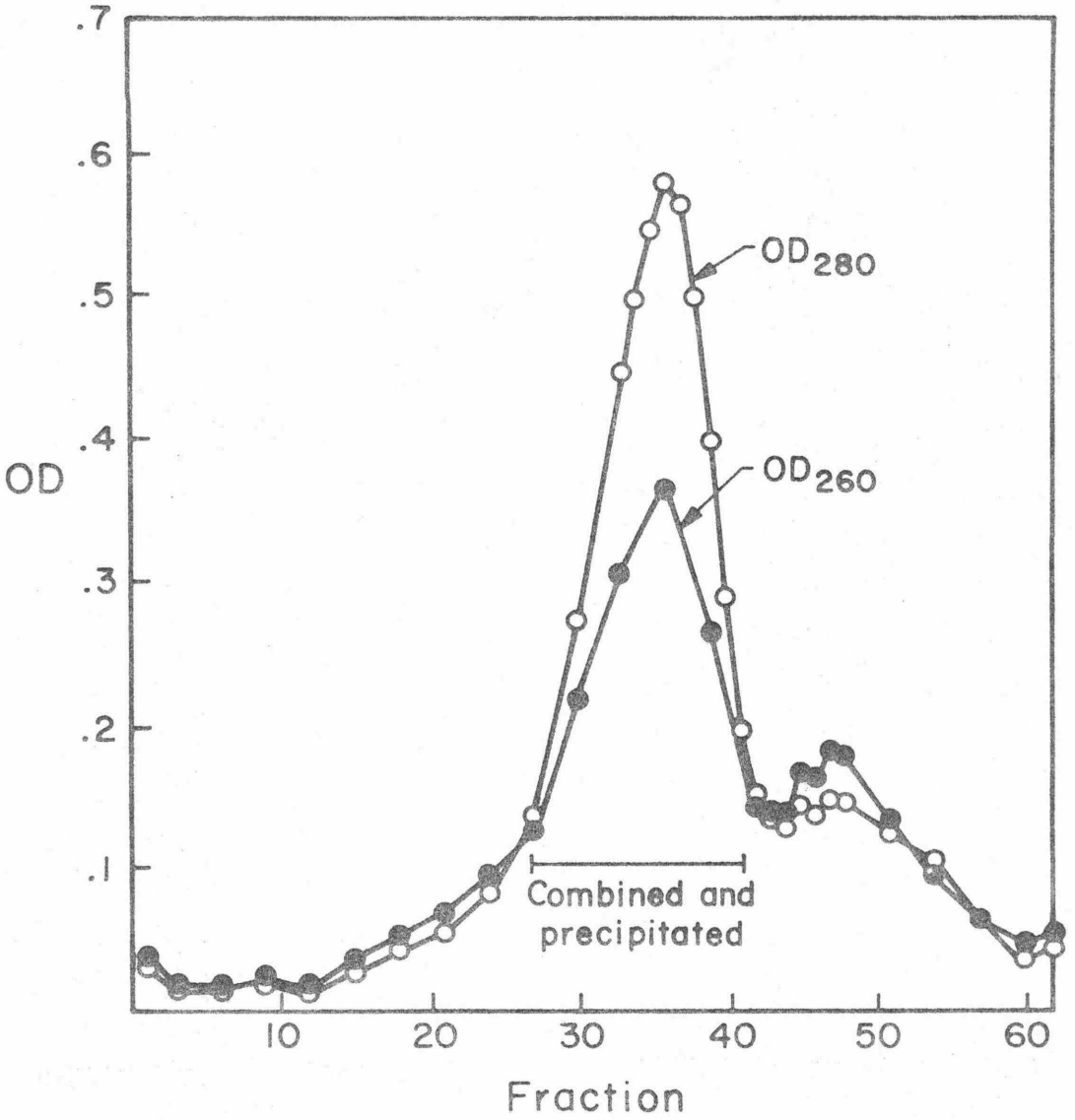

2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
OD280
14,000
12,000
10,000
8000
6000
4000
2000
Cpm
OD280
Specific activity cpm/OD280
RNA polymerase activity-cpm
Precipitated
10
20
30
40
50
60
Fraction

Figure 3. High salt glycerol gradient sedimentation of E. coli D-10 RNA polymerase, fractions 10-30 of the low salt glycerol gradient sample shown in Figure 2. 1 ml of RNA polymerase solution in LSB containing approximately 10 mg of protein were layered on 60 ml of a 10%-30% glycerol gradient in HSB (1 M KCl etc.) and centrifuged at 22.5 K for 45 hr at 4°C in the SW 25.2 rotor. Fractions 27-41 were combined and gave an enzyme of specific activity 1000 units per mg on T7 DNA.


.7
.6
.5
.4
.3
.2
.1
OD
OD280
OD260
Combined and
precipitated
10
20
30
40
50
60
Fraction

1. Soak overnight in the cold in 1 litre deionized H_2O.
2. De-fine several times from deionised H_2O.
3. Suspend in 1 litre .5 N NaOH and rapidly filter on a Buchner funnel. Repeat.
4. Wash quickly 3 times with 2 litres of deionised H_2O.
5. Suspend in 1 litre .25 N HCl, rapidly filter and wash several times with deionised water until the filtrate is greater than pH 5.0.
6. Equilibrate with buffer D, and pour the column.

The column should be washed with 2 volumes of buffer D before use. 300 mg of protein can be applied to a column of 100 mls. The sample is dialysed extensively against buffer D before being applied to the column. This is especially necessary if it has just been precipitated with ammonium sulphate which coprecipitates with the protein. After application the column is washed with buffer D until the OD 280 of the run-off peak is less than .1. Impurities are eluted with approx. 2 column volumes of buffer D .13 M KCl, and the enzyme itself with 2 column volumes of buffer D .23 M KCl. As an alternative to batch elution a gradient of KCl (0-.7 M) in buffer D can be used to develop the column. 400 mls gradient are required per 100 mls column volume.

9. Phosphocellulose chromatography

Whatman P-11 cellulose phosphate, 7.4 meq. per g is prepared as follows:

1. Suspend in deionised water in the cold overnight.
2. De-fine several times from ionised water.
3. Suspend in .1 N KOH, filter rapidly on a Buchner funnel and wash with several litres of deionised water.
4. Suspend in .1 N HCl, filter rapidly and wash with deionised water.
5. Suspend in deionised water and titrate to pH 7.9 with 1 N KOH at room temperature.
6. Equilibrate with buffer P.05 M KCl.

200 mg of protein can be applied to a 100 ml column. The sample must be well dialysed against buffer P .05 M KCl to remove all traces of magnesium and ammonium sulphate beforehand. The run-off is usually very large. The column is washed with buffer P.05 M KCl until the OD 280 is less than .1 and the enzyme is then eluted with buffer P .4 M KCl. It is important to note that enzyme purified on phosphocellulose is often deficient in the σ sub-unit. Although Burgess et al,, (1969) reports that some σ remains bound to the column at.05 M KCl, it is certainly a small amount, and insufficient to saturate the core enzyme with which it elutes at .4 M KCl. The sample must be applied in buffer P .05 M KCl, otherwise the enzyme may not stick to the column. Presumably at lower ionic strengths the cationic region of the enzyme surface is obscured.

10. Preparation of core enzyme and σ sub-unit

Phosphocellulose chromatography of purified enzyme

results in the separation of the σ sub-unit. (Burgess 1969). 10 mg of glycerol gradient purified enzyme in 5 ml of storage medium are dialysed against several changes of buffer P 0 M KCl and applied slowly to a phosphocellulose column 1 cm X 7 cm. The column is washed with 5 ml of buffer P.05 M KCl followed by 100 ml of gradient, .05 M KCl → .4 M KCl in buffer P. There is a small run off, and a large peak at .38 M KCl. At intermediate salt concentrations protein is continually removed, and a further small amount is eluted when the column is washed with buffer P 1.0 M KCl (Figure 4). Total protein recovery is 60% - 70%. In the case of Figure 4, the eluate was divided into seven fractions (PCI-PCVII) as shown, precipitated from 60% saturated ammonium sulphate, and dissolved in storage medium. 5-10 µg samples were applied to SDS-polyacrylamide gels (Figure 5). The ratio of β to σ rises steadily through the gradient. PCII has a large excess of σ, while PCVI and PCVII have almost none. A band appearing very close to the σ position may not be σ, but another polypeptide running slightly faster. However in the detailed analysis (Part II Chapter II Table 1) it is assumed to be σ.

11. DNA cellulose chromatography

DNA cellulose is prepared essentially as described by Litman (1968). Commercial Calf Thymus DNA (Sigma, Type I, highly polymerised) is dissolved at 2-3 mg/ml in .01 M tris

Figure 4. Separation of the σ sub-unit and the core enzyme of RNA polymerase by phosphocellulose chromatography. 10 mg of protein (glycerol gradient purified enzyme) were applied in buffer P (5 ml) to a phosphocellulose column 1 X 7 cm equilibrated with buffer P, and eluted with 100 ml of gradient from .05 → 1.0 M KCl in buffer P. Fractions were combined as shown, precipitated with ammonium sulphate. Figure 5 is a photograph of SDS-polyacrylamide gels for these samples PC 2.1→PC 2.7.

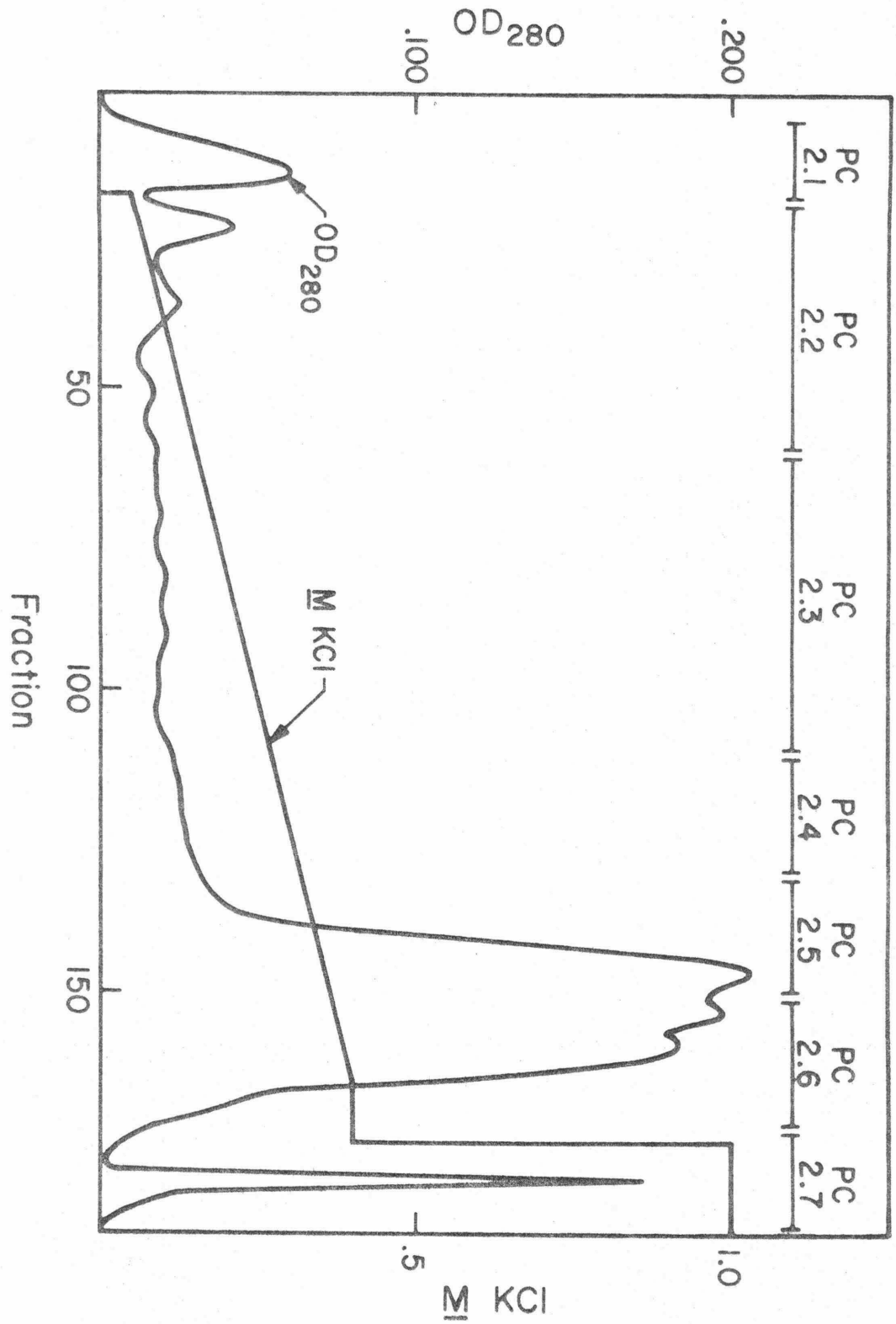
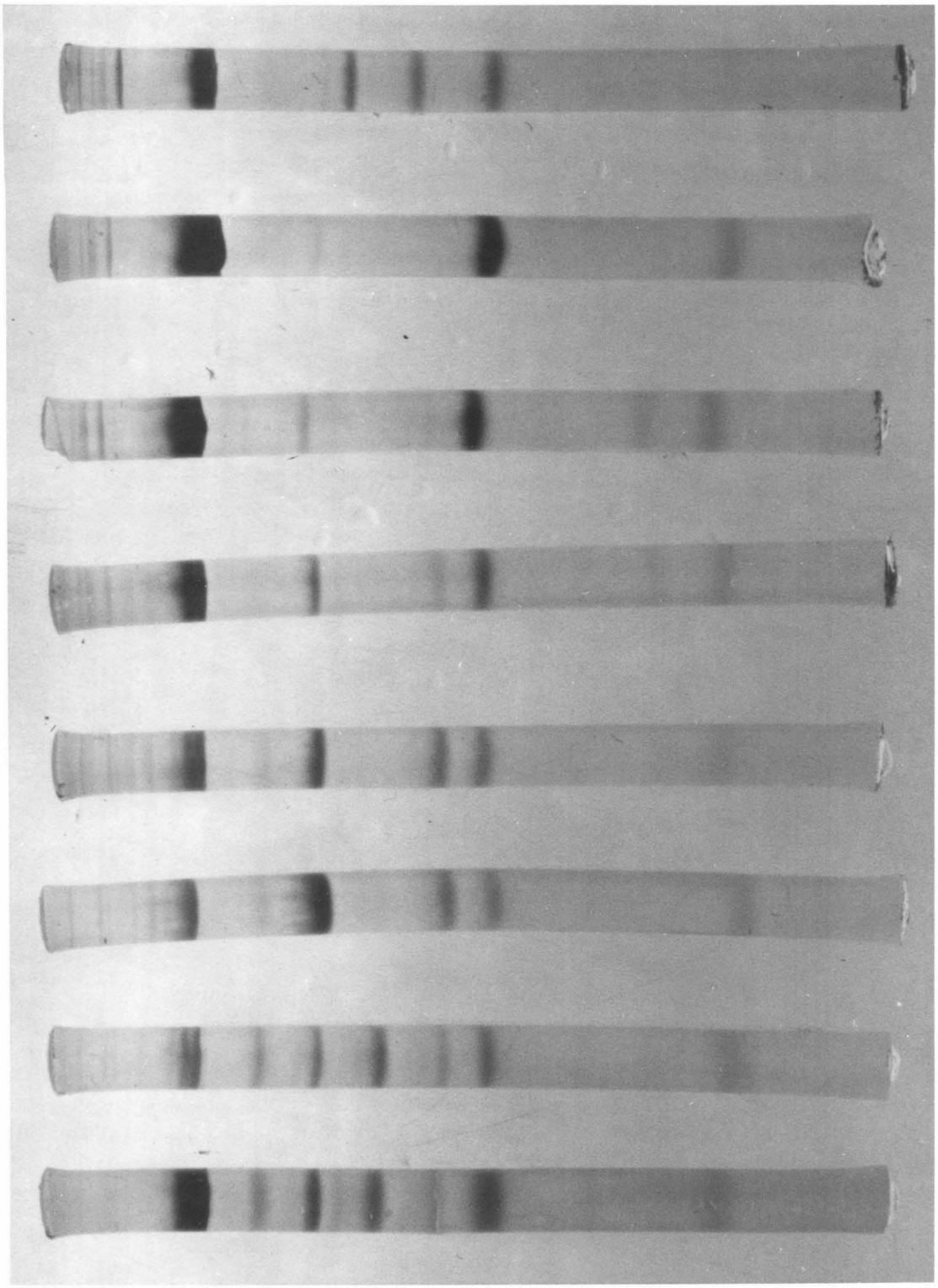

OD280
.100
.200
PC 2.1
PC 2.2
PC 2.3
PC 2.4
PC 2.5
PC 2.6
PC 2.7
OD280
M KCl
50
100
150
Fraction
.5
1.0
M KCl

Figure 5. SDS-polyacrylamide gels of the samples from the phosphocellulose column (Figure 4), after staining with Coomassie Blue. Electroporesis at 45 Volts for 75 minutes in 5% acrylamide, .13% bisacrylamide, and .1% SDS gels. Samples were left to right 10 µg of glycerol gradient enzyme (GGC), and phosphocellulose enzymes PC 2.1 to PC 2.7. Reading from top to bottom the bands are referred to as β(β1 β2 or ββ') σ1σ2σ3σ4 α α1 ω.



pH 8.0. A sufficient amount of dry acid washed cellulose (Whatman CF 11) is added to produce a thick slurry. This is thinly and evenly spread around a beaker and lyophilised. 10 volumes of 100% ethanol are added, the cellulose is then well dispersed and irradiated with an ultraviolet lamp (2 General Electric G15 T8 15 watt Germicidal Tubes) with constant stirring for 30 minutes at a distance of 5 cm. The depth of the suspension should be 3 cm or less. After irradiation the slurry is filtered on a Buchner, washed with 1 M KCl and then extensively with water. The resin is dried exhaustively on the Buchner, the cake is broken up well and lyophilised. All the procedure and storage are at room temperature.

The amount of DNA bound is determined by hydrolysing a known dry weight of DNA cellulose in 5% percholic acid for 10 minutes at 100°C. The OD 260 of the supernatant is read using a straight cellulose blank and the amount of DNA calculated from the relationship that one OD 260 unit is equivalent to a concentration of approximately 35 µg/ml of denatured DNA. An average of 8-10 mg of DNA were bound per gram of cellulose. The most important limiting factor to raising this amount is the initial concentration at which the DNA is dissolved.

The resinis suspended in buffer DC 1.0 M KCl, the column is poured and washed with buffer DC 1.0 M KCl until the OD 260 of the eluant is less than .02. There may be some

free DNA in the resin which is removed by this wash. The column is then washed with buffer DC .15 M KCl until equilibrated. The sample is applied in a small volume after extensive dialysis against DC .15 M KCl. The slower the application the better the binding of the enzyme. The column is washed until the run-off has emerged, and developed by a gradient at .5 M KCl, although the activity is somewhat skewed to higher salt concentrations (Figure 6). Where the activity is skewed the leading edge appears deficient in σ when analysed on SDS gels. Figure 7 shows gels of samples of enzyme prepared from the leading and trailing edges of the DNA cellulose peak (Figure 6). 400 mg of F3 can be applied to a column 2 x 40 cm and eluted by a gradient of 2 x 150 mls of gradient. 40 mg of nearly pure enzyme have been successfully purified on a column of 1.5 x 30 cm. Hence the capacity of the resin for enzyme is considerable. One column has been used 8 times without a noticeable loss of capacity. I am grateful to Stanley C. Froehner who prepared the resin used in these experiments.

12. Storage

Enzyme is stored at a concentration of 5 mg/ml in SMB at -20°C and is apparently completely stable. Some activity may be lost for templates which require σ such as T7 or T4 DNA but this is minimal e.g. 10-20% loss over one year.

Figure 6. DNA cellulose chromatography of a sample of E. coli B RNA polymerase which had a specific activity of 350 before this step. 40 mg of protein were applied in buffer DC .15 M KCl to a column 1.5 x 30 cm and eluted with a linear gradient (300 ml) from .15 → 1.0 M KCl in DC at 15 ml/hr flow rate. Fractions were assayed for RNA polymerase activity (10 λ aliquots) in the presence of 35 μg of ascites tumour DNA in the standard assay + .15 M KCl, + .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 sulphate precipitation.

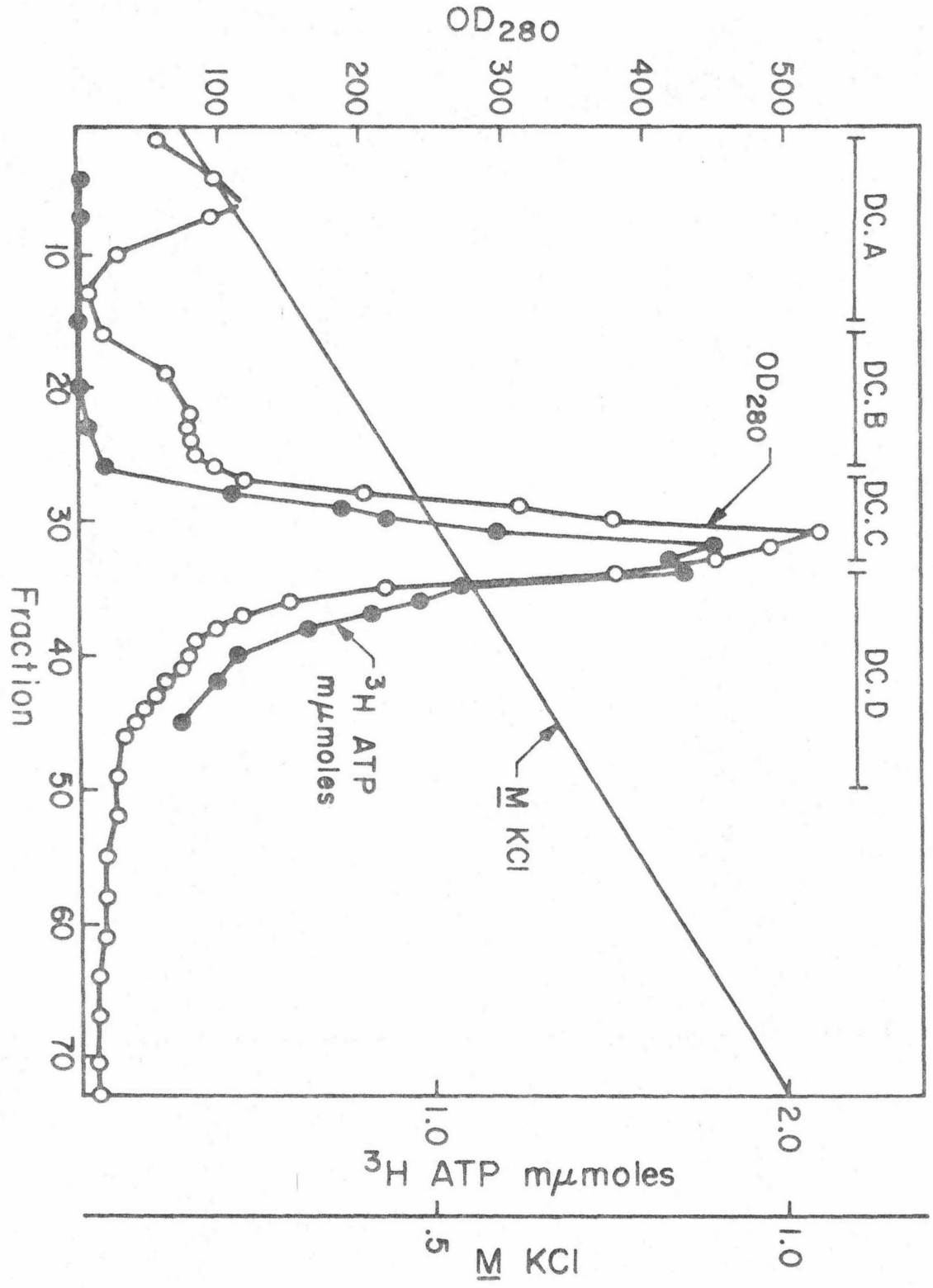
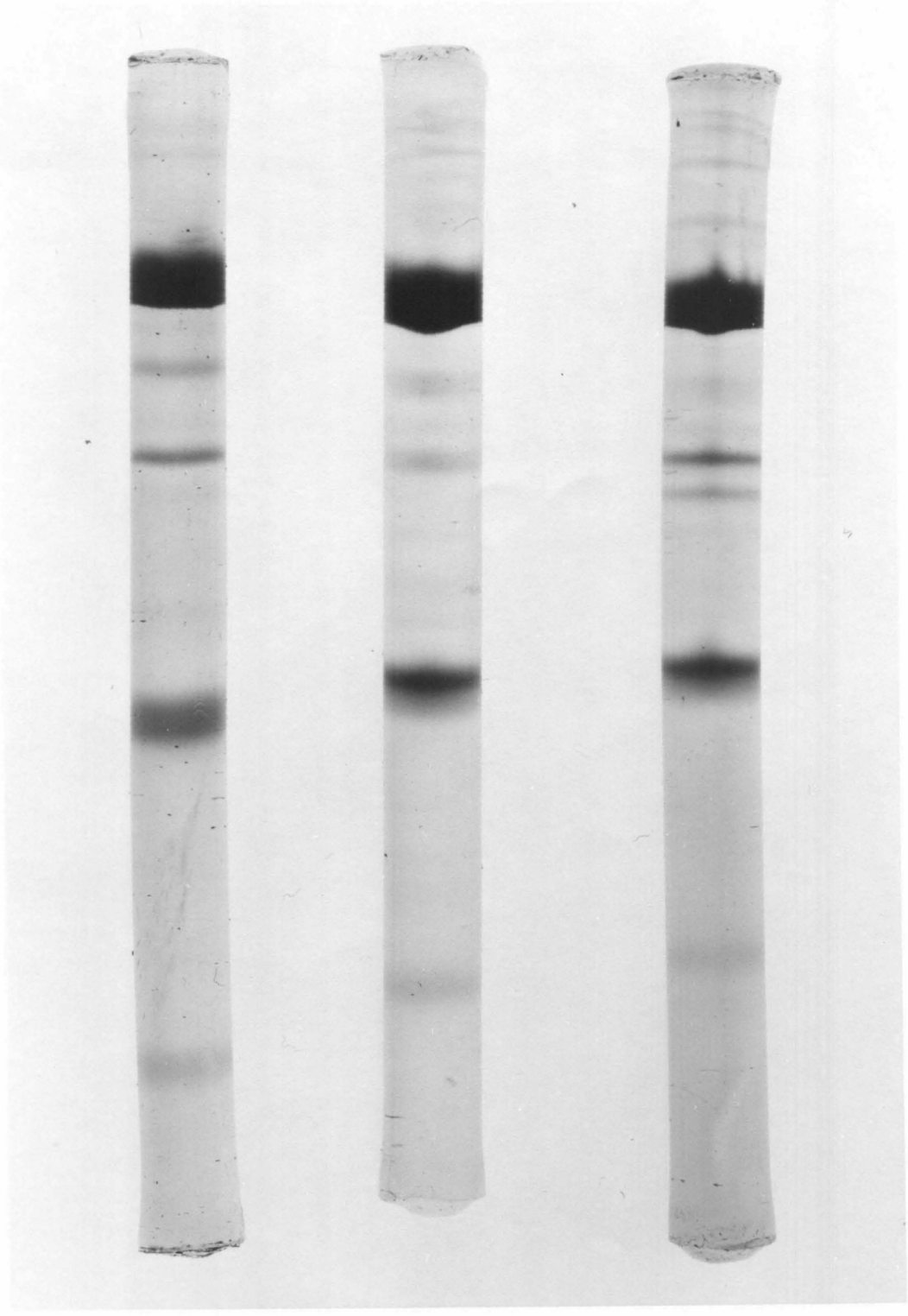

OD280
100
200
300
400
500
DC. A
DC. B
DC. C
DC. D
OD280
10
20
30
40
50
60
70
Fraction
3H ATP mμmoles
M KCl
1.0
2.0
3H ATP mμmoles
.5
M KCl
1.0

Figure 7. SDS-polyacrylamide gels of RNA polymerase preparations (left to right) GGA, DC.D., DC.E. The final steps in purification of these enzymes were high salt glycerol gradient sedimentation (GGA) shown in Figure 3, and DNA-cellulose chromatography (DC.D, DC.E) shown in Figure 7. The left hand gel was run at a different time from the other two, GGA was prepared from E. coli D-10 and the others from E. coli B.



13. Unit of enzyme activity

One unit is that which will incorporate 1 mμmole of ATP per 10 minutes at 37°C into acid insoluble form in the low salt reaction mixture described above in the presence of excess T7 DNA.

14. Notation of enzyme preparations.

Glycerol gradient purified enzyme - GGA, GGB, GGC etc.

Phosphocellulose purified enzyme - PC 2.1, PC 2.2 etc.

DNA cellulose purified enzyme - DC 3.1, DC 3.2, DC 4.1 etc, DC.A, DC.B. etc.

(xi) Analysis of the enzyme preparation

Contamination with DNA can be estimated by reading the ratio of OD 280 to OD 260. This should be greater than 1.5. Purified enzyme has been reported to have an OD 280/OD 260 of 1.8. It should be completely dependent for activity on added DNA. Dependency and OD 280/OD 260 correlate quite well. A yellowish colour is an indication of an unidentified contaminant, possibly a flavoprotein. This may contribute to a high OD 260. It can be removed efficiently by DNA cellulose but not by phosphocellulose. The highest specific activity reported is in the region of 1000 units per mg. However, core enzyme which lacks the σ factor may be very pure but only have a specific activity of 100 or less on T7 DNA. SDS - polyacrylamide gel electrophoresis is an

excellent method for estimating purity when used in conjunction with enzyme assays, dependency assays etc., and quickly reveals the presence or absence of σ factor (Figure 6). Figure 7 shows gels of several enzyme preparations. By appropriate assays ribonuclease, deoxyribonuclease, polyphosphate kinase, polynucleotide phosphorylase and phosphatase can be routinely detected.(See below.)

(xii) Assay for the synthesis of polyadenylic acid (Poly A)

The synthesis of homopolymer by RNA polymerase has been well described. (Chamberlin and Berg, 1962; Stevens, 1964). It occurs in the absence of a complete complement of nucleotide triphosphates, generating Poly A, Poly U, Poly C but not Poly G. The reaction mixture is the same as that used for RNA synthesis, except that one or more of the nucleotide triphosphates is absent. Acid-insoluble ^{14}C, or ^{3}H ATP is collected as described in the previous assay.

(xiii) Assay for ribonuclease (RNAase) activity

Activity is measured as the amount of radioactive RNA made soluble in 10% TCA. A simple procedure to test the presence of RNAase in DNA or RNA polymerase preparations is to generate labelled RNA, using the DNA or the enzyme to be assayed. After 15 minutes at 37°C, the reaction is made .01 M EDTA, and incubated for a further two hours. At intervals 50 λ aliquots are removed, diluted in 1 ml .01 M tris and precipitated with 10 mls 10% TCA as usual. The

EDTA prevents further synthesis of RNA, but not the activity of RNAase. If RNAase is present the amount of acid precipitable counts falls with time (Table 1.)

(xiv) Assay for deoxyribonuclease (DNAase) activity

A most sensitive assay measures the conversion of unknicked, closed circular super-coiled SV-40 DNA (component I) to the knicked form (Compenent II), which has no super-coiling and sediments more slowly than component I (Bauer and Vinograd, 1968). The relative amounts of the two forms can be calculated conveniently after synthetic boundary sedimentation in the Beckman Model E Analytical Ultracentrifuge fitted with automatic scanner. 20 µg of SV-40 DNA is incubated at 37°C for 15 minutes with 50 µg RNA polymerase preparation to be assayed. A control contains no added RNA polymerase. After 15 minutes reactions are cooled, EDTA may be added to prevent further activity (of DNAase I for example) and the samples quickly loaded in the synthetic boundary cells and sedimented at 25°C through 2.8 M CsCl pH 7.0. The traces from the scanner usually show two peaks even in the control. If DNAase is present the amount of the slow moving component II in the sample is higher than the control. The assay is most sensitive since the effect of a single knick can be observed. The precise number of bonds broken can be calculated from the Poisson Distribution.

(xv) Assay for polynucleotide phosphorylase.

Table 1. Assay for ribonuclease activity in RNA polymerase

Time after addition of .01 M EDTA (minutes)	TCA precipitable counts
1	3273
2	3057
5	3157
10	2956
20	2987
30	3076
60	3293
90	3237
120	3150
240	3244
Time after addition of ribonuclease (minutes)	
5	372
10	329
20	265
60	222

100 µg of E. coli D-10 enzyme (GGA) were incubated at 37°C in .5 ml of a standard high salt RNA polymerase assay with 100 µg of E. coli DNA for 15 minutes. The reaction was made .009 M EDTA and incubated for a further 240 minutes during which time aliquots of 20 λ were removed at intervals and TCA precipitated. At 240 minutes 80 µg pancreatic ribonuclease were added to .35 ml of the reaction, and aliquots (20 λ) were taken and precipitated at the times shown.

This enzyme generates polynucleotides from ribonucleoside diphosphates (Grunberg-Manago and Ochoa, 1955). Since all samples of nucleoside triphosphates which I have tested contain about 10% diphosphate these can be used as substrate for the reaction. The reaction only requires a single nucleotide, does not require a template, shows autocatalytic kinetics in the absence of a template and is completely inhibited by low concentrations of phosphate. These facilitate the assay. 50 µg of RNA polymerase preparation are added to .25 ml of .01 M tris pH 7.9, .004 M $MgCl_2$, .01 M β mercaptoethanol, .08 M KCl, .2 mM ATP-^{3}H (10^5 cpm per mumole). This is incubated at 37°C and 50 γ aliquots are removed at 15 minute intervals and precipitated with 10% TCA. The results of such an assay are shown in Figure 8.

(xvi) Assay for the synthesis of polyphosphate

Polyphosphate is synthesised from the γ phosphate of ATP. The standard assay is for 50 µg of enzyme in .25 mls of .01 M tris pH 7.9, .01 M $MgCl_2$, .01 M β mercaptoethanol, .2 mM γ ^{32}P ATP. The reaction is carried out at 37°C and 50 λ aliquots are taken and precipitated with 10% TCA at 15 minute intervals. Results of such an assay are shown in Figure 9.

(xvii) Assay for phosphatase, ATPase, and GTPase.

This assay follows the procedure of Conway and Lipmann (1964) and Martin and Doty (1949). The reaction is carried

Figure 8. Assay of RNA polymerase for the presence of polynucleotide phosphorylase, and the effect of the concentration of KCl on the reaction. 50 µg of glycerol gradient purified E. coli D-10 enzyme were assayed in .25 ml of .004 M $MgCl_2$, .01 M tris pH 7.9, .01 M β mercapto-ethanol, and .2mM ^3H ATP (100,000 cpm/mumole), at 37°C. At the times shown aliquots of 50 λ were removed and precipitated with 10% TCA. Samples contained from zero to .08 M KCl. A reagent blank contained no enzyme.

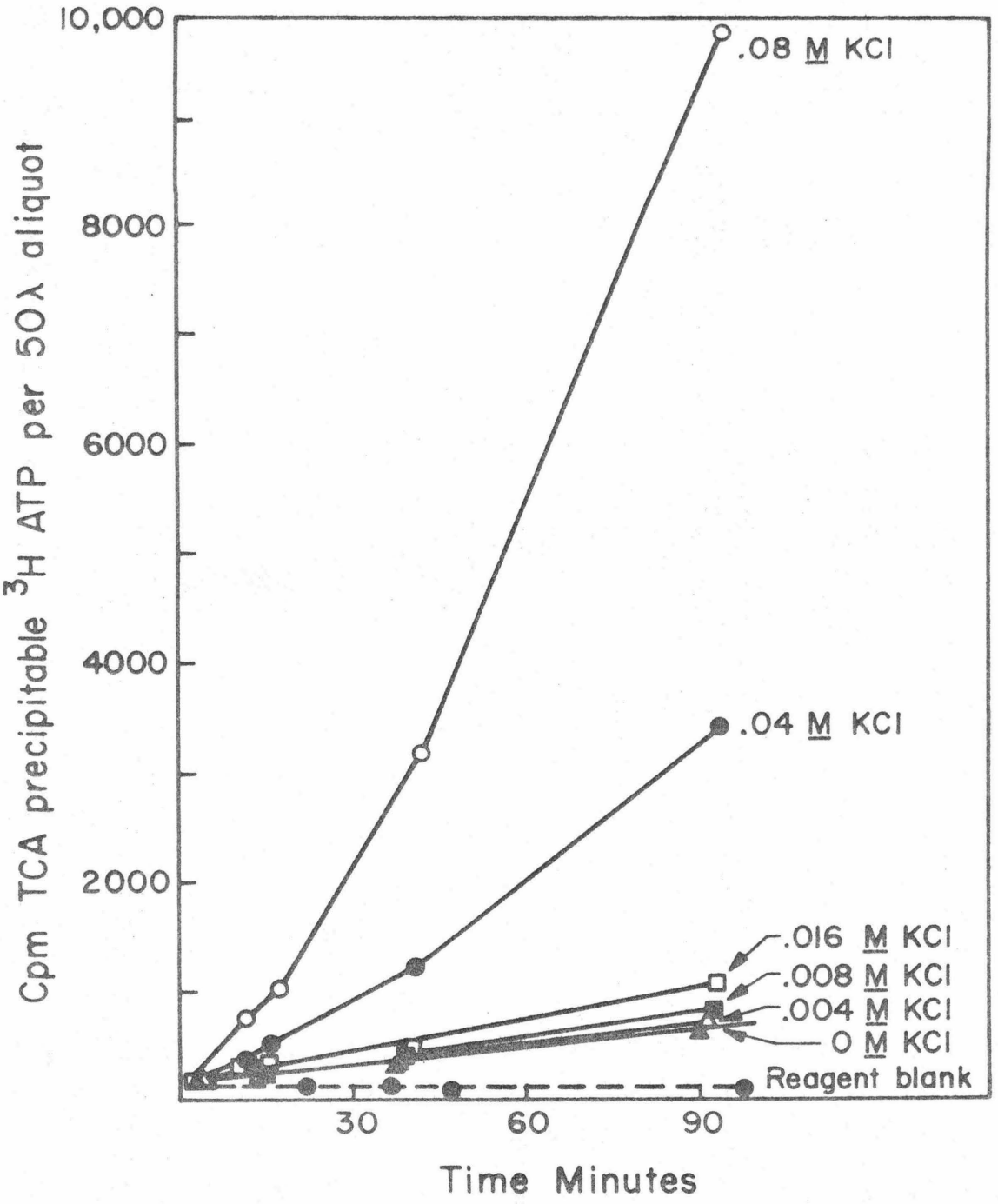
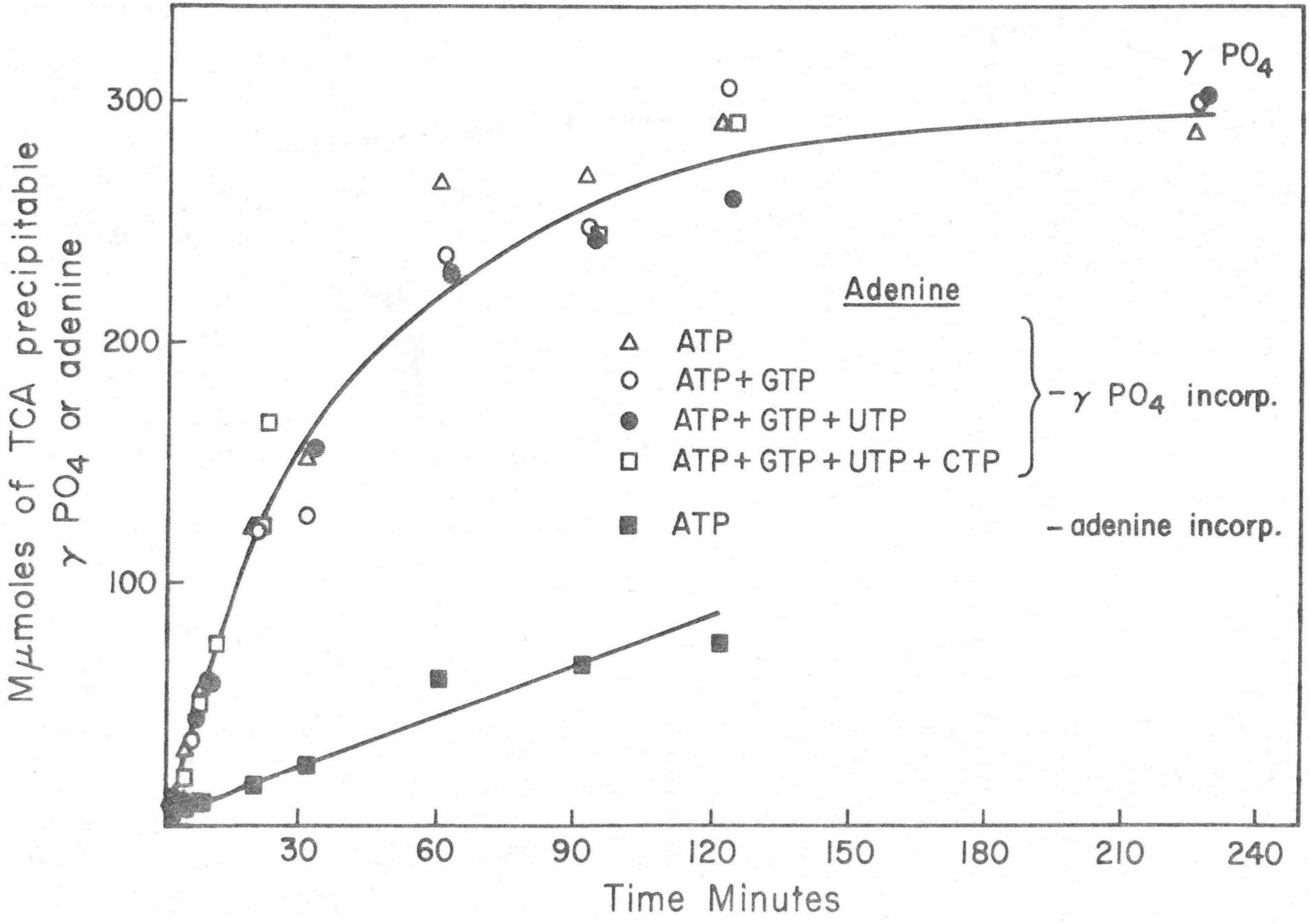

10,000
8000
6000
4000
2000
Cpm TCA precipitable ^{3}H ATP per 50λ aliquot
.08 M KCl
.04 M KCl
.016 M KCl
.008 M KCl
.004 M KCl
0 M KCl
Reagent blank
30
60
90
Time Minutes

Figure 9. Assay of polyphosphate kinase in an RNA polymerase preparation. 200 µg of glycerol gradient purified D-10 enzyme were assayed in .5 ml of .1 M tris pH 7.9, .004 M $MgCl_2$, .01 M β mercaptoethanol, and .2 mM γ ^{32}P and ^{3}H ATP, at 37°C, in the presence of various nucleoside triphosphates. Samples were: 1. ATP alone 2. ATP + GTP 3. ATP + GTP + UTP 4. ATP + GTP + UTP + CTP. Additional nucleotides were each .2mM. At times shown aliquots of 50 λ were removed and precipitated with 10% TCA. Time courses are plotted for γ PO4 incorporation for each reaction, for adenine for reaction 1. (rates were very similar for 1,2,3 and 4) Specific activities were 8000 and 12,000 cpm/mµmole for γ ^{32}P and ^{3}H adenine respectively.


γ PO4
300
200
100
Mμmoles of TCA precipitable
γ PO4 or adenine
Adenine
ATP
ATP+GTP
ATP+GTP+UTP
ATP+GTP+UTP+CTP
- γ PO4 incorp.
ATP
- adenine incorp.
30
60
90
120
150
180
210
240
Time Minutes

out in .25 mls of .05 M tris, .008 M $MgCl_2$, .0008 M K_2HPO_4, .15 M KCl, .0001 M dithiothreitol, .0001 M EDTA, .2mM γ ^{32}P GTP, (or ,2 mM ATP) containing 40-50 µg of enzyme. It is incubated at 37°C, .4 mls of isobutanol: benzene (1:1),.08 mls of silicotungstate reagent, and .08 mls of molybdate are added and shaken successively. The suspension is centrifuged and .2 ml aliquots of the supernatant are counted in 10 mls of Dioxane Scintillation fluid. The free phosphate is quantitatively extracted into the organic phase.

(xviii) Assay for the presence of σ

The presence of σ as determined from SDS-polyacrylamide gels can be confirmed by the relative activity of enzyme on DNA from phage and higher organisms. It has been shown that σ stimulates the transcription of T4 DNA (Burgess 1969) and ø E DNA (Losick and Sonenshein, 1969). Similar results were obtained for T7 DNA (Chapter II). 2 µg of enzyme, are assayed in the standard high salt reaction mixture in the presence of .05 M ADP and .008 M KH_2PO_4, for 10 minutes at 37°C. For assay (1), 5 µg of phage DNA (either T4 or T7) acts as template, for assay (2) 5 µg of higher organism DNA (ascites tumour, pea bud, rat liver etc.). If the enzyme contains σ total incorporation in the presence of phage DNA will be greater than that in the presence of higher organism DNA. When a saturating amount of σ is present there is 8

times more incorporation for T7 DNA compared to ascites tumour DNA. In the absence of Mg^{++}, ascites tumour DNA is a better template.

RESULTS.

(i) The assay for RNA polymerase

The standard assay as described was carried out in low salt. Many assays were carried out at high ionic strengths. The effect of raising the ionic strength by the addition of higher concentrations of magnesium chloride is shown in Figure 10. The essential observation is that RNA synthesis continues at a greater rate for a longer period of time as the ionic strength is raised. This is in accord with the results of Fuchs et al., 1967; So et al., 1967; Millette and Trotter, 1970, and Richardson, 1970. A second effect also reported by these authors was the presence of a lag phase in the initiation of RNA synthesis, which increases in length with ionic strength. This has also been observed.

A most important variable in the assay is the nature of the DNA used as template. Phage DNA has a higher efficiency than either E. coli DNA or higher organism DNA. This is shown in Table 2.

(ii) Purification of RNA polymerase

RNA polymerase has been purified 4 times from E. coli D-10 and 6 times from E. coli B. There was great variation between lots in the yield of enzyme, the single lot of E. coli D-10 being far superior to all lots of E. coli B. Recent lots of E. coli B have however been consistently good.

Figure 10. The effect of ionic strength on the RNA polymerase assay. 5 μg of glycerol gradient purified enzyme (GGC) were assayed in .25 ml .06 M tris pH 7.9, .0001 M EDTA, .0001 M dithiothreitol, .18 M KCl, .48 mM of ^{3}H ATP (10,000 cpm/mumole), GTP, CTP and UTP and 10 μg T7 DNA, at increasing concentrations of $MgCl_2$ from .004 M to .02 M. Aliquots of 50 λ were removed at the times indicated and precipitated with 10% TCA.

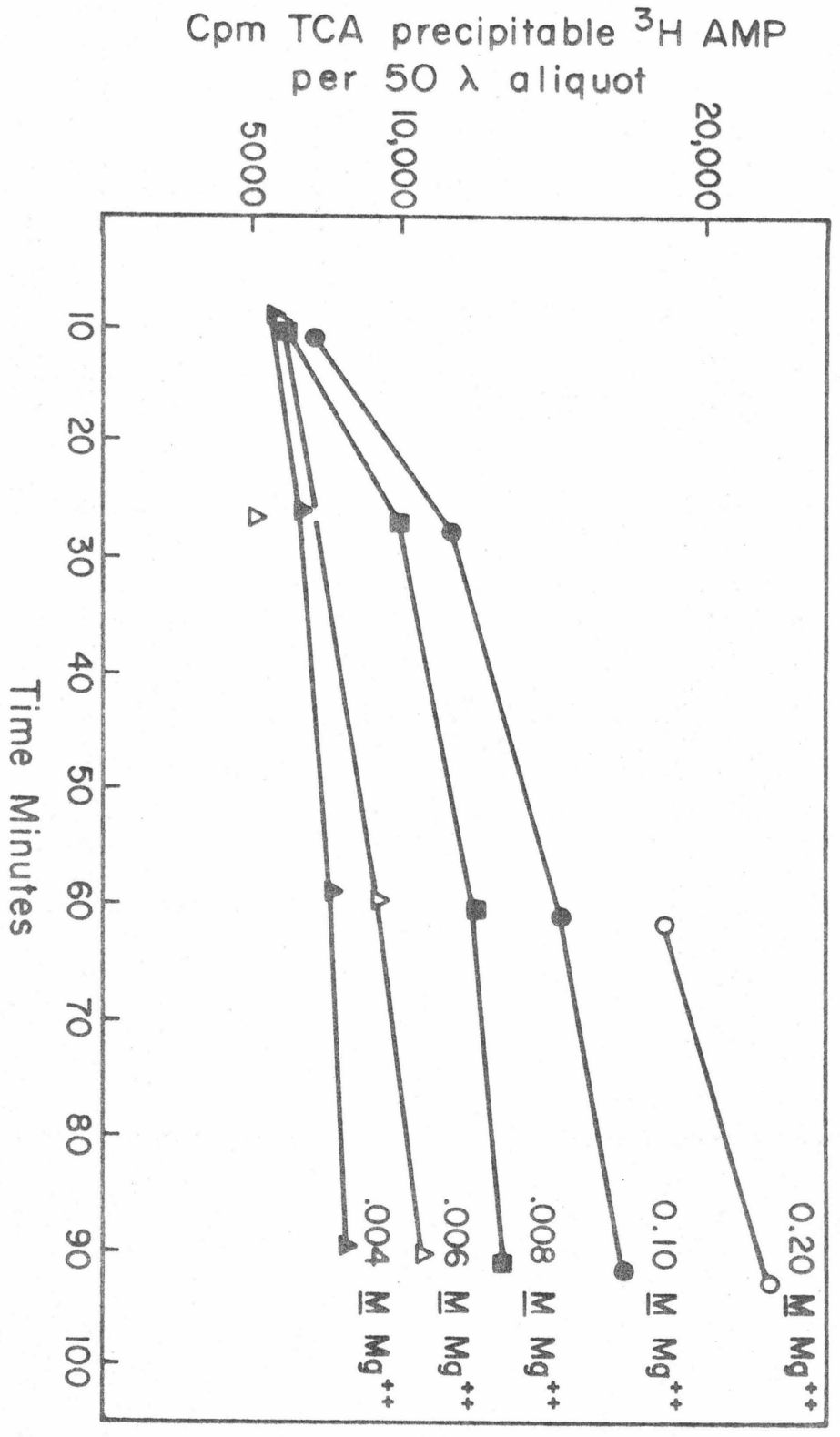

Cpm TCA precipitable ^{3}H AMP
per 50 λ aliquot
5000
10,000
20,000
Time Minutes
10
20
30
40
50
60
70
80
90
100
.004 M Mg++
.006 M Mg++
.008 M Mg++
0.10 M Mg++
0.20 M Mg++

Table 2. Effect of source of DNA on RNA polymerase activity

DNA	Exp.1.	Exp.2.	Exp.3.	Average
T7	100	100	100	100
Ascites Tumour	40	21	9	23
Pea bud		30	9	20
E. coli	14	37	-	25

In each case activity was assayed in .25 ml .04 M tris pH 7.9, .01 M $MgCl_2$, .01 M β mercaptoethanol, .8 mM K_2HPO_4, .02 mM ADP, .15 M KCl, .2 mM each of ^{14}C ATP, CTP, UTP and GTP., and 5 μg of the DNA indicated. The enzyme preparation was 2 μg of PC 2.6 core enzyme to which 2 μg of PC 2.2 σ-rich fraction were added.

The reactions were carried out at 37°C for 30 minutes where TCA was added, and the precipitate collected as described in Materials and Methods, and counted. Values are normalized to those of T7 DNA.

With the introduction of DNA cellulose chromatography it is now convenient to prepare good enzyme from strain B in large amounts.

600 g of cells are handled routinely at one time. In the case of D-10 these yield 200 mg of protein in F3, and this can be conveniently applied to one SW 25.2 rotor for a low salt glycerol gradient (Figure 2.). The enzyme sediments at 20-23 S under these conditions and appears as a distinct peak of OD 280 and activity. Recovery of activity is estimated as 90%. On a high salt glycerol gradient (Figure 3.) the enzyme sediments at 13S and moves away from some smaller material with high OD 260 which had probably aggregated during the low salt centrifugation. This enzyme has a specific activity of 1000 on T7 DNA, and contains the sub-units β1, β2 σ α ω as well as τ. τ is not required for activity and may be a contaminant but often runs with the enzyme. The yield of enzyme is about 10 mg per 100 g of cells, representing an estimated 60% recovery of activity. The results of a preparation are summarised in Table 3 .

The major difference for cells of E. coli B is the high yield of protein of low specific activity obtained at the F3 stage (Table 3). The yield is 1-2.5 g of a yellow protein solution per 600 g of cells which is far in excess of the amount which can be handled by glycerol gradients in a single rotor. In early preparations, chromatography on DEAE cellulose followed by phosphocellulose proved an effective way of

Table 3. Comparison of the yield of enzyme from E. coli D-10 and E. coli B. Data for 100 g wet weight of cells.

STAGE	mg protein	Units of total activity	Units per mg Specific activity	Estimated purity
		E. coli D-10		
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%
		E. coli B		
F1		7,000		
F3	400	5,000	10	
DEAE	80			
Phospho-cellulose	16	3,500	200	
High salt glycerol gradient	8	2,600	300	75%
DNA cellulose	3	2,600	850	85%

I unit is the number of mumoles of ATP incorporated in 10 minutes at 37° C in the presence of excess T7 DNA. Purity was estimated from absorption scans of SDS-polyacrylamide gels after staining with Coomassie Blue.

reducing the protein to manageable proportions. However, this resulted in a loss of the σ sub-unit some of which elutes in the run-off of the phosphocellulose column. Consequently even after a further step of a high salt glycerol gradient, the enzyme had a specific activity of only 300 units per mg on T7 DNA despite a purity of 75% as estimated from polyacrylamide gels. The yellow colour was still present at this stage. The specific activity was raised to 850 by chromatography on DNA cellulose (Figure 6) which has the ability to separate partially, active and inactive enzyme. The final preparation was colourless.

Subsequent preparations from E. coli B have been applied to DNA cellulose at the F3 stage. No loss of σ results and the enzyme has a specific activity of up to 300 on ascites tumor DNA. This method is now used routinely in our laboratory. If further purification is required a high salt glycerol gradient is appropriate, since much of the protein which co-chromatographs with the enzyme on DNA cellulose is $<$ 13S. Alternatively, a DEAE cellulose column will retain the enzyme while most of the other proteins should be positively charged and therefore eluted in the run-off. This last procedure has not been tried, however.

Final preparations were always colourless solutions of OD 280/OD 260 greater than 1.5 and had an absolute dependency on added DNA for activity.

The differences between E. coli D-10 and E. coli B are

impossible to evaluate since only one lot of D-10 cells were analysed. However, none of 5 lots of E. coli B was as good as D-10, all giving more than 5 times as much protein at the F3 stage without an equivalent increase in activity. The yield from E. coli B was always smaller.

The preparations were routinely analysed by SDS-polyacrylamide gel electrophoresis. Examples of such gels are shown in Figures 5 and 7.

The yield of activity from D-10 was 65% of that present in the F1. For B the recovery was only 37%, which reflects the number of steps required to obtain good enzyme.

(iii) Analysis for ribonuclease and phosphatase.

The results of an assay for ribonuclease are shown in Table 1. 100 µg of D-10 enzyme (specific activity 1000 units/mg) were incubated with 100 µg of E. coli DNA under conditions described in the legend. After 15 minutes of synthesis at 37°C, the reaction was made .009 M EDTA stopping RNA synthesis. Aliquots were precipitated at 0,10,20,30,120, and 240 minutes. No fall in the amount of RNA was observed, showing that neither the E. coli DNA nor the RNA polymerase contained significant amounts of ribonuclease.

Phosphatase was assayed by the method of Martin and Doty (1949) as described in Materials and Methods. 42 µg of benzyme (850 units per mg) were assayed for the ability to hydrolyse high specific activity $\gamma\,^{32}P$ ATP or $\gamma\,^{32}P$ GTP. No

hydrolysis was observed after 20 minutes at 37°C.

(iv) Activity of DNAase in polymerase preparations.

Activity was assayedat very high concentrations of RNA polymerase (220 µg/ml) and SV-40 DNA component I (100 ug/ml). (Most experiments on the enzyme were conducted at or below these concentrations). The assay is based on the fact that closed circular SV-40 (component I) is super-coiled and sediments more rapidly than component II which contains one or more single-stranded knicks.(Bauer and Vinograd, 1968). Hence the effect of a single knick per molecule can be observed. In the assay described, 50% of the SV 40 component I were converted to component II in 20 minutes at 37°C. 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 = .69.

(v) The synthesis of poly A by RNA polymerase.

Chamberlin and Berg, (1962), Ibid, (1964), Stevens, (1964), and Niyogi and Stevens, (1965) have reported the synthesis of poly A and have demonstrated that this reaction is catalysed by the same enzyme responsible for synthesizing RNA. Their observations have been confirmed using a highly purified polymerase containing only β1 β2 τ σ α and ω

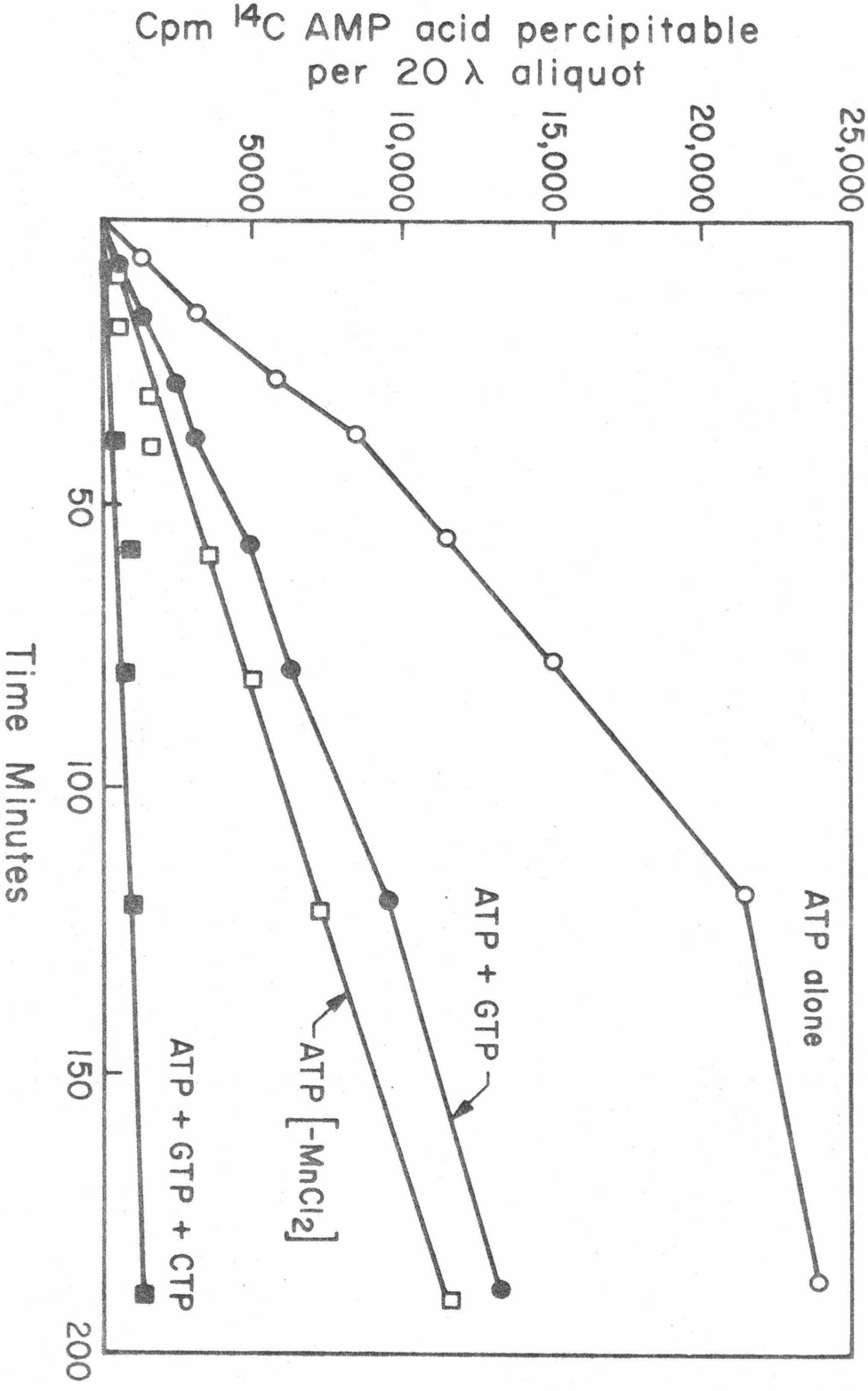
sub-units, with a specific activity of 1000. Several factors have been reported to affect the rate of synthesis of poly A: (1) other nucleotides (Chamberlin and Berg, 1962; Stevens, 1964), (2) Mn^{++} (Stevens, 1964), (3) high salt (So et al., 1967), and (4) double- or single-stranded DNA (Chamberlin and Berg, 1964; Stevens, 1964). These have been analysed. Furthermore the length of the chains of poly A has been calculated from the rate of incorporation of $\gamma\,^{32}P$ ATP and ^{14}C ATP.

1. The effect of additional nucleoside triphosphates on the synthesis of poly A.

50 µg of glycerol gradient purified RNA polymerase (GGA) were assayed for the ability to catalyse the synthesis of poly A in the presence of 25 µg of E. coli DNA at 37°C. Acid precipitable ^{14}C ATP was measured. The medium (C & B .16) contained .04 M tris pH 7.5, .004 M $MgCl_2$, .001 M Mn Cl_2, .012 M β mercaptoethanol .16 M KCl, and either (i) ATP alone .8 mM or (ii) ATP (.8 mM) and GTP (.8 mM) or (iii) ATP (.8 mM), GTP (.8 mM) and CTP (.8 mM). A time course for each reaction is presented in Figure 11(a). The total volume of each reaction was .25 ml and 50 microlitre aliquots were precipitated with 10% TCA at the times shown. The rate of synthesis is considerably reduced by the presence of GTP, or of GTP and CTP, and it is linear for at least 120 minutes. The rates are:

	Conditions	
(i)	ATP	.086 mµmoles acid precipitable ATP per

Figure 11(a). The synthesis of poly A by RNA polymerase and the effect of adding other nucleoside triphosphates. The 50 µg of glycerol gradient purified enzyme (GGA) were assayed at 37°C in .25 ml of .04 M tris pH 7.5, .004 M $MgCl_2$, .001 M $MnCl_2$, .012 M β mercaptoethanol, .16 M KCl, .8mM of the nucleotides as shown, and 25 µg of E. coli DNA. The specific activity of the ^{14}C ATP was 2220 cpm/mµmole. Aliquots of 20 λ were removed at the times shown and precipitated with 10% TCA. Three samples contained ATP alone, or ATP + GTP, or ATP + GTP + CTP. A blank sample without enzyme gave 50 cpm background. In sample 4., all of $MnCl_2$ was replaced by $MgCl_2$.


Cpm ^{14}C AMP acid percipitable per 20 λ aliquot
5000
10,000
15,000
20,000
25,000
50
100
150
200
Time Minutes
ATP alone
ATP + GTP
ATP [-MnCl2]
ATP + GTP + CTP

		minute per 50 λ (100)
(ii)	ATP + GTP	.036 mumoles acid precipitable ATP per minute per 50 λ (42)
(iii)	ATP + GTP + CTP	.0036 mumoles acid precipitable ATP per minute per 50 λ (4.2)

2. The effect of the concentration of KCl and the presence of Mn++ on the synthesis of poly A.

The effect of KCl was first reported by So et al., (1967). For the sake of comparison the reactions were carried out in a medium closely resembling that of So et al.: .1 M tris pH 7.5, .012 M $MgCl_2$, .0048 M β mercaptoethanol. Three tubes were set up each containing 50 µg RNA polymerase (glycerol gradient purified), 25 µg E.coli DNA, .8 mM ATP and either (i) O M KCl.

(ii) .16 M KCl

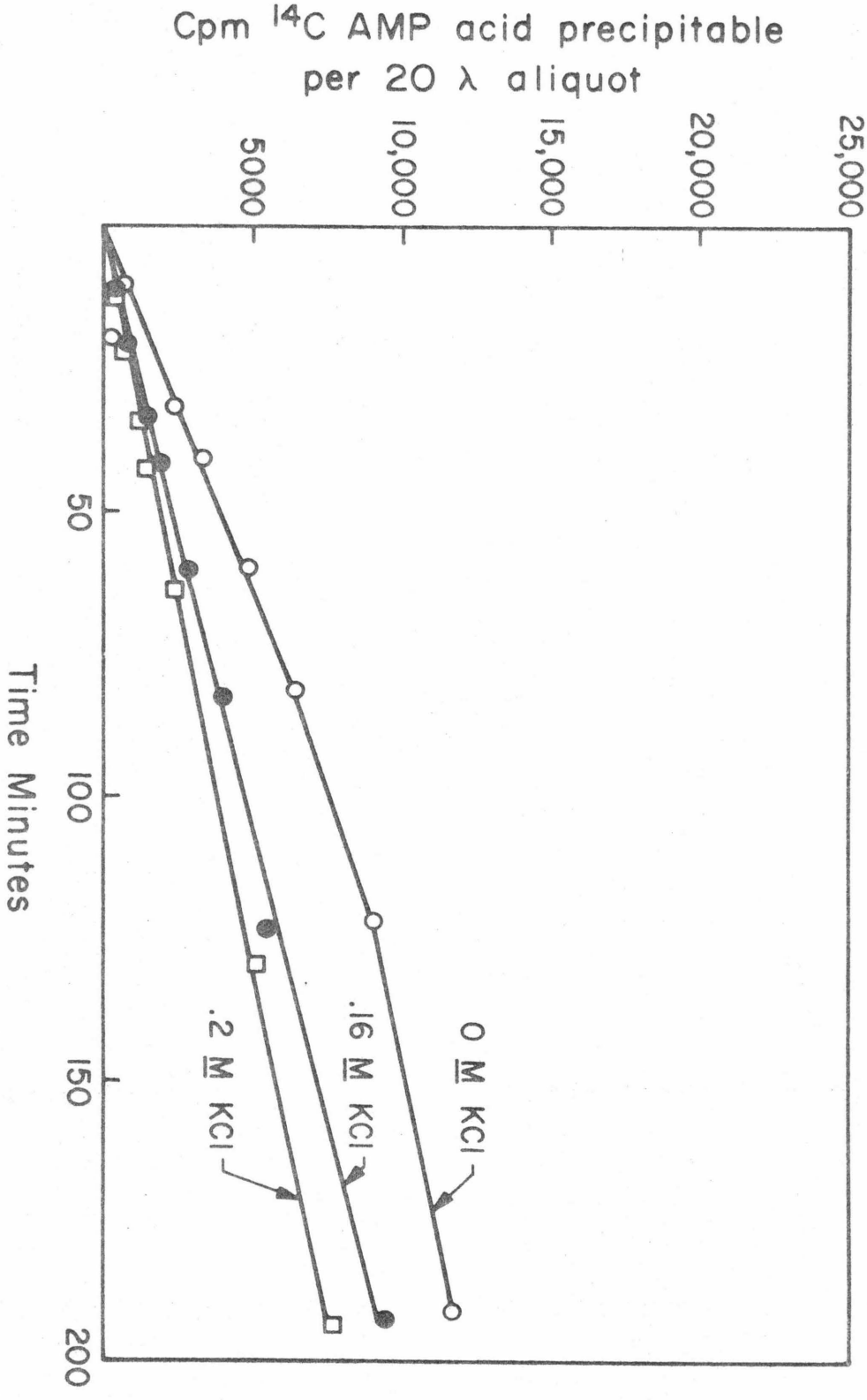
(iii) .2 M KCl

Figure 11 (b) shows a time course for each reaction. As in the previous experiment the rate of synthesis is linear for at least 120 minutes. The increased salt concentration does reduce the rate of reaction though the effect is not so marked as the effect of added nucleotides:

	KCl	Mumoles ATP incorporated per minute per 50 λ	
(i)	0	.036	(100)
(ii)	.16	.023	(63)
(iii)	.20	.018	(50)

There is a striking difference however between the rate

Figure 11 (b). The effect of KCl on the synthesis of poly A by RNA polymerase. 50 µg of glycerol gradient purified enzyme (GGA) were assayed in .25 ml .1 M tris pH 7.5, .012 M β mercaptoethanol, .0008 M ^{14}C ATP (2220 cpm/mµmole) and 25 µg E. coli DNA. 20 λ aliquots were precipitated with 10% TCA at the times shown. Three samples contained either zero M, .16 M, or .20 M KCl.


Cpm ^{14}C AMP acid precipitable per 20 λ aliquot
5000
10,000
15,000
20,000
25,000
50
100
150
200
Time Minutes
0 M KCl
.16 M KCl
.2 M KCl

of synthesis in this medium and in the medium (C & B .16) used for the previous experiment. The comparable tubes are tube (i) in the first experiment and tube (ii) in the second, in which the major differences are the level and type of divalent cation; the rate for the latter case is only 30% of the former. If all of the Mn++ in the first medium is replaced by Mg++ the rates become almost identical as shown in Figure II (a).

	RATE
(i) C & B .16	.086 mumoles ATP/minute/50 λ
(ii) C & B .16 No Mn++	.027 mumoles ATP/minute/50 λ
(iii) So medium .16 M KCl	.023 mumoles ATP/minute/50 λ

3. The effect of template on poly A synthesis.

The reaction was carried out in the presence of 25 µg of RNA polymerase and 10 µg of either T7, ascites tumour, or E. coli DNA, under the conditions described in Table 4. TCA precipitable counts were assayed after 10 minutes. The rates are normalised to the value for ascites tumour DNA which is about 2 times as efficient a template as E. coli DNA and 40 times as effective as T7 DNA. Mn++ was absent, the KCl concentration was .15 M and .008 M K_2HPO_4 was present to inhibit polynucleotide phosphorylase. The incorporation observed was .008 mµmoles per 10 minutes per .0004 mµmoles of T7 DNA. This is the five to six orders of magnitude less than the expected rate of RNA synthesis. It is

Table 4. Effect of source of DNA on synthesis of poly A

DNA	cpm ^{3}H ATP incorporated	Normalized %
Ascites tumour	14,626	100
E. coli	6,958	47.5
Ø T7	351	2.4
Ø T7 (+GTP+CTP)	212	1.5

25 µg of RNA polymerase (GGC) were incubated in .25 ml of .05 M tris pH 7.9, .005 M $MgCl_2$, .15 M KCl, .0008 M K_2HPO_4 .01 M βmercaptoethanol .8 mM ^{3}H ATP (22,500 cpm/mµmole) containing 10 µg of the DNA as shown, at 37°C for 20 minutes. The reaction was stopped by adding 10% TCA and the TCA precipitable material was collected on nitrocellulose membranes as described in Materials and Methods. The background was 85 cpm and has been subtracted. One reaction contained GTP (.8 mM) and CTP (.8 mM) as shown.

difficult to prepare DNA from E. coli or higher organisms without shear or knicking, while this preparation of T7 as analysed by alkaline sedimentation contained less than 1 knick per single strand. Stevens, (1964), and Chamberlin and Berg, (1964) have observed that the rate of poly A synthesis is greatly increased by denaturing the DNA, and that the rate catalysed by double stranded DNA can be reduced considerably by treatment with E. coli exonuclease I (Chamberlin and Berg, 1964), which digests single-stranded DNA selectively, so cleaning up the DNA preparation. Of all the methods tested to reduce poly A, the effect of the absence of single-stranded DNA is clearly the most significant, while the effect of ionic strength is almost insignificant. Mn++ has been shown to cause DNA polymerase to incorporate abnormal nucleotides, as well as stimulating the synthesis of poly A by RNA polymerase. Since these may be considered artifactual activities, Mn++, although it does lead to increased RNA synthesis, has been left out of the reaction mix.

4. The rate of initiation of synthesis of poly A and the rate of chain extension.

High specific activity $\gamma^{32}P$ ATP was used to follow the number of chains of poly A which were initiated, and ^{14}C ATP to follow the total amount of ATP incorporated. The results of such an experiment, for two cases, ATP alone, and ATP + GTP are shown in Figure 12 (a) (b). The graphs

Figure 12 (a). The rate of initiation and propagation of poly A synthesis by RNA polymerase. Glycerol gradient purified enzyme, GGA, (50 µg) was assayed in .01 M tris pH 7.5, .004 M $MgCl_2$, .01 M β mercaptoethanol, .2mM ATP, .25 mls total volume containing 25 µg of E. coli DNA. The ATP was labelled in the γ PO_4 (340,000 cpm ^{32}P per mµmole) or the adenine (2166 cpm ^{14}C/ mµmole). Aliquots of 5 λ were precipitated with 10% TCA at the times shown.

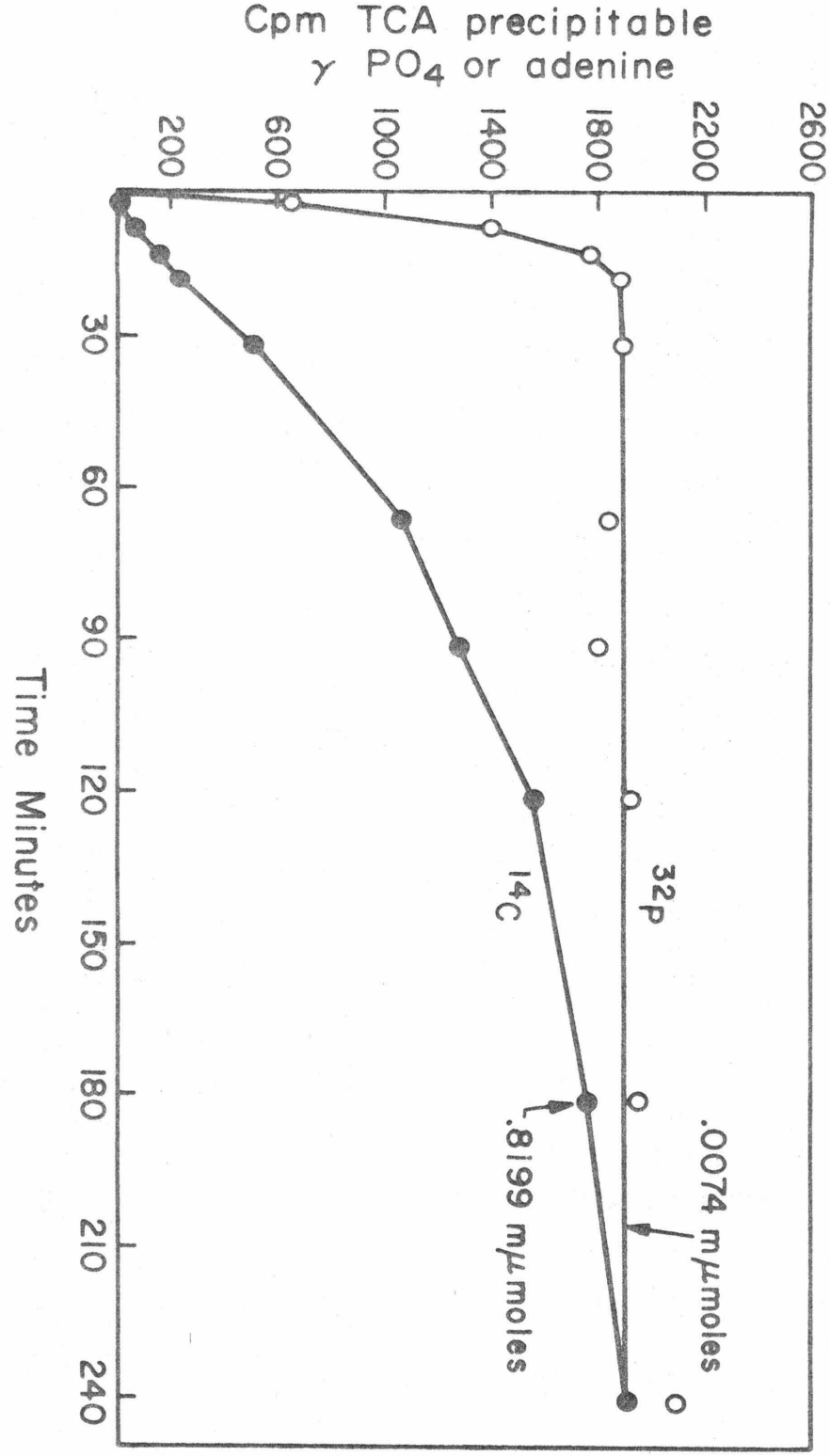

Cpm TCA precipitable
γ PO4 or adenine
200
600
1000
1400
1800
2200
2600
30
60
90
120
150
180
210
240
Time Minutes
14C
32P
.8199 mμmoles
.0074 mμmoles

Figure 12 (b). The effect of the presence of GTP on the rate of initiation and propagation of poly A synthesis by E. coli RNA polymerase. Conditions were exactly as Figure 12 (a) except that .2 m$\underline{M}$ GTP was also present.

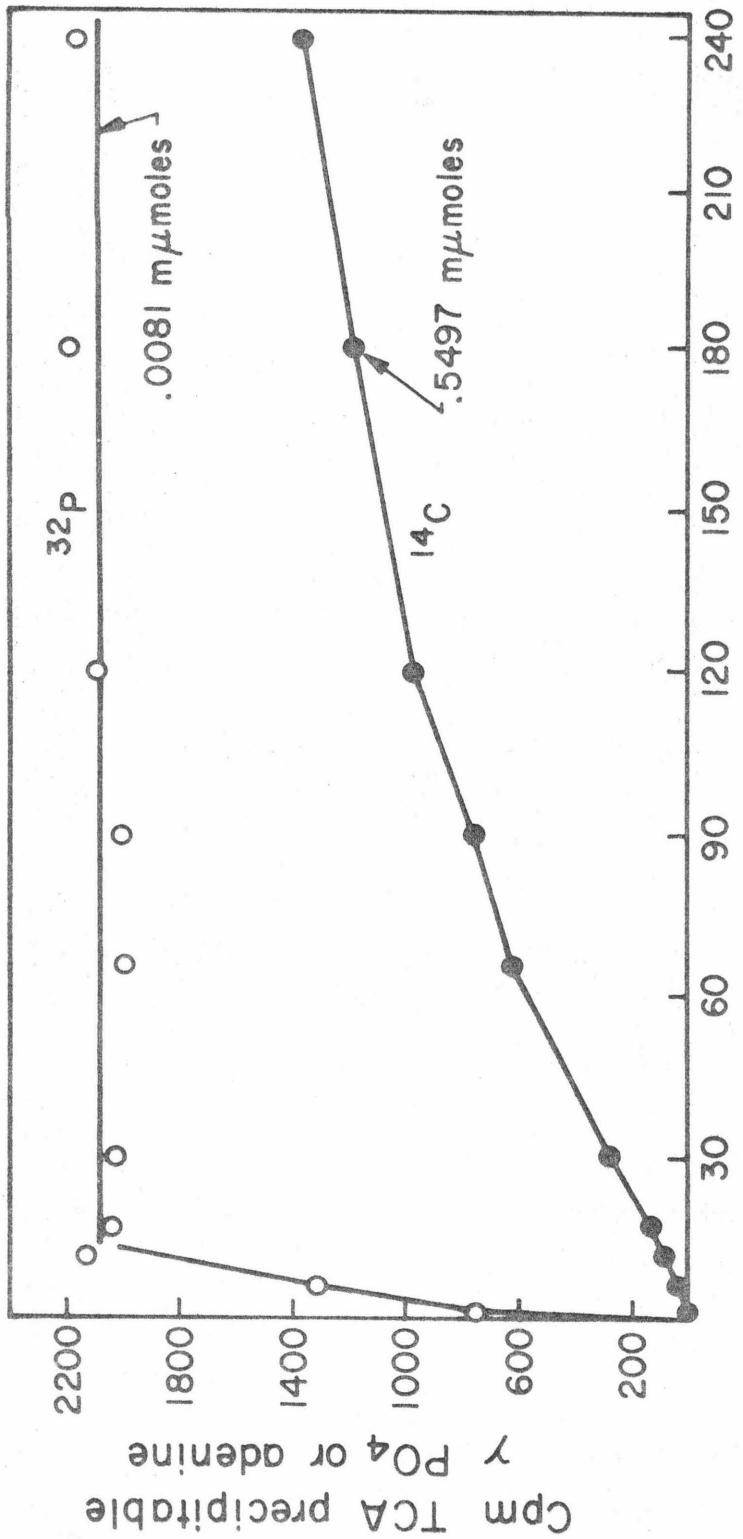
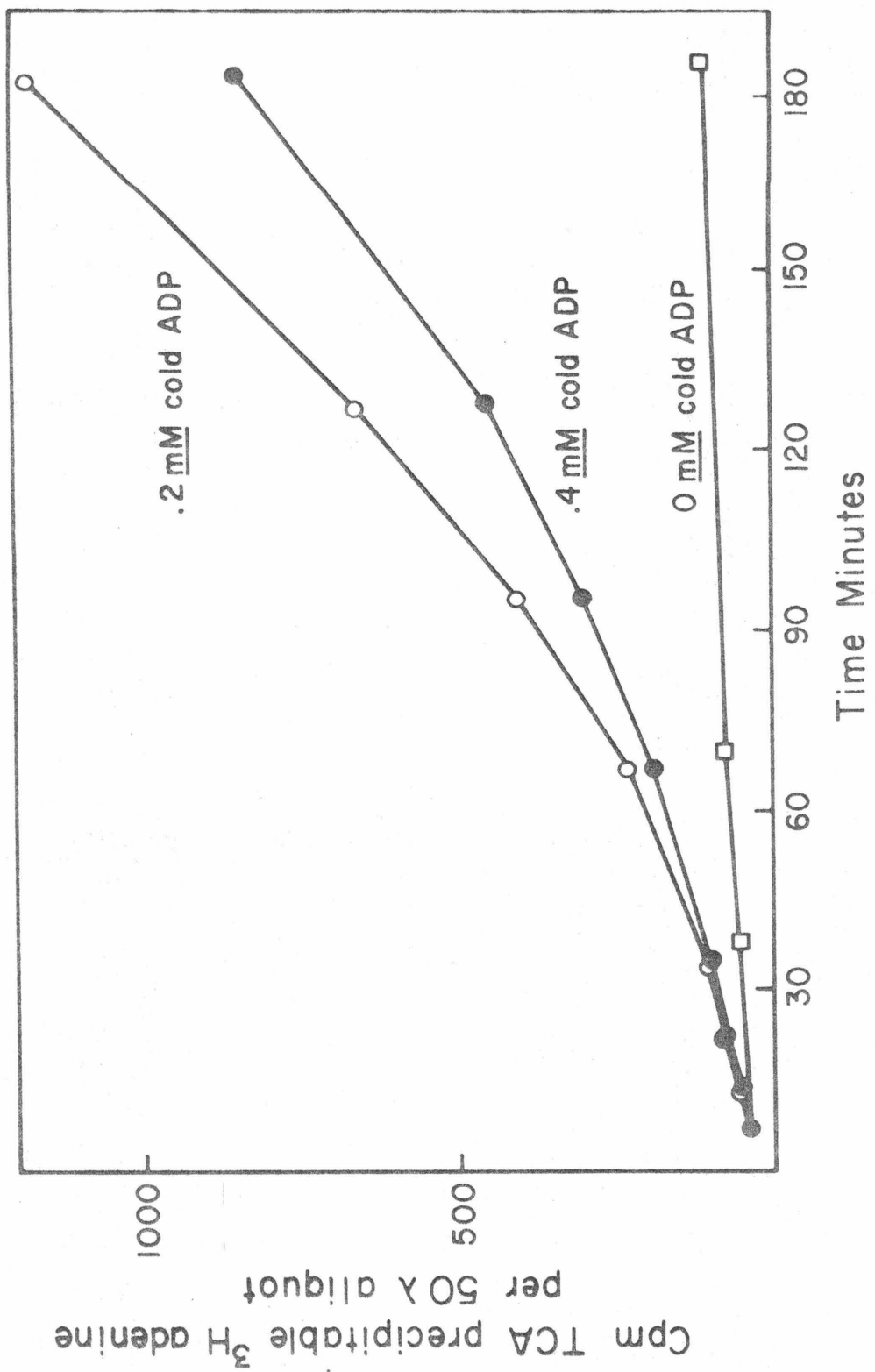

Cpm TCA precipitable
γ PO4 or adenine
2200
1800
1400
1000
600
200
30
60
90
120
150
180
210
240
Time Minutes
32P
.0081 mμmoles
14C
.5497 mμmoles

Figure 12. The effect of ADP on the activity of polynucleotide phosphorylase in preparations of RNA polymerase. 100 µg of glycerol gradient purified D-10 RNA polymerase were assayed at 37°C for polynucleotide phorphorylase in .5 ml .01 M tris pH 7.9, .004 M $MgCl_2$, .01 M β mercaptoethanol, .2 mM GTP, .2mM CTP and .2 mM ^3H ATP (7500 cpm/mumole). The ATP contained sufficient ^3H ADP to prime the polynucleotide phosphorylase reaction. Three samples were assayed (i) in the absence of added cold ADP (ii) with .2mM cold ADP and (iii) with .4 mM cold ADP. Aliquots of 50 λ were removed at the times shown and precipitated with 10% TCA. The precipitates were collected and washed on nitrocellulose membranes as described in Materials and Methods. Reagent background was 35 cpm.


Cpm TCA precipitable ^{3}H adenine
per 50 λ aliquot
1000
500
30
60
90
120
150
180
Time Minutes
.2 mM cold ADP
.4 mM cold ADP
0 mM cold ADP

plot incorporation versus time and the details are described in the legends. The medium has no Mn++ or KCl. It is important to note the following:

(i) The rate of initiation is rapid being 75% complete in 10 minutes, and 100% by 25-30 minutes.

(ii) There is no lag phase.

(iii) The presence of GTP does not affect the rate of initiation.

(iv) The rate of chain extension as measured by the incorporation of ^{14}C ATP is considerably reduced by the presence of GTP. This is also shown in Figure 11(a).

(v) The average chain length is small, being less than 20 nucleotides at 15" and less than 100 at 60". The average rate of chain extension is less than 1 nucleo- per minute per chain.

(vi) Assuming a molecular weight of 5×10^5 and a purity of 100%, 89% of the input enzyme molecules initiate. Under these low salt conditions re-initiation is unlikely.

This experiment does not prove that all of the acid precipitable $\gamma\ ^{32}P$ is incorporated in poly A, but in the absence of any alternative explanation, it is likely that the time allowed by most workers for the formation of a complex between RNA polymerase and DNA in the presence of ATP (15-20 minutes) is also sufficient to allow a very large number of enzyme molecules to start the synthesis of poly A.

Furthermore the attempts to control poly A synthesis by adding other nucleotides show that they do not inhibit the initiation of poly A synthesis. The apparent rate of chain extension is reduced but this is misleading since in a double label experiment using ^{3}H UTP and ^{14}C ATP in the presence of GTP, UTP was incorporated into acid insoluble material, to about 30% of the extent to which ATP was incorporated. Poly U can be synthesised by RNA polymerase, but since the amount of chain initiation by $\gamma\ ^{32}P$ ATP is not reduced by the presence of UTP, and since approximately 90% of the enzymes initiate, it is unlikely that this is occurring in this case. Nor is it likely that poly U is being synthesised on the poly A which is made, since there is no lag. The most reasonable interpretation is that a copolymer of A & U is being synthesized. It may or may not contain G.

5. Summary.

It is clear that the synthesis of poly A in an incomplete reaction mixture does occur to a significant extent, and that it cannot be entirely eliminated. The amount of synthesis in the most extreme case (C & B .16, ATP alone) was 344 mμmoles of poly A per 10 minutes per mg. of enzyme. Under optimal conditions, with T 7 DNA, this same enzyme would incorporate 1000 mμmoles of AMP as RNA. In the absence of Mn++ the rate of Poly A synthesis falls to approximately 100 mμmoles per 10 minutes per mg. The presence

of other nucleotides reduces it still further, while the most striking change is that due to differences in the DNA template.

Although some poly A synthesis occurs in the presence of RNA polymerase, T7 DNA and ATP alone, it is very little and it would be significantly reduced by the presence of other nucleotides. It is most probably catalysed by small pieces of nucleic acid in the DNA preparation which although not observed in sedimentation analysis were probably present in very small amounts.

(vi) Contamination by polynucleotide phosphorylase

The presence of this enzyme is assayed as described in Materials and Methods. Even highly purified preparations of RNA polymerase contain measurable polynucleotide phosphorylase activity, and it can be a more serious source of error than the synthesis of poly A, when T7 DNA is used as template. The substrates are nucleoside diphosphates which have been found as contaminants to a level of 5-10% in every batch of triphosphates tested. Hence the reaction mixture for RNA polymerase is adequate for polynucleotide phosphorylase unless the nucleotides have been purified (see Chapt. II).

Polynucleotide phosphorylase of Azotobacter agilis (Ochoa and Mii, 1961), E. coli (Kimhi and Littauer, 1968) and Micrococcus lysodeikticus (Singer and Guss, 1962) exhibit a lag phase in the absence of oligonucleotide primer, thereafter the reaction is autocatalytic. The

enzyme contaminating the RNA polymerase preparations displays exactly those kinetics in the absence of DNA (Figure 8). The activity is totally inhibited by .0008 M K_2HPO_4 as expected for polynucleotide phosphorylase. As shown in the same figure there is a striking effect on the reaction when the concentration of KCl is increased. The rate continues to increase to .2 M KCl and decreases slightly at .3 M KCl. This effect is serious since most reactions of RNA polymerase are thought to work most effectively at salt concentrations between .15 and .25 M (So et al., 1967; Pettijohn and Kamiya, 1967).

It has also been observed that the incorporation of label is increased when ADP is added (Figure 13). This is expected if the ADP contains oligonucleotides as reported by Beers (1961), and if the rate of synthesis is proportional to the square of the concentration of ADP. Hence dilution of labelled ADP (from the ATP solution) would be more than compensated by the increased rate. At higher ADP concentrations the apparent rate falls, presumably as the total concentration rises above the Km. This effect of ADP is again serious since some workers add it to the RNA polymerase reaction mix to inhibit polyphosphate kinase (Maitra and Hurwitz, 1967; Millette and Trotter, 1970).

Four methods can be used to limit the effect of the enzyme.

(1) The triphosphates may be purified (see part II Chapter II)

(2) The RNA polymerase preparation may be purified on phosphocellulose when all of the polynucleotide phosphorylase activity elutes in the run-off. Figure 4. shows the profile of a phosphocellulose column. The fractions were combined to give seven subfractions PC 2.1 to PC 2.7. These were precipitated, dissolved, assayed for polynucleotide phosphorylase, and samples run on SDS-polyacrylamide gels. The gels are shown in Figure 5. GGC represents the enzyme before purification of phosphocellulose. Table 5. shows the results of the assay for polynucleotide phosphorylase. 100% of the activity eluted in the run-off. There is a conspicuous band in the SDS gel PC 2.1 (which is the run-off) below σ, which is absent from all other fractions. It is the most likely candidate for polynucleotide phosphorylase, with an approximate molecular weight of 75,000 using the α, β and σ sub-units as markers. Klee (1969) reports a molecular weight of 65,000 for the sub-unit.

(3) Since some σ factor is eluted in the run-off, and since phosphocellulose columns are not perfectly reproducible, another method for removing polynucleotide phosphorylase is desirable. The DNA cellulose column which carries the same charge as phosphocellulose, may be equally effective, and DNA cellulose purified enzymes do not contain the 75,000 molecular weight band on SDS-polyacrylamide gels. (Fig.7) They have not been tested for phosphorylase activity.

(4) Inorganic phosphate is a very powerful inhibitor of

Table 5. RNA polymerase, polynucleotide phosphorylas and polyphosphate kinase activities of fractions from the phosphocellulose column (Figure 5.).

Fraction	RNA Synthesis		Polyphosphate synthesis		Polynucleotide synthesis by poly N phosphorylase	
	mμmoles of AMP per 10" per mg.		mμmoles of PO_4 per 10" per mg.		mμmoles of AMP per 60" per mg.	
PC 2.1	116	(100)	27.5	(100)	8.0	(100)
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)

The fractions from the phosphocellulose column (Figure 5) were assayed for RNA synthesis, polyphosphate synthesis and polynucleotide synthesis by polynucleotide phosphorylase. RNA synthesis was assayed in .25 ml .01 M tris pH 7.9, .01 M $MgCl_2$, .01 M β mercaptoethanol, .4mM CTP; UTP, GTP and ^{14}C ATP (5000 cpm/mμmole) with 25 μg E.coli DNA as template. The reaction was at 37°C for 10 minutes. Polyphosphate synthesis and the activity of polynucleotide phosphorylase were assayed in the same reaction (.25 ml) of .004 M $MgCl_2$ .01 M tris pH 7.9, .01 M β mercaptoethanol, .2 mM ATP (90,000 cpm ^{32}P per mμmole of γ Phosphate, and 52,000 cpm ^{3}H per mumole of adenine) incubated at 37°C. A time course from 0 to 120 minutes was plotted after taking four 50 λ aliquots and precipitating with TCA.

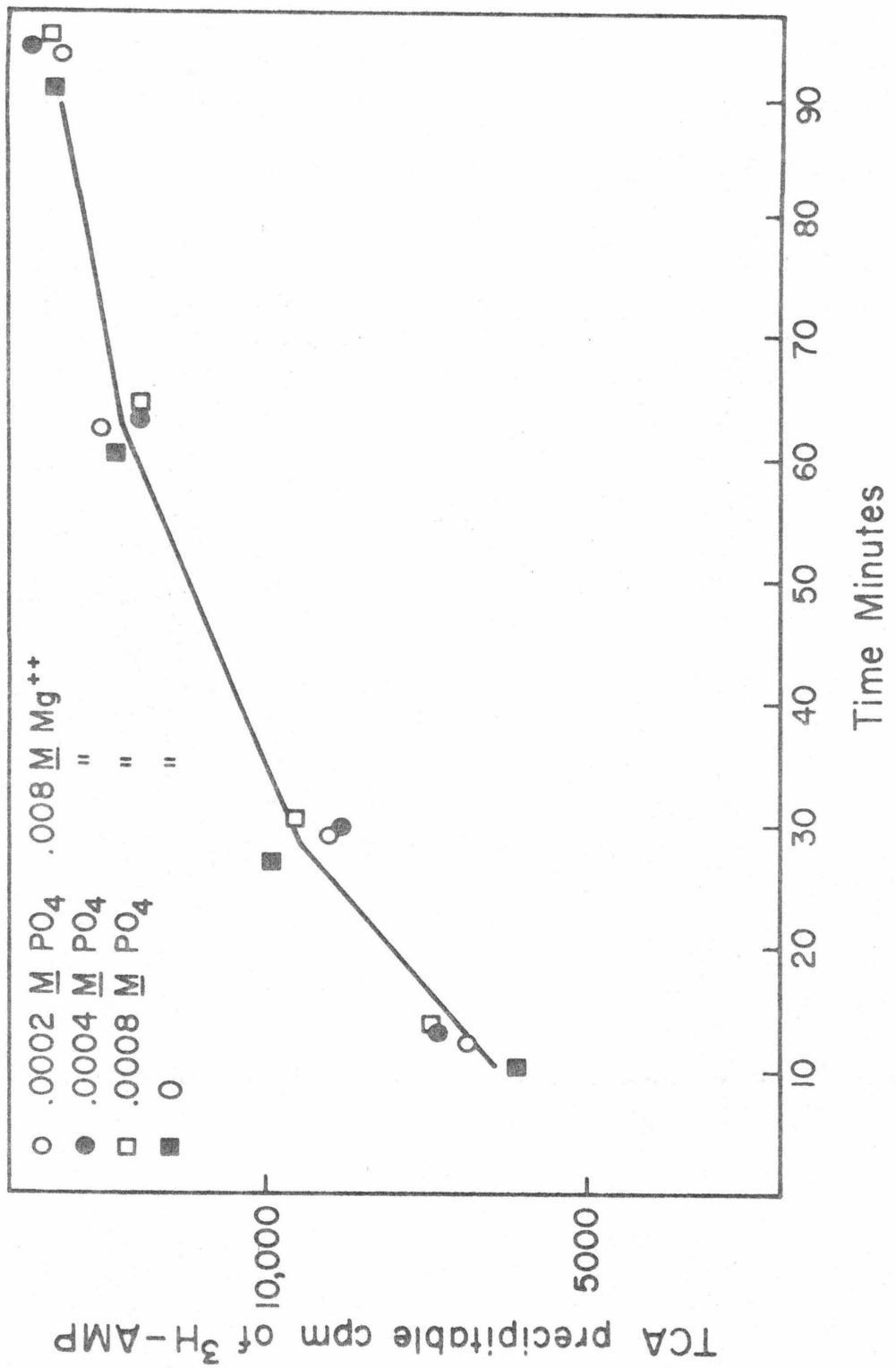
polynucelotide phosphorylase. As shown in Figure 14 it can be added to a concentration of .8 m<u>M</u> without affecting the RNA polymerase reaction. At this concentration polynucleotide phosphorylase is inhibited by 80%. In order to inhibit both polynucleotide phosphorylase and polyphosphate kinase .8 m<u>M</u> phosphate and .05 m<u>M</u> ADP are appropriate. As will be shown, however, at high salt concentrations the rate of synthesis of polyphosphate is negligible anyway, and the ADP can be left out. All RNA polymerase assays now routinely contain .8 m<u>M</u> phosphate. From Figure 14 it can be seen that this does not provide sufficient phosphate to reverse the polynucleotide phosphorylase reaction and so cause hydrolysis of RNA.

(vii) <u>The synthesis of polyphosphate</u>

1. The reaction

In the absence of DNA, highly purified preparations of RNA polymerase incorporate the γ phosphate from ATP into acid precipitable form (Figure 9). The molar incorporation of the γ phosphate is greatly in excess of the adenine in this experiment where both $\gamma\ ^{32}P$ ATP and ^{3}H ATP were present as substrates. Hydrolysis of the ATP must therefore have occurred. The incorporation of the ^{3}H label displays kinetics which are quite different from those of the γ phosphate, and since the former is totally inhibited by low concentrations of inorganic phosphate it is concluded that it represents the activity of polynucleotide phosphorylase.

Figure 14. The effect of K_2HPO_4 on the synthesis of RNA by RNA polymerase. The reaction was carried under exactly the same conditions as that described in Figure 10. Four samples contained (i) 0 M K_2HPO_4 (ii) .2mM K_2HPO_4 (iii) .4mM K_2HPO_4 and (iv) .8 mM K_2HPO_4. The specific activity of the ^3H ATP was 10,000 cpm/mμmole.


TCA precipitable cpm of ^{3}H-AMP
10,000
5000
10
20
30
40
50
60
70
80
90
Time Minutes
○ .0002 M PO4 .008 M Mg++
● .0004 M PO4 " "
□ .0008 M PO4 " "
■ 0 " "

The two activities can be separated from one another on phosphocellulose (Figure 4, Table 5.). The rate of incorporation of γ phosphate is 3.25 µmoles per 10 minutes per mg of enzyme. It rises to a saturation value between 60 and 120 minutes of 15 mµmoles or 1/12 of the γ ATP phosphate in the reaction. Maitra and Hurwitz (1967) observed a similar reaction at a similar rate (4 mµmoles/2 hrs). It is variable, however, because of differences in the concentration of ADP in the ATP samples—ADP is a powerful inhibitor of the reaction.

2. Inhibitor studies.

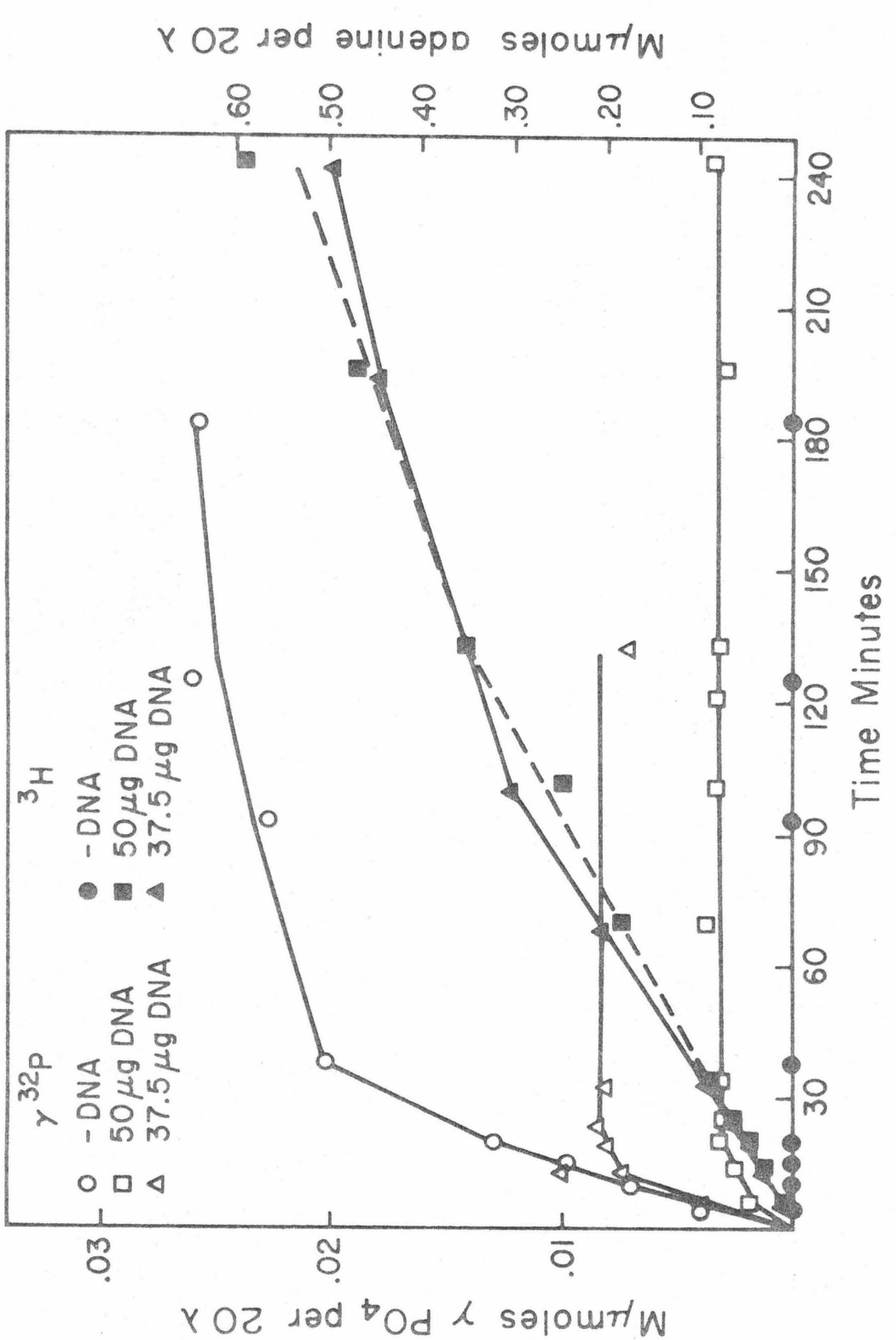
The kinetics are rapid, which does not suggest that the reaction is caused by bacterial growth during the incubation. This is confirmed by the fact that neither chloramphenicol (40 µg/ml), actinomycin D (20 µg/ml) nor sodium azide (.08%) inhibit it (Table 6). Other nucleoside triphosphates have no effect (Figure 9). 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) does not inhibit the selective incorporation of γ phosphate (Table 6). E. coli DNA (50 µg) caused inhibition, reducing the amount incorporated by 50 µg of enzyme at saturation by a factor of 8 (Figure 15). The DNA also caused the synthesis of poly A, which depleted

Table 6. Effect of antibiotics and inhibitors on polyphosphate synthesis.

Inhibitor	Concentration	Mμmoles of TCA precipitable γPO_4 per 10 minutes per mg enzyme	% activity
-	-	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	.08%	.9	75

A highly purified RNA polymerase preparation, GGA (100 ug), was assayed for contaminating polyphosphate kinase in .5 ml .01 M tris pH 7.9, .004 $MgCl_2$, .01 M β mercaptoethanol, .13 mM ^{32}P ATP, and .2 mM each of CTP and GTP at 37°C. Antibiotics were added when shown in the table. Aliquots of 50 λ were precipitated at times between 0 and 180 minutes with TCA, the precipitates collected and washed on nitrocellulose membranes, and counted. The incorporation at 10 minutes was abstracted from the time course and tabulated as a per cent of the incorporation in the absence of antibiotic.

Figure 15. The effect of DNA on the activity of polyphosphate kinase in an RNA polymerase preparation. Highly purified D-10 RNA polymerase GGA (50 μg), was assayed for polyphophate kinase in 25 ml of .01 M tris pH 7.5, .004 M $MgCl_2$, .01 M β mercapto-ethanol, .2 mM GTP, .2 mM UTP, and .2 mM ATP. The ATP was labelled in the γ phosphate (260,000 cpm ^{32}P per mμmole) and in the adenine (2150 cpm ^{14}C per mμmole). The samples contained (i) no DNA (ii) 37.5 ug DNA and (iii) 50 ug DNA. E. coli DNA was used. The reaction was carried out at 37°C and aliquots of 20 λ were removed at the times shown, precipitated with 10% TCA, and the precipitates were collected and washed on nitrocellulose membranes.


γ 32P
○ -DNA
□ 50μg DNA
△ 37.5 μg DNA
3H
● -DNA
■ 50μg DNA
▲ 37.5 μg DNA
Mμmoles γ PO4 per 20 λ
.03
.02
.01
Mμmoles adenine per 20 λ
.60
.50
.40
.30
.20
.10
30
60
90
120
150
180
210
240
Time Minutes

the ATP pool. However this cannot be responsible for all of the inhibition since 30 minutes after the incorporation of phosphate had ceased, 90% of the ATP still remained. Furthermore, 50 µg of DNA caused 88% inhibition of the incorporation phosphate at saturation while 37.5 µg of DNA caused 70% inhibition, but both gave the same amount of poly A synthesis. It is concluded therefore that the DNA has an inhibitory effect above and beyond the depletion of the ATP pool.

High concentrations of KCl inhibit the reaction completely (Table 7), while both potassium phosphate and sodium pyrophosphate are also inhibitory. When the % inhibition is plotted versus Δ ionic strength (Figure 16) it is clear that the pyrophosphate is a very effective inhibitor at low molar concentrations (.2 -.5 mM). ADP stops the reaction completely at .2 mM .

3. The 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. 100 µg of RNA polymerase were added to .5 mls of reaction mix containing .2mM γ^{32}P ATP under standard conditions. After 90 minutes, during which 4 50 λ aliquots were taken to measure the progress of the reaction, the tubes were placed in ice, and 3 further aliquots were removed and added to 50 λ of pronase (6 mg/ml), ribonuclease (4 mg/ml)

Table 7. Effect of salts on polyphosphate synthesis.

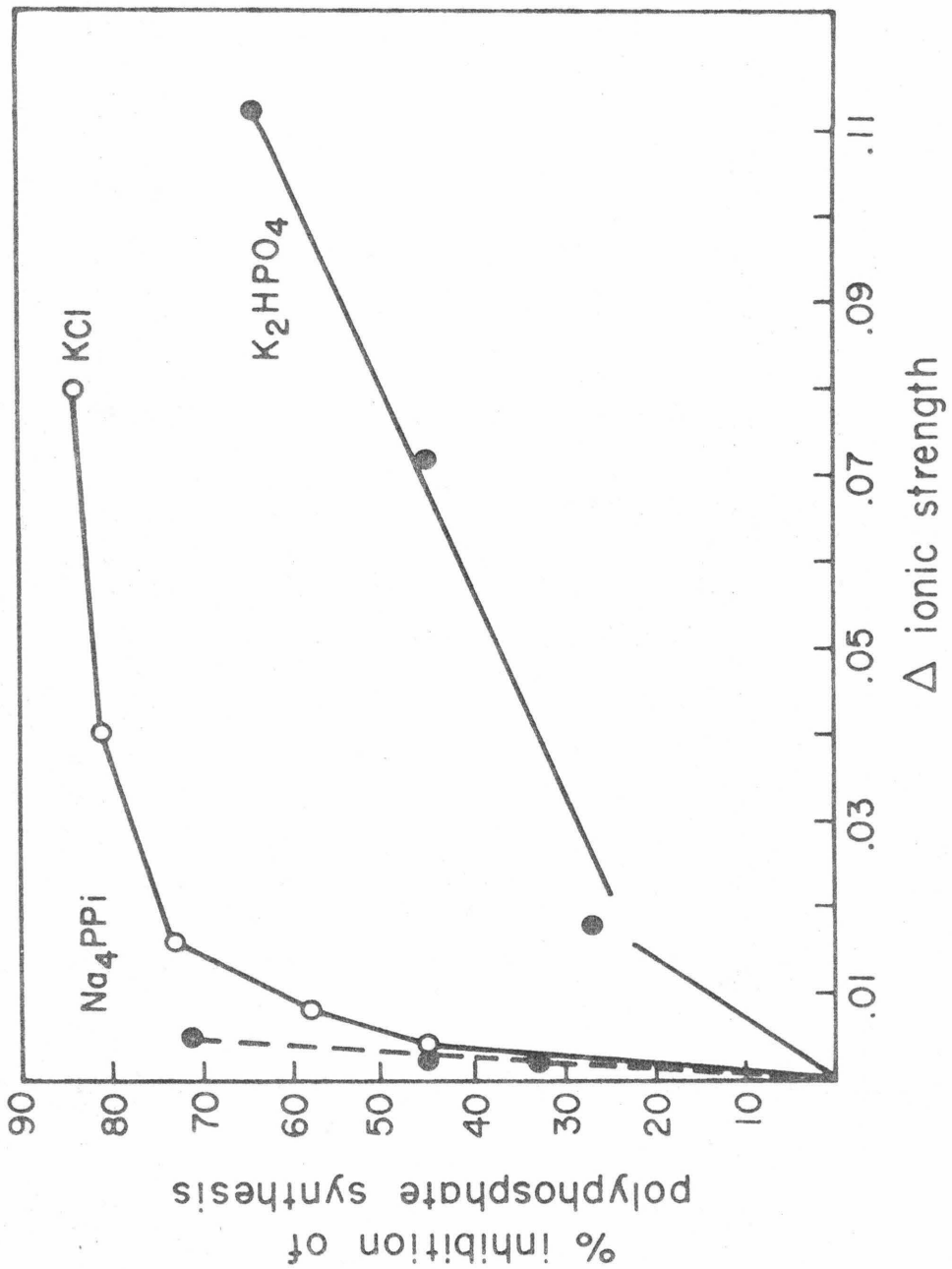
SALT	MOLARITY	NORMALISED	% INHIBITION OF POLYPHOSPHATE SYNTHESIS	CHANGE IN IONIC STRENGTH $\Delta\tau$
KCl	0	100	0	0
	.004	55	45	.004
	.008	42	58	.008
	.016	27	73	.016
	.040	19	81	.040
	.080	16	84	.080
	.100	16	84	.100
	.200	13	87	.200
	.300	0	100	.300
	.400	0	100	.400
K_2HPO_4/	0	100	0	0
KH_2PO_4	.004	73	27	.018
	.008	55	45	.072
	.012	36	64	.108
Na_4PPi	.0002	67	33	.002
	.0005	29	71	.005

The conditions of synthesis are described in the legend to Figure 16.

Figure 16. The effect of KCl, K_2HPO_4 and sodium pyrophosphate on the activity of polyphosphate kinase in highly purified RNA polymerase preparations. For the effect of KCl and K_2HPO_4, 50 ug of glycerol gradient purified enzyme (GGA) was incubated at 37°C in .25 ml .01 M tris pH 7.9, .004 M $MgCl_2$, .01 M mercaptoethanol, and .2 mM γ ^{32}P ATP in the presence of increasing concentrations of KCl (0-.08 M) or K_2HPO_4 (0-.012 M). Aliquots of 50 λ were TCA precipitated at times between zero and 90 minutes, and the precipitates collected and washed as usual. The incorporation was calculated for the 10 minute point and values were normalized setting the zero KCl or zero K_2HPO_4 point equal to 100. These values are tabulated in Table 7. This figure graphs the % inhibition versus the change in ionic strength due to the added salt.

The effect of sodium pyrophosphate was measured in a different experiment in which the conditions were the same as those above except that the concentration of ATP was .13 mM, and both GTP (.2 mM) and CTP (.2mM) were present. These do not effect the reaction (see Figure 9) of polyphosphate kinase. Values of incorporation after 10 minutes at 37°C were extracted

Figure 16. (cont'd). from a time course, and normalised to the value for zero sodium pyrophosphate as shown in Table 7. % inhibition is again plotted versus the change in ionic strength.


% inhibition of
polyphosphate synthesis
90
80
70
60
50
40
30
20
10
.01
.03
.05
.07
.09
.11
Δ ionic strength
KCl
K_2HPO_4
Na_4PPi

or water. These were returned to 37°C and incubated for a further 30 minutes. Pronase caused a 25% reduction in TCA precipitable ^{32}P, while the ribonuclease treated sample showed a 10% increase, neither of which can be judged to be very significant in view of the high concentration of enzyme added. If DNAase is added at zero time to a reaction, no effect is observed. The product is degraded 100% by alkaline phosphatase or phosphodiesterase.

4. The size of the product.

Three approaches were made to analyse the size of the phosphate compound, glycerol gradient sedimentation, gel filtration and SDS-polyacrylamide gel electrophoresis.

For band sedimentation 50 µg of RNA polymerase preparation were added to a standard reaction mix .1 mM $\gamma^{32}P$ and ^{3}H ATP, .1 mM GTP, .1 mM UTP, .01 M tris pH 7.9, .004 M $MgCl_2$, .01 M β mercaptoethanol, in .25 mls. After 90 minutes at 37°C, the reaction was cooled and dialysed exhaustively for 7½ hours against 2 changes of 2 litres each of .01 M tris pH 7.9, .004 M $MgCl_2$, .01 M β mercaptoethanol. Of the counts remaining after dialysis 20% were precipitable by 10% TCA, and the material was completely stable when stored at 4°C for 18 hours. A total of 6000 counts were applied to each of two glycerol gradients, 10-30% glycerol, .01 M tris pH 7.9, .004 M $MgCl_2$, .01 M β mercaptoethanol, one of which was .5 M KCl. A third tube contained a sample of RNA polymerase in the low salt

gradient. After 10 hrs at 50 K at 4°C, the tubes were dripped 37 fractions collected per tube and assayed for polymerase activity (tube 3.) and γ ^{32}P counts. 70% of the counts applied were recovered, and for both the high and low salt gradient 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.

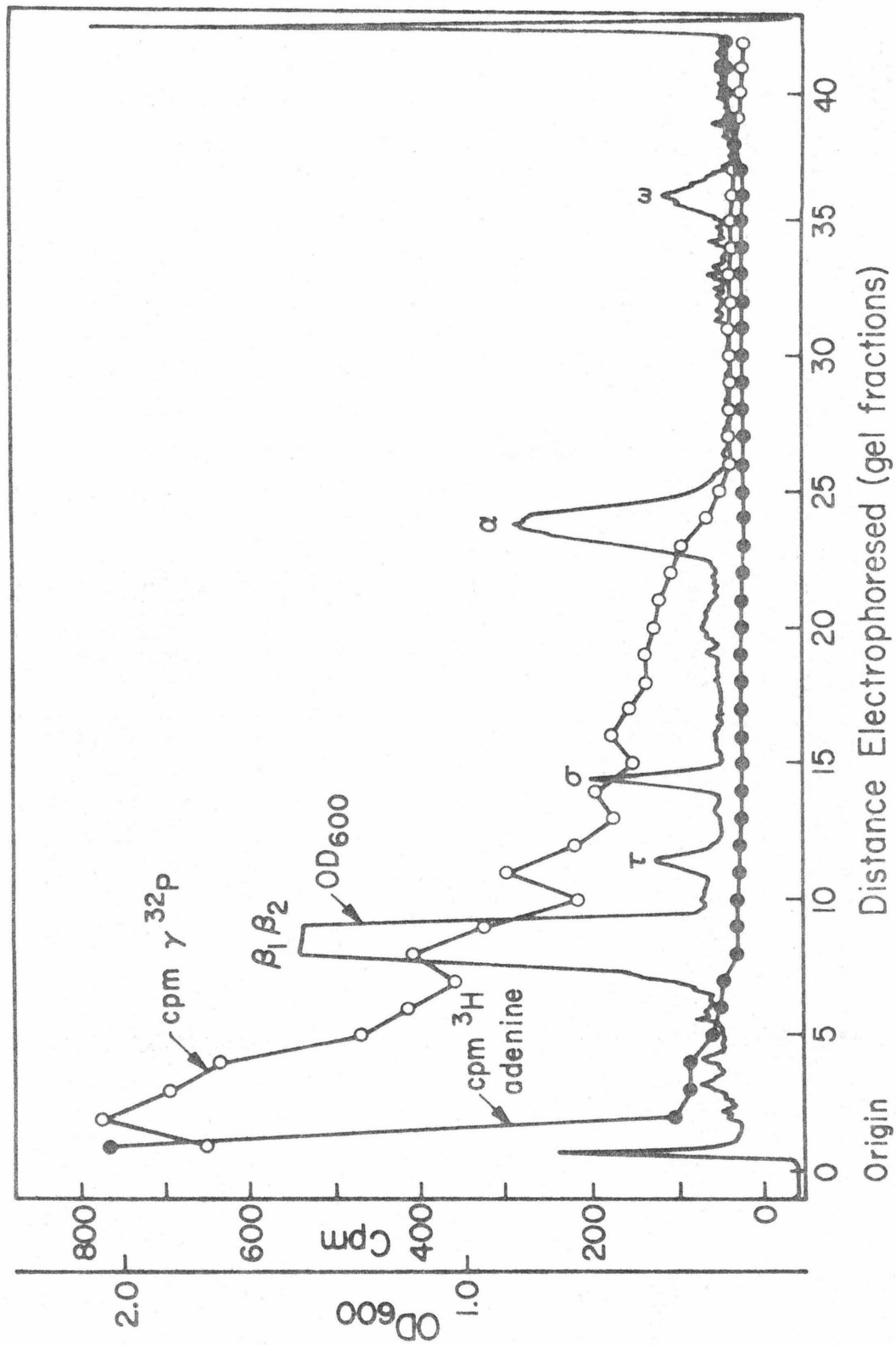
A similar sample was applied to a P-60 column, 45 x .7 cm, without dialysis, and eluted with .01 M tris pH 7.5, .01 M β mercaptoethanol .004 M $MgCl_2$. 68% of the TCA precipitable γ phosphate was eluted in the void volume with the peak of OD 280 of the RNA polymerase. Since from the glycerol gradients, it is known that the enzyme is not associated with the phosphate it is concluded that a high proportion of the phosphate has a high molecular weight.

Aliquots of the peak fraction in the void volumes were electrophoresed on SDS-polyacrylamide gels (5%) for 75 minutes at 45 volts, as described in Materials and Methods. The gels were stained with Coomassie Blue to locate the sub-unit of RNA polymerase, then fractionated and counted. Figure 17 shows the analysis of such a gel. The ^{32}P is distributed heterogeneously and is not associated exclusively with any sub-unit of the RNA polymerase. The ^{3}H label represents poly A synthesised by polynucleotide phosphorylase. It does not run with the phosphate or the enzyme

Figure 17. SDS-polyacrylamide gel electrophoresis of the products of polyphosphate kinase and polynucleotide phosphorylase. 500 µg of glycerol gradient purified RNA polymerase preparation (GGA) were incubated in .25 ml of .02 M tris pH 7.9, .008 M $MgCl_2$, .02 M β mercaptoethanol, 2 mM ATP (36,000 cpm ^{32}P/mµmole and 60,000 cpm ^{3}H/mµmole) for 90 minutes at 37°C. .2ml of the reaction was chromatographed on a P-60 column (45 x .7 cm) and eluted with .01 M tris pH 7.5 .004 M $MgCl_2$, .01 M β mercaptoethanol. 68% of the input TCA precipitable $\gamma^{32}P$ and 75% of the ^{3}H eluted in the void volume with the enzyme. Aliquots of the peak fraction containing 6000 counts of $\gamma^{32}P$ and 1000 counts of ^{3}H were electrophoresed on each of three standard SDS-polyacrylamide gels for 75 minutes at 45 volts (see Materials and Methods). In addition 5 µg of GGA enzyme were applied to gel 2 to act as a marker. Gel 1 was sectioned immediately after electrophoresis (to minimise elution of the sample in the staining and destaining solution) and the fractions were counted in the NCS/Toluene cocktail. Gels 2 and 3 were stained in Coomassie Blue, destained, fractionated and counted. All gels gave the same profile of counts shown in this figure for gel 1, and no loss occurred

Figure 17. cont'd.

during staining and destaining. An OD 600 profile taken on the Gilford Spectrophotometer is superimposed to show the relationship of the stained bands to the distribution of the counts. 90% of the counts applied were recovered after fractionation.


OD600
2.0
1.0
Cpm
800
600
400
200
0
cpm γ 32P
β1 β2
OD600
cpm 3H adenine
α
σ
τ
ω
0
5
10
15
20
25
30
35
40
Origin
Distance Electrophoresed (gel fractions)

sub-units. 100% of the counts applied were recovered.

5. Separation of the phosphate incorporation from RNA polymerase activity.

The activities of phosphate incorporation and RNA synthesis co-chromatograph on DEAE cellulose. They do not separate completely on a high salt glycerol gradient where the RNA polymerase activity moves a little faster. Chromatography on phosphocellulose (Figure 4.) was a very effective method of separation. Sub-fractions PC 2.1 to PC 2.7 were assayed for RNA synthesis and phosphate incorporation, (Table 5.) and were analysed on SDS-polyacrylamide gels (Figure 5). The phosphate incorporation by 2.7 was extremely high representing a specific activity of 250 mμmoles per 10 minutes per mg. This is 30 times the specific activity of the enzyme before phosphocellulose chromatography and more than 209 times the specific activity of any of the other fractions from the column. The SDS-polyacrylamide gel of fraction 2.7 revealed 2 prominent bands between the σ and α sub-units of RNA polymerase which do not show up on any of the other gels. The ω sub-unit is completely absent and σ is almost so. It is not known which of these features are responsible for the phosphate incorporation.

The phosphocellulose fractions all contained the α and β sub-units 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 α, β, and ω had least σ, but it is clear from what was observed, and from the gels that specific activity increases from 2.1 to 2.6 and then falls slightly at 2.7. Some fractions showing significant RNA synthesis show no phosphate incorporation e.g. 2.2 and 2.3. These are the fractions with the highest population of σ.

6. Summary.

The analysis is consistent with the explanation that the incorporation of $\gamma\,^{32}P$ from ATP by RNA polymerase in the absence of DNA represent the synthesis of polyphosphate (Kornberg et al., 1956). It is certain that RNA polymerase is not phosphorylated since the phosphate counts do not co-sediment with RNA polymerase on a glycerol gradient nor do they co-electrophorese with any specific band on SDS-polyacrylamide gels. The product was stable under the conditions of the sedimentation.

The question remains if this activity is caused by the presence of polyphosphate kinase, (Kornberg et al., 1956; Maitra and Hurwitz, 1967; Millette and Trotter, 1970) or by RNA polymerase in the absence of saturating amounts of σ or ω sub-unit. The former is the most likely explanation for the pattern of inhibition by pyrophosphate and ADP is identical to that reported by Kornberg et al., (1956) for polyphosphate kinase, and the specific activity of the RNA

polymerase preparations for polyphosphate synthesis is very similar to that of a crude lysate of E. coli kinase.

Discussion

(i) 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°C. This final yield of enzyme is 27 mg per 100 g of cells. 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 and in which the recovery was estimated at 56%. Large differences have been observed for the yield and recovery between D-10 and B preparations (Table 2). It seems likely that K-12 is significantly better than either of these. Burgess (1969) suggests that the yield reflects the rate of cell division, being optimal for rapidly dividing cells. His preparations were made on cells grown to three quarters log phase, while these E. coli D-10 cells were grown to one quarter log phase and E. coli B to one half. Since the yield from D-10 is greater than from B but less than K-12, the variation cannot be attributed only to changes in growth rate. Media and strain differences

may be expected to cause some variation. These data suggest that K 12 is superior to D-10 which is better than B.

There are three methods described for the separation of nucleic acids from the polymerase preparation: 1. Streptomycin and/or protamine precipitation (Chamberlin and Berg, 1962); 2. phase separation in polyethylene glycol-dextran sulphate (Babinet, 1967); 3. treatment with DNAase (Burgess, 1969). Streptomycin and protamine have been used extensively (Furth et al., 1962; Chamberlin and Berg, 1962; Stevens and Henry, 1964; Richardson, 1966) and routinely in this laboratory until recently. The method has been criticised on the grounds that it is not reproducible and this is certainly true for different preparations of cells. However, the two-step protamine procedure is completely reproducible for a given batch of cells, and is convenient, requiring only one assay. In spite of the fact that Burgess (1969) has shown that his method of purification removes all of the DNAase as measured by a sensitive assay, I have avoided adding the DNAase because the protamine is effective and rapid, and removes some protein as well as nucleic acid.

After ammonium sulphate fractionation large amounts of F3 are most conveniently handled by a DNA cellulose column. 60% of the protein which sticks at .15 M KCl is RNA polymerase, similar to the observation of Bautz and Dunn (1969). The specific activity after DNA cellulose chromatography of F3 is greater than 500 on T7 DNA.

No loss of σ occurs, as on phosphocellulose which has not been used extensively for this reason. After DNA cellulose chromatography, high salt glycerol gradient sedimentation or agarose gel filtration (Burgess, 1969) are effective means of final purification.

For large preparations it is reasonable to treat 600 g of cells in one day, carrying the procedure up to the low salt glycerol gradient sedimentation, or to the DNA cellulose column whichever was appropriate, Since the sample must be dialysed before either of these steps, it is a good point at which to stop and to dialyse overnight.

Ribonuclease and phosphatase were not observed in any of the highly purified preparations. The DNAase activity was very low. Polynucleotide phosphorylase was a measurable contaminant, but several procedures are easily employed to deal with it. The addition of .8 mM phosphate to assay was used as the most convenient method. As an unrelated observation the phosphocellulose column may prove a very efficient method for purifying polynucleotide phosphorylase from which it elutes quantitatively in the run-off. From SDS-polyacrylamide gels, a polypeptide of molecular weight of 75,000 electrophoresing between σ and α sub-units of RNA polymerase may represent a sub-unit of polynucleotide phosphorylase.

The incorporation of γ phosphate from ATP by RNA polymerase preparations was investigated thoroughly as

possibly representing the formation of a phosphoenzyme intermediate. Since the product of the reaction is heterogeneous, does not co-sediment with RNA polymerase in a glycerol gradient, does not electrophorese with any of the sub-units in the presence of SDS, and represents an average of eight phosphates incorporated per polymerase molecule this is unlikely. Furthermore the activity can be almost completely separated from RNA polymerase on phosphocellulose although they do co-chromatograph on DEAE cellulose. The reaction bears many resemblances to that catalysed by polyphosphate kinase.

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. 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 .05 m<u>M</u>.

Highly purified enzyme catalyses poly A synthesis. The most important method for reducing this reaction is the employment of DNA templates which do not have single-stranded regions. The fact that a perfect DNA molecule does

not support poly A synthesis to an appreciable extent is strong evidence that it is an artifactual reaction, although clearly we do not know the configuration of DNA molecules in the cell. In fact considering the high rate of RNA and DNA synthesis during phage and bacterial growth, and the presence of several repair systems, there may be some single-stranded DNA available for poly A synthesis in vivo. The reaction is further reduced by the presence of other nucleotides, but as observed for the case of UTP, the other nucleotide is then incorporated at a compensatory rate, and the overall rate of initiation by ATP is not reduced at all. All studies in which DNA and enzyme are incubated in the presence of ATP and one or two of the other nucleotides, to investigate the initiation of synthesis, or to examine the initiation complex must take into account the possibility of poly A synthesis. High salt has a small inhibitory effect, while Mn++ is a strong stimulant, providing further support for including the former and excluding the latter from the reaction.

Anthony, D. D., Zeszotek, E., and Goldthwait, D. A., Acad. Sci., Proc. Nat. Wash., 56, I026. (1966)

Babinet, C., (I967). Biochim. Biophys. Res. Commun ., 36, 925.

Bauer, W., and Vinograd, J., (I968). J. Mol. Biol., 33, I4I

Bautz, E.K.F., and Dunn, J.J., (I969). Biochim. et Biophys. Acta, 34, 230.

Beers, R.F., Jr., (I96I). J. Biol. Chem., 236 2703.

Burgess, R.R., (I969). J. Biol. Chem., 244, 6I60.

Burton, K., (I956). Biochem. J., 62, 3I5.

Chamberlin M., & Berg P.,(I962). Proc. Nat. Acad. Sci., Wash., 48, 8I.

Chamberlin, M., and Berg, P., (I964). J. Mol. Biol., 8, 708

Conway, T.W., and Lipmann, F., (I964)Proc. Nat. Acad. Sci. Wash. 52,I462.

Dahmus, M.E., and McConnell, D.J., (I969). Biochemistry, 8, I524.

di Mauro, E., Snyder, L., Marino, P., Lamberti, A., Cappo, A., Tocchini-Valentini, G.P.(I969). Nature, 222, 533.

Dische, Z., and Schwarz, K., (I937). Mikrochim. Acta, 2, I3

Fuchs, E., Millette, R. L., Zillig, W; and Walter G., (I967 European J. Bioch., 3, I83.

Furth J.J.,Hurwitz, J., and Andeks M. (I962). J. Biol. Chem 237, 26II.

Grunberg-Manago, M., and Ochoa, S., (I955). J. Am. Chem. Soc., 77, 3I65.

Hartmann, G., Hanikel, K., Kniisel, F., and Niiesch, J., (I967).Biochem. et Biophys. Acta, I45, 843

Hyman, R.W., and Davidson, N., (I970). J. Mol. Biol., 50, 42I.

Kimhi, V., and Littauer, U.Z., (I968). J. Biol. Chem., 243, 23I.

Kirby, K.S., (I962). Biochim. Biophys. Acta, 55, 545.

Klee, C.B., (I969). J. Biol. Chem., 244, 2558

Kornberg, A., Kornberg, S.R., and Simms, E.S., (I956). Biochim. et Biophys. Acta, 20, 2I5.

Litman, R.M., (I968). J. Biol. Chem., 243, 6222.

Losick, R., and Sonenshein, A.L., (I969). Nature, 224, 35.

Maitra, U., and Hurwitz, J., (I967). J. Biol. Chem., 242, 4897.

Marmur, J., (I96I). J. Mol. Biol., 3, 208.

Martin, J.B., and Doty, P.M., (I949). Anal. Chem. 2I, 965.

Millette, R.L. and Trotter, C.D., (I970). Proc. Nat. Acad. Sci. Wash., 66, 70I.

Niyogi, S.K., and Stevens, A., (I965). J. Biol. Chem., 240, 2587.

Ochoa, S., and Mii, S., (I96I). J. Biol. Chem., 236, 3303.

Pettijohn, D., and Kamiya, T., (I967). J. Mol. Biol., 29, 275.

Richardson, J. P., (I966). Proc. Nat. Acad. Sci., Wash., 55 I6I6.

Richardson, J.P., (I970). Nature, 225, II09.

Shapiro, A.L., Vinuela, E. and Maizel, J.V. Jr. (I967).

Biochem. Biophys. Res. Comm. 28, 8I5.

Singer, M.F., and Guss, J.K., (I962). J. Biol. Chem., 237 I82.

Sippel, A., and Hartmann, G., (I968). Biochim. et Biophys. Acta, I57, 2I8.

So, A.G., Davie, E.W., Epstein, R., and Tissieres, A., (I967). Proc. Nat. Acad. Sci., Wash., 58, I739.

Stevens, A, (I964). J. Biol. Chem., 239, 204.

Stevens A., and Henry J., (I964). J. Biol. Chem., 239,I96.

Studier, F.W., (I965). J. Mol. Biol., II, 373.

Walter, G., Zillig, W., Palm,P.and Fuchs, E., (I967). European J. Bioch., 3, I94.

Part II

Chapter II

Initation of synthesis by RNA polymerase on T7

INTRODUCTION

The identification of the regions of DNA involved in the control of gene expression, such as the operator and promoter sequence of the operon, is one of the most interesting problems of molecular genetics (Yarus, 1969). The promoter in molecular terms is regarded as the site at which RNA polymerase initiates transcription (Silverstone et al., 1969; Roberts, 1969), and as in the case of the recognition of the operator by the repressor (Gilbert and Muller-Hill, 1966; Ptashne, 1967), a sequence of bases must be recognised by a protein molecule in this process.

The reaction catalysed by E. coli RNA polymerase can be divided into five stages, and the first three stages are expected to take place in close proximity to the promoter site.

1. Binding

   $E + DNA \leftrightarrows E.\ DNA$

2. Activation

   $E.\ DNA + \text{purine triphosphate} \leftrightarrows E.\ DNA.\ PuTP$

3. Initiation

   $E.\ DNA.\ PuTP + XTP \leftrightarrows E.\ DNA.\ PuTP\text{-}XMP + P\text{-}Pi$

4. Propagation

   $E.\ DNA.\ PuTP\text{-}XMP + XTP \leftrightarrows E.\ DNA.\ PuTP\text{-}XMP\text{-}(XMP)_n + n\ P\text{-}Pi$

5. Termination

E. DNA. RNA → E + DNA + RNA

Initiation of synthesis in vitro has been shown clearly to reproduce the specificity exhibited in vivo (Summers and Siegel, 1969; Travers, 1970), which is partly conferred on the enzyme by the presence of the σ sub-unit (Burgess et al., 1969; Summers and Siegel, 1969; Travers, 1970; Sugiura et al., 1970). In its absence the core enzyme composed of β β' α and ω sub-units transcribes both sense and anti-sense strands. For example in the case of ø T4 and T7 only the early genes are transcribed in vitro in the presence of σ, while in its absence all sequences are read.

From this work it has been deduced that all operons do not carry the same promoter, and that other promoters respond either to a totally different RNA polymerase as in the case of the late genes of T7 (Chamberlin et al., 1970) or to a different σ factor as in the case of T4 late genes (Travers, 1970). Recently it has been reported that an E. coli polypeptide which is partially responsible for the replication of the ø Q β plus strand, and which has been purified, causes complete E. coli RNA polymerase to transcribe the ribosomal genes of E. coli DNA, (Kamen, 1970; Travers and Kamen, 1970), which are not transcribed by the normal enzyme preparation. These observations provide a detailed molecular model of a positive control mechanism, which explains the

nature of pleiotrophic genes such as gene 1 in T7 (Chamberlin et. al., 1970) and gene Q in λ (Naono and Tokuyama, 1970). The transcription of SPO1 involves a similar control mechanism (Wilson and Geiduschek, 1969; Geiduschek et al., 1969).

It is noteworthy that selective transcription of the DNA of higher organisms and animal viruses by E. coli RNA polymerase has been observed for the case of chromatin (Paul and Gilmour, 1966; Bekhor et al., 1969; Tan and Miyagi, 1970), ribosomal DNA (Reeder and Brown, 1970), SV40 (Westphal, 1970), adenovirus (Green et al., 1970) and vaccinia (Kates and Beeson, 1970), and that for several of these cases the sequences transcribed in vitro have been completely competed out in RNA-DNA hybridisation by RNA synthesised in vivo. This presents the fascinating possibility that the RNA polymerase initiation site, or at the very least the strand selection site has been conserved.

Szybalski and co-workers have observed the presence of pyrimidine-rich sequences thought to be 15-40 nucleotides long in all DNAs examined (Szybalski et al., 1966; Taylor et al., 1967; Summers and Szybalski, 1968(a); ibid., 1968 (b); Szybalski, 1969 (a), Szybalski, 1969 (b)). The pyrimidine clusters were detected on those strands which are transcribed in vivo, and where both strands are transcribed, as in ø λ the pattern of transcription correlates closely with the distribution of poly G binding sites. Szybalski has proposed that such clusters form part of the mechanism of either

initiation or termination of transcription. Since point mutations of promoters have been described (Martin, 1969) and there is considerable evidence that there are several classes of promoters binding different RNA polymerases, it seems likely that the situation is more complex (Szybalski, 1969 (a)) but probably involves these pyrimidine clusters.

While the fidelity of transcription is sometimes maintained in vitro, RNA polymerase is clearly capable of initiating synthesis under conditions which are artifactual. For example, synthesis can be carried out on single-stranded nucleic acid, both RNA and DNA, neither of which presumably acts as template in vivo (Straat and T'so, 1970). The absence of σ leads to the transcription of the anti-sense strand of closed-circular Ø fd FI replicative form, (Sugiura, et al., 1970), and the generation of some self-complementary RNA on T4 (Bautz et al., 1969). It has also been observed that under some conditions (e.g. low ionic strength) the enzyme will bind indiscriminately to DNA limited only by physical space along the DNA molecule (Pettijohn and Kamiya, 1967), though it is not clear if all such enzymes can initiate synthesis. Vogt (1969) has shown that the introduction of single-stranded knicks by DNAase I increases the template activity of Ø DNA, and the enzyme is known to interact with the ends of DNA molecules (Berg et al., 1965). Concerning the heterogeneity of initiation sites, the 5' terminal of RNA synthesised both in vivo and in vitro can be any one of

the four nucleotides although it is most commonly A or G (Maitra and Hurwitz, 1965; Stevens, 1969). The heterogeneity of the terminal nucleotide may reflect heterogeneity of the binding site. As a first approach to the identification of the DNA to which the enzyme binds, several groups have studied the extent to which it binds in the absence of nucleotide triphosphates (Kadoya et al., 1964; Fox et al., 1965; Richardson, 1966; Pettijohn and Kamiya, 1967; Stevens, 1969). A binary complex of enzyme and DNA is formed, with approximately one enzyme per 200 - 1000 base pairs, assuming a molecular weight for the active enzyme of 400,000. The binding of the enzyme is sensitive to ionic strength ($\mathcal{T}$ /2). At low values the binding is essentially infinite, limited only by space on the DNA molecule (Pettijohn and Kamiya, 1967). At $\mathcal{T}/2 = .2$ there is a sharp transition and binding falls to a small number of the order of one per 500 base pairs. At high ionic strength $\mathcal{T}/2 > .35$ no binding is observed by a co-sedimentation assay. It follows that initiation of synthesis is also dependent on ionic strength. The fact that at intermediate ionic strength the number of enzymes bound is limited, suggests but does not prove that the enzyme is attached to specific sites under these conditions.

A complex formed between DNA and enzyme in the presence of ATP and/or GTP at low ionic strength is stable to .4 M $(NH_4)_2SO_4$ and can initiate synthesis when UTP and CTP are

added (Hyman and Davidson, 1970; Anthony et al., 1966). Initiation does not otherwise occur at ,4 M $(NH_4)_2SO_4$. The formation of this complex may represent an activation step. One of the early steps is sensitive to the antibiotics rifamycin and rifampicin (di Mauro et al., 1969). The inhibition may be overcome by pre-incubation with ATP and GTP. Thus if T4 DNA, enzyme and ATP, GTP and CTP are pre-incubated together, on the addition of UTP and rifampicin, there is little inhibition of synthesis. However, if the rifampicin is added during the pre-incubation, inhibition is 100%. This same pre-incubation step is required for the complex to be resistant to .4 M $(NH_4)_2SO_4$ (Anthony et al., 1966; Hyman and Davidson, 1970). The experiments of di Mauro et al., suggested that the activation step is distinct from the initiation step. RNA synthesised in vitro on the T4 DNA template has as 5' terminals pppApU or pppGpU (Maitra and Hurwitz, 1965; Travers, quoted in di Mauro et al., 1969). Preincubation of T4 DNA with enzyme and ATP plus GTP, clearly should not allow the synthesis of the first phosphodiester bond, but it does stabilise the complex to high ionic strength and to the inhibitory action of rifampicin. It is generally true that ATP or GTP do stabilise the DNA-enzyme complex, whereas UTP or CTP are much less effective (Anthony et al., 1966; Stead and Jones, 1967; Hyman and Davidson, 1970). However in none of these cases has it been possible to distinguish the effect as on activation rather than initiation

and for the case of the experiments of di Mauro et al., (1969) no attempt was made to rule out the presence of impurities in the nucleotide preparations. The idea that GTP and ATP have a special role in the early stages of synthesis has been supported by other lines of evidence. The predominant 5" terminal nucleotides in RNA synthesised in vivo and in vitro are ATP and GTP (Maitra et al., 1965). It has been shown for various DNAs that those nucleotides which initiate most of the chains for a given DNA have high apparent Kms, i.e., of the order of .15 mM while the other nucleotides have apparent Kms. of .015 mM (Anthony et al., 1969 (b)). Studies on the binding of nucleotides and rifamycin to RNA polymerase in the absence of DNA by fluorescence spectroscopy have shown that ATP, GTP and rifamycin bind to the same site or to closely related sites, while UTP or CTP do not. (Wu and Goldthwait, 1969 (a)). An apparent dissociation constant of .14 mM was calculated for both ATP and GTP, similar to the apparent Km obtained in previous experiments by the same group (Anthony et al., 1969 (b)). Equilibrium dialysis confirmed the values for the dissociation constants for ATP and GTP, and showed thatrifamycin completely inhibited the binding of either to the enzyme. In the presence of Mg++ two binding sites for purine nucleoside triphosphates were observed, a weak site with a Ks of .15 mM, which was competed for by rifamycin, and a strong site with a Ks of .015 mM, which was not (Wu and Goldthwait, 1969 (b)). CTP and UTP had

weak binding sites only (Ks of .23 mM and .37 mM respectively) and they were not competed by rifamycin. The critical point to be drawn from these data concerns the difference between the purine and pyrimidine nucleotide triphosphates and the relationship of rifamycin to the former.

The nature of the activation step is unknown. Fuchs et al., (1967) have proposed that it involves the unwinding of a short region of double helix. They observed a lag phase before the initiation of RNA synthesis, which was lengthened by low temperature and high ionic strength and which was absent when single stranded DNA was used as template. Moreover it was not affected by pre-incubating the enzyme and the DNA in the absence of substrate, indicating that binding was not limiting. They calculated an activation energy of 180 K cals./mole. for the lag phase event by comparison to a value of 30 k cals./mole. for the synthesis reaction. Bautz and Bautz (1970) have studied the amount of rifampicin-resistant enzyme bound to DNA in low salt in the absence of nucleotides. Such a complex has a short half-life of about 15 seconds at 17°C, but it has allowed the estimation of the number of initiation sites.

The instability of the binary complex of enzyme and DNA impedes analysis of the binding site on the DNA and draws into doubt the value of experiments in which it was studied (Matsukage et al., 1969; Kameyama et al., 1970).

The formation of the stable ternary complex of enzyme-DNA-nucleotide should overcome this objection; if only a

single nucleotide is provided, the enzyme should not be able to move far along the DNA.

T7 DNA is an ideal template for the reaction since the phage can be grown readily, purified by banding in Cs Cl, and the DNA isolated almost undamaged. It does not have single-stranded ends, is not glucosylated (Lunan and Sinsheimer, 1956), neither is it circularly permuted (Ritchie et al., 1967).

Genetic and biochemical evidence suggests that only a small number of sites are available to E. coli RNA polymerase for initiation. Hausmann, R., and Gomez, B., (1967),Studier (1969),, and Studier and Maizel (1969) have identified 19 cistrons by complementation tests. Mutations in gene 1 have a pleiotrophic effect on most of the other cistrons. Autoradiographs of SDS-polyacrylamide gels reveal 17 distinct phage induced proteins, of total molecular weight equal to 80% of the coding capacity of T7. Using amber mutants 12 of these bands have been assigned to different genes. Mutants in gene 1 only show the three earliest polypeptides and none of the others (Studier and Maizel, 1969).

Summers (1969, a) and Siegel and Summers (1970) have extended the analysis to RNA synthesis directed by T7. 12 or 13 phage specific bands are separated by polyacrylamide electrophoresis, and one of these of molecular weight 1.1×10^6 corresponds to the largest protein coded by gene 1 of molecular weight 1.1×10^6 (Chamberlin et al., 1970). In the presence of chloramphenicol, or of an amber mutation in gene

1., only a few (3 or 4) distinct T7 RNA species are observed.

The most reasonable interpretation of these results is that only a small number of the early cistrons can be transcribed by E. coli RNA polymerase producing no more than 4 T7 RNA and protein species. One of these proteins, the product of gene 1., is a T7 RNA polymerase (Chamberlin et al., 1970) which subsequently transcribes the late cistrons. It had been speculated that the product of gene 1 was a T7 σ factor which would induce the bacterial core enzyme to initiate at the late promoters, (Studier and Maizel, 1969; Summers and Siegel, 1969) but Chamberlin et al., (1970) have clearly excluded this.

Because of the small number of postulated initiation sites and because the DNA can be prepared in excellent condition in high yield, T7 was chosen as the template for a study of the process of initiation and the nature of the DNA-enzyme complex. The effect of GTP on the formation of this complex was examined most closely since it contributes little if any to the artifacts caused by homopolymer and polyphosphate synthesis.

MATERIALS AND METHODS

(i) Growth and purification of bacteriophage T7

Strains: E. coli B/5 was used as host for wild type T7 M. Stocks were provided originally by Professor R.S. Edgar and the late Dr. J.J. Weigle. The cells are grown in medium K. 1 l. of this medium contains:

7 g Na_2HPO_4	)
3 g KH_2PO_4	)
.5 g NaCl	) SALTS
1.0 g NH_4Cl	)
40 ml 10% glucose	
350 ml 3% casamino acids	
5 ml 1 M $MgSO_4$	
.2 ml .5 M $CaCl_2$	

The four major salts are autoclaved together, the other components separately. $CaCl_2$ is added last with shaking to prevent precipitation of Ca SO_4. A 50 ml starter culture is inoculated from a slant and allowed to stand overnight at room temperature. This is used to inoculate large cultures the following morning, approximately 1 ml of starter per 100 ml of fresh medium. Incubation is at 30°C. Growth is monitored by reading the optical density at 600 millimicrons. One OD 600 corresponds to a cell density of approximately 10^9/ ml. At OD 600 = .4, the culture is infected at a multiplicity of infection of 2. A further 40 ml of 10%

glucose is added per litre and the culture is vigorously aerated. The first lysis occurs approximately 15 minutes after infection. Incubation continues until the OD 600 is $<.05$.

The culture should be aerated as strongly as possible as the yield of phage is strongly dependent on O_2 supply. To prevent contamination of the laboratory with phage, the operation is carried out in the hood. The air outlet from the culture is passed through a large flask of H_2O to reduce release of Ø. Aeration of the culture is by the laboratory air supply which is filtered by a Koby Air Purifier, and by passage twice through sterile water. A large amount of foam is generated on lysis. It is important not to add Anti-Foam, since it prevents reinfection. However, after 20 minutes, the addition of a few drops of 2-octanol reduces the foam and does not seem to affect the yield of phage. The yield is estimated from the relationship A260 = 1 corresponds to 10^{12} Ø phage per ml. The average yield was 8×10^{10} phage per ml of culture.

Phage are collected by centrifugation at 30K for 2½ hours in a Beckman 30 rotor. A cohesive sticky pellet must be scraped from the tubes and suspended by stirring vigorously overnight in the cold in 1 M NaCl, .01 M $MgSO_4$, .01 M tris pH 7.5. 2 volumes of 6 M CsCl, .01 M $MgSO_4$, .01 M tris pH 7.5 are added to give a refractive index of approximately 1.381, and the solution is centrifuged to

at 35 K in the Beckman SW41 rotor for 48 hours. The SW41 tubes are long and provide the best opportunity for clear separation of the Ø which band at a density of 1.516 (Rubenstein, 1968). The Ø can be clearly seen with the eye as a whitish translucent band. It is easy to remove gently the top layers with a tissue culture pipette, then to use a long tipped Pasteur pipette to take out the Ø. Other nucleic acids will be found at higher densities so it is critical to avoid mixing the Ø with the solution below. The suspension is dialysed against .5 M NaCl .01 M $MgSO_4$ .01 M tris pH 7.5. The phage are stored over chloroform at $4^{o}C$.

(ii) Labelling of cells for the isolation of ^{3}H T7 DNA

5 mc. ^{3}H thymidine were added to a 500 ml culture in early log phase. In mid-log phase 20 ml 10% glucose were added and it was infected with Ø at a MOI of .1. ^{3}H Ø T7 were isolated as described. Thymidine is taken up more quickly than thymine. It is added early to ensure complete incorporation. The bacterial DNA is degraded after infection, and the nucleotides are incorporated in the phage DNA (Labaw, 1951; ibid, 1953; Putnam et al., 1952). In this experiment the specific activity of the labelled DNA was 100,000 cpm per microgram.

(iii) Preparation of T7 DNA

A suspension of Ø, approximately 10^{12} per ml in 10 ml of .5 M NaCl .01 M $MgSO_4$, .01 M tris pH 7.5 is extracted

with an equal volume of freshly distilled phenol, saturated with .01 M tris pH 8.0 and adjusted to pH 8.0, by shaking gently at 4°C in a flask or beaker. The solution is extremely viscous and it is necessary to centrifuge at 30,000 g. for 20 minutes to move all of the protein to the interface. the supernatant is removed carefully with a wide-nosed tissue culture pipette and re-extracted with phenol. This is followed by three extractions with di-ethyl ether to remove the phenol and more protein before dialysing two times against 6 litres of .01 M tris pH 8.0 .0001 M EDTA. The final solution is stored at 4°C over chloroform. The yield of DNA is between 80% and 100%. 10^{14} Ø give approximately 4 mg DNA. It is important to keep the volume reasonably low, that is, less than 3 ml per mg otherwise the final concentration will be too low to be useful, and further manipulation to reduce the volume should be avoided to prevent shear or knicking. Phenol must be re-distilled to lower the concentration of quinones, products of oxidation of phenol, which may cause single stranded breaks in DNA. OD 260/280 should be $>$ 1.9 (Freeman and Jones, 1967; Stead and Jones, 1967) and OD 260/230 should be $>$ 2.3. The concentration of DNA was calculated assuming an $E^{1\%}_{260} = 200$. (Richardson et al., 1964). All preparations of DNA gave S20, w of 32S, and contained no low molecular weight material on UV scans of analytical runs.

(iv) Sedimentation coefficients and molecular weight of of T7 DNA

The methods used were as described by Studier, (1965). Analysis was carried out at both neutral and alkaline pH. The S20,w of native DNA was 32S. For alkaline DNA the value was 38 S. These are in good agreement with the results reported by Studier (1965) and Schmid and Hearst, (1969).

Molecular weights between 21 and 26 x 10^6 have been reported. The most recent studies have given a number of 23 x 10^6 (Schmid and Heast, 1969; Gray and Hearst, 1968) which is used in this paper.

From the alkaline sedimentation profile 70% of the DNA single strands behaved as complete, unknicked molecules, therefore 60% of the double-stranded molecules carry an average of one knick.

(v) Preparation of RNA polymerase

This was described in part II Chapter I. The enzyme preparations were analysed for ribonuclease, phosphatase, DNAase, polynucleotide phosphorylase and polyphosphate kinase as described. Total RNA synthesis was assayed in .05 M tris pH 7.9, .008 M $MgCl_2$, .0008 M $K2HPO_4$, .15 M KCl, .0001 M dithiothreitol, .0001 M EDTA, .2 mM each of ATP, GTP, UTP and CTP, and 50-100 ug/ml DNA. at 37° for 10 minutes. Collection of the TCA precipitate is described in Part II Chapter I. Initiation of RNA synthesis on T7 DNA was assayed by following the incorporation of $\gamma\ ^{32}P$ ATP and

γ ^{32}P GTP into acid precipitable form under two conditions, low and high salt. The low salt incubation medium was as described above except the KCl concentration was .06 M. For the high salt medium the KCl concentration was .15 M and the $MgCl_2$ was raised to .028 M. Both contained .08 mM ADP to inhibit polyphosphate kinase, and .0008 M K_2HPO_4 was present to inhibit polynucleotide phosphorylase.

Two enzyme preparations were used in this work, one having a specific activity of 850 units per mg (DCD) and the other used less with a specific activity of 350 (GGC). Figures 5 and 7 (Part II, Chapter I of this thesis) show photographs of SDS-polyacrylamide gels of these enzymes. The preparation DCD had a very low amount of DNAase activity. At high concentrations of enzyme (220 µg/ml) and SV-40 DNA (100 µg/ml), 50% of the SV40 DNA molecules were unknicked after 20 minutes at 37°C. This represents an average of .69 knicks per DNA molecule. GGC was not measured.

(vi) Growth of E. coli Bb for the isolation of labelled RNA polymerase

E. coli Bb was obtained from the collection of Professor W. Wood. It grows well on minimal medium m9, yielding approximately 2 g cells wet weight per litre. The medium contains 7 g Na_2HPO_4, 3 g KH_2PO_4, and 1 g NH_4Cl in one litre to which are added 40 ml 10% glucose, 1 ml of 1 M $MgSO_4$, 25.6 ml 25% NaCl, .1 ml .01 M $FeCl_2$, and .2 ml .5 M $CaCl_2$. In order to reduce the manipulations of ^{14}C labelled

cells a 15 litre cold culture was grown in parallel with one of 500 mls to which .85 millicuries of ^{14}C amino acids were added in early log phase. Cells were grown to late log phase and collected by centrifugation at 10K for 10 minutes. The labelled and unlabelled cells were mixed, and RNA polymerase was isolated according to the methods described in Part II Chapter II. 38 g of cells gave approximately 2 mg of RNA polymerase of specific activity 560 units per ug, after DNA cellulose chromatography. It is clear in retrospect that RNA polymerase represents a higher proportion of the cellular proteins at early log phase and a more efficient use of isotope would be obtained if a large quantity of cells was collected in stationary phase and then put in fresh medium. As soon as logarithmic growth started the isotope should be added, the cells would be harvested in mid log phase and the enzyme isolated.

(vii) Sedimentation analysis of complex formation

T7 DNA has an S20,w of 32.0 ± .8 (Studier, 1965; Gray and Hearst, 1968). It sediments more rapidly through a 10-30% glycerol gradient in .4 M $(NH_4)_2SO_4$ than RNA polymerase which has an S20,w of 13S under these conditions (Richardson, 1966,b). RNA polymerase/T7 DNA complex is not separated from the free T7 DNA but is well separated from free RNA polymerase. The most convenient rotor to use is the Beckman SW-41. 6 tubes may be run, each containing 12.5 ml glycerol gradient, 10-30% .4 M $(NH_4)_2SO_4$, .01 M tris pH 7.9,

.0001 M EDTA, .01 M $MgCl_2$, .0001 M dithiothreitol (buffer S). Up to .5 ml of sample can be layered gently on top, and then covered with a thin layer of paraffin oil to prevent evaporation and surface denaturation. Sedimentation is for 12 hours at 41 K at 4°C, not using the brake at the end of the run. The tubes are then dripped in the cold, to give 30 fractions. Optical density at 230, 260 and 280 mμ is read, and they are assayed for RNA polymerase activity adding excess T7 DNA and taking care to reduce the concentration of ammonium sulphate to less than .07 M. If complex is present two peaks of activity will be observed, one coinciding with a fast peak of OD 260 near fraction 16, and the other with a slower peak of OD 230 near fraction 23. Unless the final salt concentration of the assay is reduced to.07 M ammonium sulphate, the amounts of activity in the two peaks are not comparable. Above .07 M the rate of synthesis for uncomplexed enzyme falls off very quickly, becoming zero at .2 M.

(viii) Millipore filter method for assaying complex formation.

The method was developed by Jones and Berg (1966), and has been modified by Anthony et al., (1966) and Hyman and Davidson, (1970). Millipore Filters type HAWP, pore size .45μ have the ability to adsorb a complex of RNA polymerase and DNA, but do not bind either DNA or enzyme alone. The amount of cold enzyme bound can be monitored by measuring

the enzyme activity retained on the filter, or by the amount passing through. A more convenient and accurate method measures the amount of labelled polymerase on the filter. Labelled DNA can be used to assay the retention of nucleic acid. Enzyme and DNA are mixed in appropriate proportions and reaction mixture. Filters (25 mm in diameter) are soaked in buffer for at least 10 minutes at room temperature, then washed with 10 ml of buffer. The sample is applied and filtered at a rate of 5 ml per minute. The filters are washed with 5 x 10 ml volumes of buffer, dried at room temperature for 15 minutes and at 80°C in vacuo for 10 minutes, then counted in a Beckman Scintillation Counter. Non-specific binding of ^{3}H T7 DNA (10 μg) in a control lacking enzyme is less than 2% of input.

A complex between RNA polymerase and DNA can be formed at low salt $\tau/2$ .2 and is relatively stable. It will be retained on Millipore membranes provided the salt concentration of the washing buffer is not greater than .1. However if the salt concentration is raised most of it is dissociated and eluted. A salt stable complex is assayed by washing the filters with .4 M $(NH_4)_2SO_4$, .05 M tris pH 7.9, .004 M $MgCl_2$, .0001 M EDTA, .0001 M dTT.

The preparation of ^{14}C RNA polymerase was used to check binding of the enzyme alone to the filters. Before chromatography on DNA-cellulose the enzyme, or a contaminant, stuck strongly (> 80%) to the filters at a range of ionic strengths.

After chromatography, < 1% was retained on the filters.

(ix) Assay for complex by resistance to rifampicin

RNA polymerase and enzyme are incubated at 37°C for 20 minutes in the presence of different nucleotides. The solutions are cooled on ice, and rifampicin is added at a high concentration (>100 µg/ml). The missing components are added and the reaction returned to 37°C. A time course of RNA synthesis is followed by removing 50 λ aliquots at intervals and precipitating them with 10% TCA. If an initiation complex has formed before the addition of rifampicin synthesis is observed. Rifampicin is a specific and potent inhibitor of chain initiation (di Mauro et al.,1969). Rifampicin was a gift from Prof. D. McMahon.

Streptolydigin, a second antibiotic used in some studies was a gift from the Upjohn Company, Kalamazoo, Michigan.

(x) Purification of nucleoside triphosphates

The procedure is a modification of those described by Hurlbert, et al., (1954). Dowex-1x8 is suspended in 4 M formic acid 4 M ammonium formate, washed and de-fined with this buffer and washed with glass distilled water to the pH of water. A column, 55 cm x .8 cm., is used to analyse small amounts of XTP (1-5 µmoles). The sample is dissolved in H_2O or neutral buffer of low ionic strength, applied to the column and eluted at room temperature by a linear gradient of 2 x 250 ml of 1.0 M formic acid to 2.0 M formic acid

1.0 M ammonium formate. The triphosphates come off in the order C-A-U-G and are well separated from one another. They are identified by their position of elution and their characteristic OD 280/OD 260. For preparative purposes 200 µmoles of GTP or ATP were applied to completely separate columns 2.2 cm x 22.5 cm and eluted by 2 x 500 ml of gradient from 1.5 M formic acid to 2.0 M formic acid 1.0 M ammonium formate. The XTP peak is desalted by chromatography on Dowex 50-H^+, 3 cm x 30 cm eluting with glass distilled water. The resin turns yellow as it is converted to the NH_4^+ form. The formic acid is removed from the eluate by lyophilisation. The Dowex 50 resin should be well washed with 4 M formic acid 4 M ammonium formate, followed by 4 N HCl until all NH_4^+ is removed and then with H_2O to neutral pH. Unless this is done the resin will yield contaminating material of high OD 230.

(xi) Oligonucleotide analysis

This is carried out according to Hatlen et al., (1969), by chromatography on a DEAE Sephadex A-25 column. The resin (30 g) is suspended in 3 l H_2O, stirred overnight, de-fined several times and filtered through Whatman No.5 paper. It is washed twice with 250 ml 95% ethanol and once with 200 ml of 100% ethanol. It is dried in a dessicator and stored there. The resin is suspended in .02 M tris pH 7.6, 7 M urea, and decanted three times, before pouring the column, 58 cm x .6 cm. The radioactive sample is made .02 M tris

pH 7.6, 7 M urea, 5 mg of soluble RNA (Nutritional Biochemicals) pre-digested for 30 minutes at 37°C with 40 ug of pancreatic ribonuclease are added, it is applied to the column and eluted with 300 ml of a linear gradient from zero to .4 M NaCl in .02 M tris pH 7.6, 7 M urea. Peaks are resolved up to the hexanucleotides. 1 ml aliquots of fractions are counted in the presence of 1 ml NCS and 10 ml of toluene scintillation fluid. Nucleotides are separated primarily on the number of phosphate groups but inorganic phosphate, XDP and XTP run slightly ahead of the mononucleotide (sp) dinucleotide (xpxp), and trinucleotide (xpxpxp) peaks respectively (Hatlen et al., 1969; Stevens, 1969)

RESULTS.

(i) The requirement of σ for the transcription of T7 ascites tumour and E. coli DNA.

In order to determine if σ is required for the initiation of transcription of T7 DNA by E. coli RNA polymerase, core enzyme was separated from the sub-unit on phosphocellulose according to Burgess (1969). The elution profile of the column and the SDS-polyacrylamide gels of the seven fractions are shown in Figures 4 and 5 of Part II Chapter I. The gels were stained with Coomassie Blue, de-stained and scanned at OD 600 in a Gilford Recording Spectrophotometer. Since the amount of stain is nearly linearly related to the amount of protein it is possible to calculate the relative amounts of protein in the different bands (de St. Groth et al., 1963; Elgin, 1970). Table 1 presents an analysis of the protein content of each band in each fraction. The molar ratios of β, α and σ were calculated assuming molecular weights of 160,000, 40,000 and 95,000 respectively (Travers and Burgess, 1969). The σ sub-unit elutes over a broad range of salt concentration, but mostly in fraction PC 2.2 which contains 46% σ and has a σ/β molar ration > 6. In contrast PC 2.6 has only .6 weight % σ and a σ/β molar ratio of $< .03$.

All of the fractions were tested for their ability to transcribe T7, ascites tumour, and E. coli DNAs (Table 2.) The reactions contained 2 µg of protein and 10 µg of DNA in

Table 1

The sub-unit composition of phosphocellulose enzymes PC2.1 to PC2.7. The fractions from the phosphocellulose column shown in Part II, Chapter I, Figure 4, were analyzed by SDS-polyacrylamide gel electrophoresis [Part II, Chapter I, Figure 5]. The gels were stained with Coomassie Blue, scanned at 260 mμ by a Gilford Spectrophotometer and the area under each peak calculated by cutting out the tracings and weighing them. The results were converted to percentages and tabulated. The molecular ratios tabulated for σ/β and α/β were calculated assuming molecular weights for β, σ and α of 160,000, 95,000 and 40,000 respectively (Travers and Burgess, 1969).

Table 1. Sub-unit composition of phosphocellulose enzyme preparations PC2.1 to PC2.7

	Weight % of each band								
Enzyme	β+β'	τ	σ	σ_1	σ_2	σ_3	σ_4	α	ω
PC2.1	27.7	7.1	12.9	-	19.2	-	6.6	15.4	11.0
2.2	24.0	1.1	45.6	-	-	-	12.1	11.0	6.0
2.3	42.1	2.1	20.5	-	-	-	15.5	14.7	5.1
2.4	58.1	-	11.4	-	-	-	4.1	20.1	5.8
2.5	67.7	-	1.8	-	-	-	-	24.2	5.3
2.6	68.3	-	.6	-	-	-	-	26.5	4.7
2.7	64.6	-	-	10.9	-	10.5	-	14.0	-
GGA	64.7	3.9	5.8	< .5	< .5	< .5	-	18.9	4.6

	Weight ratio	Molecular ratio	Weight ratio	Molecular ratio
Enzyme	σ/β+β'	σ/β	α/β+β'	α/β
PC2.1	.47	1.532	.558	4.464
2.2	1.90	6.194	.458	3.664
2.3	.49	1.597	.349	2.792
2.4	.20	.652	.357	2.863
2.5	.03	.098	.357	2.863
2.6	.008	.026	.388	3.104
2.7	-	-	.217	2.136
GGA	.09	.302	.291	2.328

Table 2

Stimulation by σ of transcription on T_7, ascites tumour and E. coli DNAs. The conditions of synthesis were .04 M tris pH7.9, 0.1M Mg Cl_2, .01M β mercaptoethanol, .8mM K_2HPO_4, .02mM ADP, .15M KC_L and .2mM each of CTP, UPT, GTP and ^{14}C ATP (2000 cpm/mμ mole) in a total volume of .25 ml. [A] Each assay contained 10 μg of the DNA and 2 μg of RNA polymerase preparation. [B] To assay the effect of σ, each preparation (2 μg) was added to sample PC2.6 (2 μg) which contained .6 weight % (2.6 molarity %) σ. [C] The stimulation is expressed as the ratio of the amount of synthesis when two enzyme preparations are added to the same reaction to the sum of the syntheses observed when each was added to a separate reaction.

Table 2. Stimulation of transcription of T_7, ascites tumour and E.coli DNAs by σ factor

[A]

Enzyme	µg added per assay	µg σ per assay (calculated)	RNA synthesis cpm TCA precipitable ^3H adenine on T_7 DNA	ascites tumour DNA	E. coli DNA
PC2.1	2.0	.258	3211	1165	591
2.2	2.1	.958	1606	522	388
2.3	2.3	.672	4341	1703	739
2.4	2.1	.239	8160	4291	1595
2.5	2.0	.039	2898	3470	2301
2.6	2.0	.012	2967	3868	3277
2.7	2.0	-	603	1088	856

[B]

µg Enzyme per assay	µg σ added	RNA synthesis cpm TCA precipitable ^3H adenine on T_7 DNA	ascites tumour DNA	E. coli DNA
2.0 µg PC2.6 + 2.0 µg PC2.1	.258	17,280	8,630	3,972
" 2.1 µg PC2.2	.958	36,591	13,643	5,112
" 2.3 µg PC2.3	.672	22,724	11,795	4,706
" 2.1 µg PC2.4	.239	16,600	9,630	5,009
" 2.0 µg PC2.5	.039	7,788	6,004	5,287
" 2.0 µg PC2.7	-	3,861	3,779	3,731

[C]

Enzymes	STIMULATION T_7 DNA	Ascites tumour DNA	E.coli DNA
PC2.1/PC2.6	2.79	1.71	1.03
PC2.2/PC2.6	8.00(8.3)*	3.10(2.8)*	1.39(1.04)*
PC2.3/PC2.6	3.24	2.12	1.17
PC2.4/PC2.6	1.49	1.18	1.03
PC2.5/PC2.6	1.32	.82	.95
PC2.7/PC2.	.92	.76	.90

*results of a second different experiment

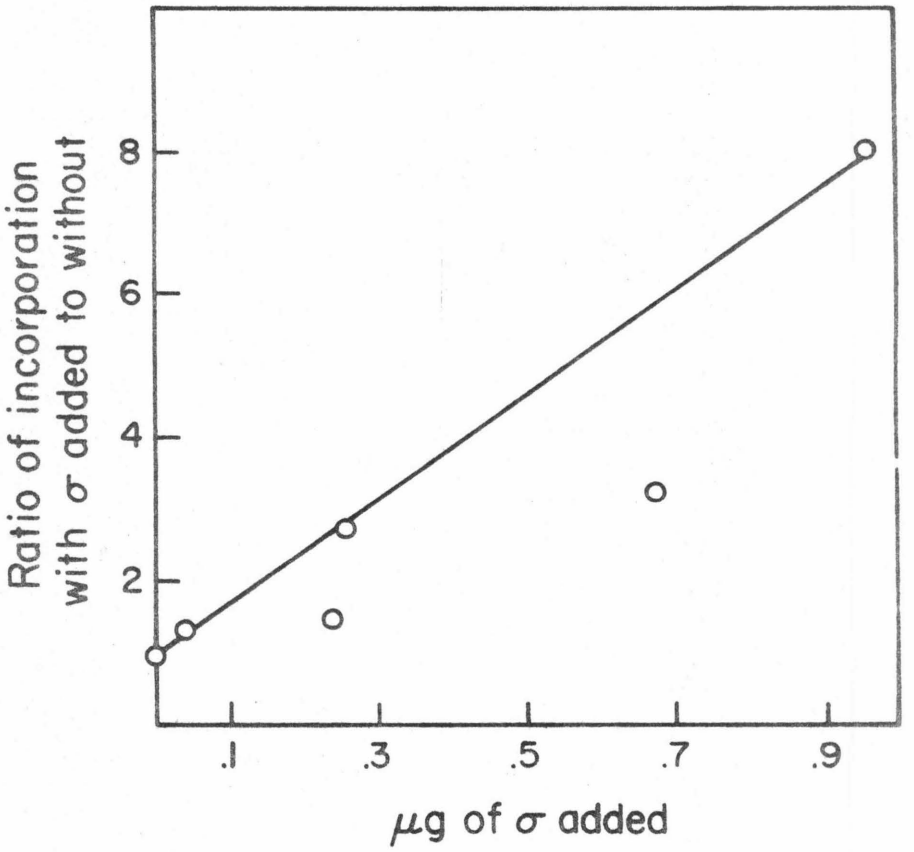
.25 ml of a high salt medium. Phosphate (.8 mM) and ADP (.02 mM) were added to inhibit polynucleotide phosphorylase (identified as σ2 on the gels) and polyphosphate kinase (identified as σ1 and/or σ3 on the gels) respectively. Each fraction from the column was assayed alone, and each in combination with PC 2.6 which has almost no σ sub-unit, to test which fractions stimulated it to transcribe T7 DNA. The original enzyme preparation GGC (before phosphocellulose chromatography) was also assayed. The results are shown in Table 2 and Figure 1. PC 2.6 is stimulated by every fraction except PC 2.7 which showed no σ on SDS-polyacrylamide gels. The amount of stimulation is proportional to the amount of σ added as shown in Figure 1.

The specific activity of PC 2.6 on T7 DNA in the absence of σ is very low (approximately 30 units per mg) although it is clearly a very pure enzyme preparation. When σ (.96μg) is added to PC 2.6 (2 μg) the specific activity expressed per mg of core enzyme increases to 370. We conclude that σ stimulates the transcription of T7 DNA by core enzyme and that considering the very low level of synthesis when it is completely absent (PC 2.7 specific activity 8 units per mg) it is a required co-factor in the reaction.

The activity of the enzyme is always greater on T7 DNA than on ascites tumour or E. coli DNA when σ is present and nearly equal when σ is completely absent (PC 2.7). The ratio of incorporation on T7 DNA to that on ascites DNA is

Figure 1

The stimulation of transcription of T_7 DNA as a function of the amount of σ added to core enzyme. The experiment was conducted as described in Table 2. The stimulation is the ratio of incorporation when σ and core [PC2.6] are present in the same assay to the sum of the incorporation when each is in a separate assay. The amount of σ added to each assay was calculated from the % σ present in each of the phosphocellulose enzymes used as a source of σ [PC2.1 to 2.5 and PC2.7]. These values are given in Table 1.


Ratio of incorporation with σ added to without
8
6
4
2
.1
.3
.5
.7
.9
μg of σ added

approximately proportional to the amount of σ present, increasing to a high value of 3 to 4. Ascites DNA is a better template for E. coli RNA polmerase when σ is present suggesting that σ-specific initiation sites exist on the DNA of higher organisms. This is supported by the wealth of evidence that E. coli RNA polymerase does transcribe the sense strand of animal virus DNA and higher organism DNA (Bekhor et al., 1969; Reeder and Brown, 1970; Westphal, 1970; Tan and Miyagi, 1970; Kates and Beeson, 1970).

In contrast E. coli DNA is always a poor template for E. coli RNA polymerase whether or not σ is present. In the absence of σ it is about as good a template as ascites or T7 DNA, but there is almost no stimulation where σ is added. This is somewhat unexpected, and leads to the conclusion that there are few, if any, σ specific initiation sites on E. coli DNA. Recently Kamen and Travers (1970) have reported the discovery of another polypeptide factor ψ, coded for by the E. coli genome, which is associated with the $\varphi\beta$ RNA polymerase, and which stimulates E. coli RNA polymerase to transcribe the ribosomal genes on E. coli DNA when σ is present.

(ii) <u>The effects of rifampicin and streptolydigin on the activity of RNA polymerase</u>.

Rifampicin and streptolydigin are specific inhibitors of E. coli RNA polymerase (Sippel and Hartmann, 1968; Schleif, 1969; Chamberlin et al., 1970). Rifampicin is effective at

low concentrations, 99% of RNA synthesis being blocked by .4 µg/ml. However, as shown in Figure 2., the remaining 1% of activity is much less sensitive to rifampicin, and unless very high concentrations are used there will always be some "leakiness". The order of addition of the XTP and RIF has little effect. If the nucleoside triphosphates are added at zero degrees, immediately before the rifampicin instead of being added simultaneously, the amount of resistant synthesis is the same. Leakiness probably represents the small number of molecules of enzyme which are in the correct configuration at specific initiation sites and which by chance interact with nucleotides before exposure to rifampicin. As shown in Figure 3, once the enzymes have initiated synthesis they are resistant to rifampicin. In this experiment the modes of action of streptolydigin and rifampicin were compared. Schleif (1969) had reported that streptolydigin, as chromomycin A3 (Sippel and Hartmann,1968) and cordycepin (Sentenac et al., 1968) inhibits chain propagation by E. coli RNA polymerase. Figure 4 shows the results of an experiment similar to that of Schleif (1969). The two antibiotics were added at zero time or at the times indicated in the figure. Aliquots of 50 λ each were precipitated with TCA at the times shown and the precipitates collected and counted as usual. The addition of streptolydigin $.44 \times 10^{-4}$ M after synthesis had begun caused propagation to cease 20-30 seconds later (curves 3 and 4 in Figure 3). In

Figure 2

Inhibition of transcription of T_7 DNA by Rifampicin. RNA polymerase activity was measured for 20 µg of enzyme (850 units/mg), 10 µg of T_7 DNA in .25 ml .05M tris pH7.9, .008M Mg CL_2, .8mM K_2HPO_4, .15M KC_L, .1mM EDTA, .1mM dithiothreitol, .2mM each of ATP, CTP, UTP and ^3H GTP (5000 cpm/mµ mole), incubating for 10 minutes at 37°C. TCA precipitable counts were measured as usual. The reactions contained between zero and 160 µg/ml rifampicin. The rifampicin was added before the nucleotides which were added last.

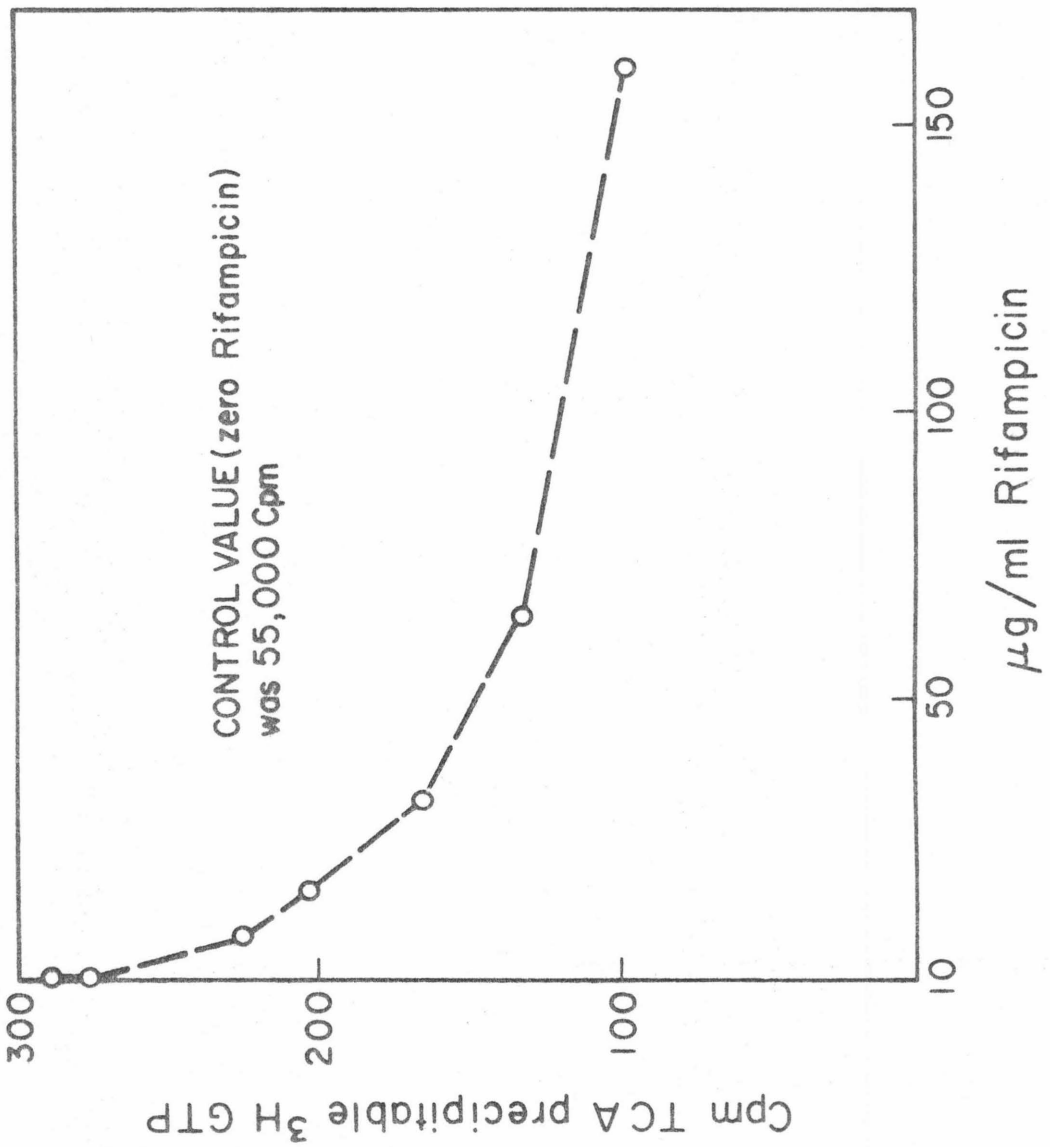
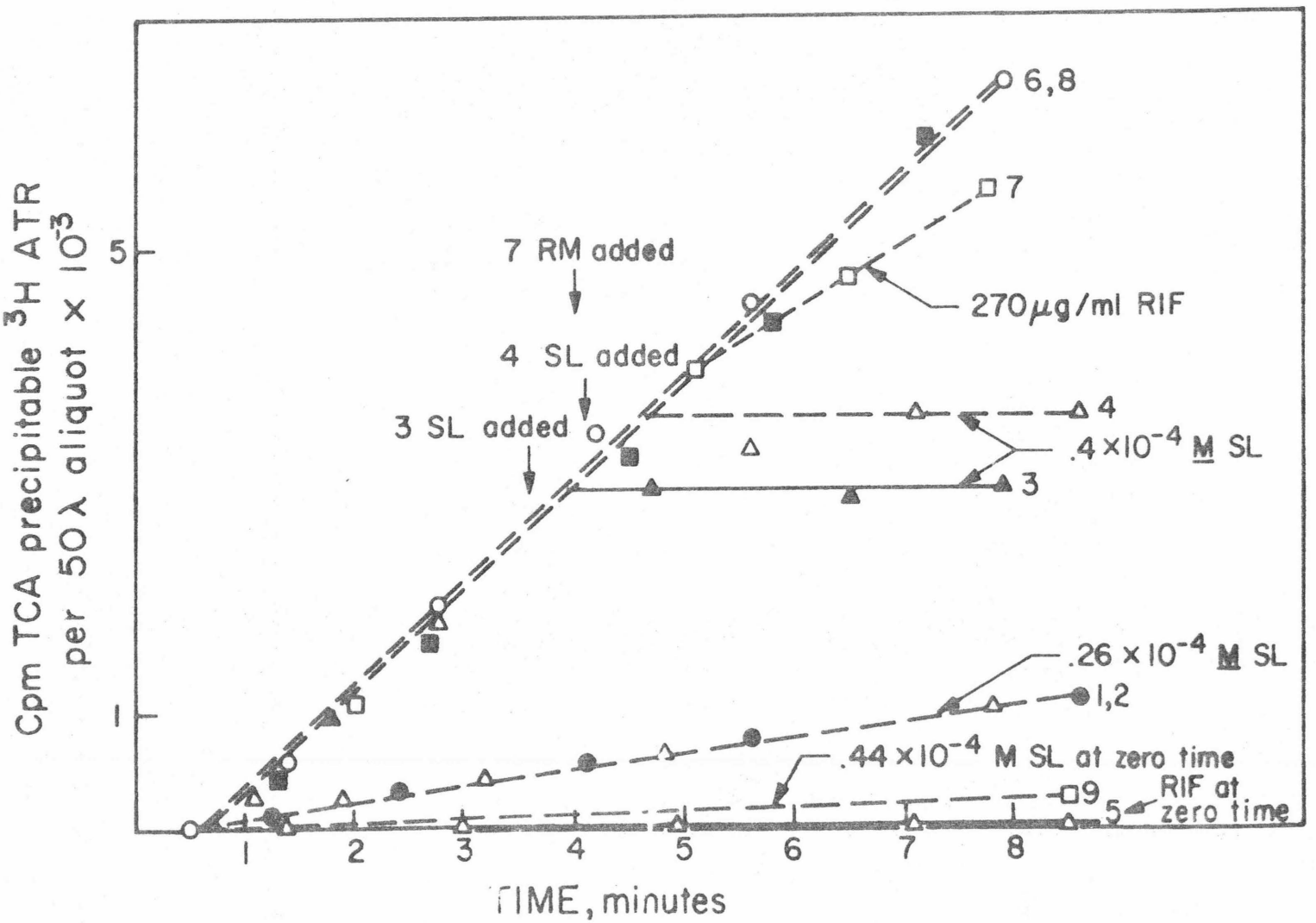

CONTROL VALUE (zero Rifampicin)
was 55,000 Cpm
300
200
100
10
50
100
150
μg/ml Rifampicin
Cpm TCA precipitable ³H GTP

Figure 3

The effect of streptolydigin and rifampicin on RNA synthesis RNA polymerase [5 µg GGC] was assayed in the presence of 44 µg of T_7 DNA in .25 ml .05M tris pH7.9, .004M Mg CL_2, .1mM EDTA .1mM dithiothreitol, .008M K_2HPO_4 .15M KC_L, .02mM ADP, .2mM each of CTP, UTP, GTP and ^3H ATP (13,000 cpm/mµ mole) at 37°C. Aliquots of 50λ were removed at various times and TCA precipitable counts per minute were assayed. 9 reactions were set up, and rifampicin, or streptolydigin was added at the times and concentrations shown. Samples 1 and 2 had $.26 \times 10^{-4}$M streptolydigin added at zero time. Samples 3 and 4 had $.4 \times 10^{-4}$M streptolydigin added at 3.6 and 4.1 minutes after the start of the reaction. Rifampicin (160 µg/ml) was added at zero time to Sample 5, and [210 µg/ml] at 4 minutes to Sample 7. Streptolydigin [$.44 \times 10^{-4}$M] was added at zero time to Sample 9. No antibiotics were present in Samples 6 and 8.


Cpm TCA precipitable ^{3}H ATR per 50λ aliquot × 10^{-3}
5
1
6,8
7
7 RM added
270μg/ml RIF
4 SL added
3 SL added
4
.4×10^{-4} M SL
3
.26 × 10^{-4} M SL
1,2
.44 × 10^{-4} M SL at zero time
9
5
RIF at zero time
1
2
3
4
5
6
7
8
TIME, minutes

contrast, rifampicin (120 µg/ml only caused a gradual reduction in synthesis if added 4 minutes after initiation (curve 7, Figure 3). This is consistent with the theory that rifampicin does not inhibit enzymes in the course of propagation but does after they terminate and before re-initiation. If rifampicin (80 µg/ml) is added at zero time no synthesis occurs (Curve 5). Some synthesis does occur when $.26 \times 10^{-4}$ M streptolydigin is added at zero time, and this is reduced by about 4 times at $.44 \times 10^{-4}$ M which is the concentration at which propagation was stopped in samples 3 and 4.

(iii) Co-sedimentation of RNA polymerase and T7 DNA in high salt glycerol gradients.

Sedimentation through high salt glycerol gradients has been used as a method to detect the RNA polymerase-T7 DNA complex. It is much less convenient than the Millipore assay, but permits recovery and further analysis of the complex. It is not as useful for quantitative analysis since many steps are involved in the preparations of the samples, all of which lead to errors.

The formation of the complex requires the presence of nucleoside triphosphates and is sensitive to rifampicin (Figure 4). RNA polymerase (100 µg) and T7 DNA (100 µg) were incubated in .25 ml .05 M tris, .004 M $MgCl_2$, .15 KCl, .0001 M EDTA and .0001 M dithiothreitol for 20 minutes at 37°C. Three samples contained (i) nothing added (ii) ATP and GTP (.2 mM each) or (iii) ATP, GTP, and rifampicin

Figure 4

Co-sedimentation of RNA polymerase activity and T_7 DNA through high salt glycerol gradients. RNA polymerase [100 µg, GGC 350 units/mg] and T_7 DNA [100 µg] were preincubated as described in RESULTS, Section (iii). Three samples contained (i) no nucleotides, (ii) ATP and GTP (.2mM each) or (iii) ATP + GTP + Rifampicin (40 µg/ml). After preincubation they were sedimented through 10-30% glycerol gradients (8 ml) with a 75% glycerol shelf 4 ml all in Buffer S (.4M$(NH_4)_2SO_4$, .01M Mg CL_2, .1mM EDTA, .1mM dithiothreitol .01M tris pH7.9) for 17 hours at 41K at 4°C. Fractions were collected and assayed for OD_{260}, OD_{230} and RNA polymerase activity, making certain to reduce the $(NH_4)_2SO_4$ concentration below .07M. Excess (70 µg/assay) ascites DNA was added to each assay.

Figure 4(a) No nucleotide in preincubation

Figure 4(b) ATP and GTP in preincubation

Figure 4(c) ATP, GTP and Rifampicin in preincubation

Figure 4(a)

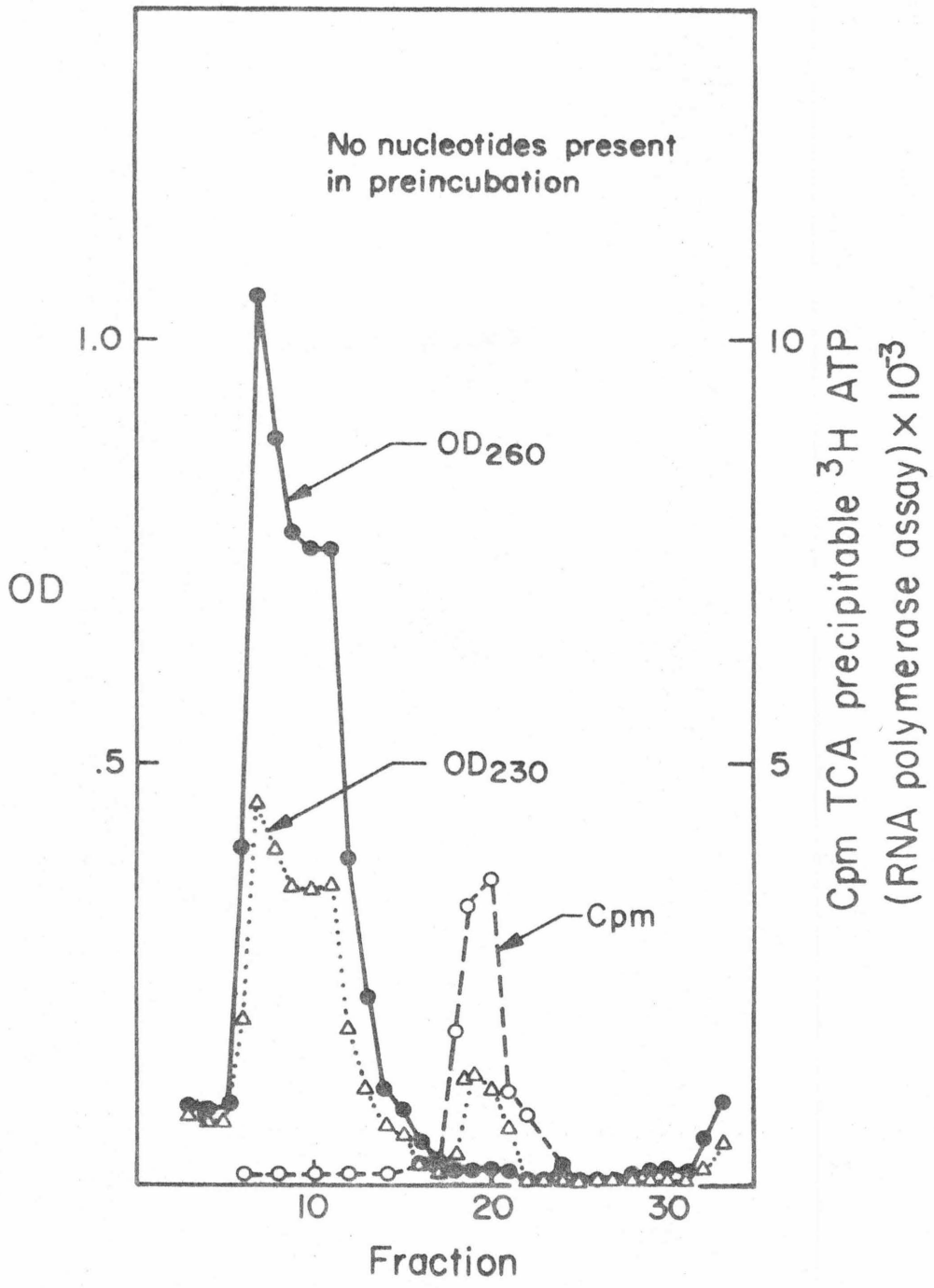

No nucleotides present
in preincubation
1.0
.5
OD
OD260
OD230
Cpm
10
5
Cpm TCA precipitable 3H ATP
(RNA polymerase assay) X 10-3
10
20
30
Fraction

Figure 4(b)

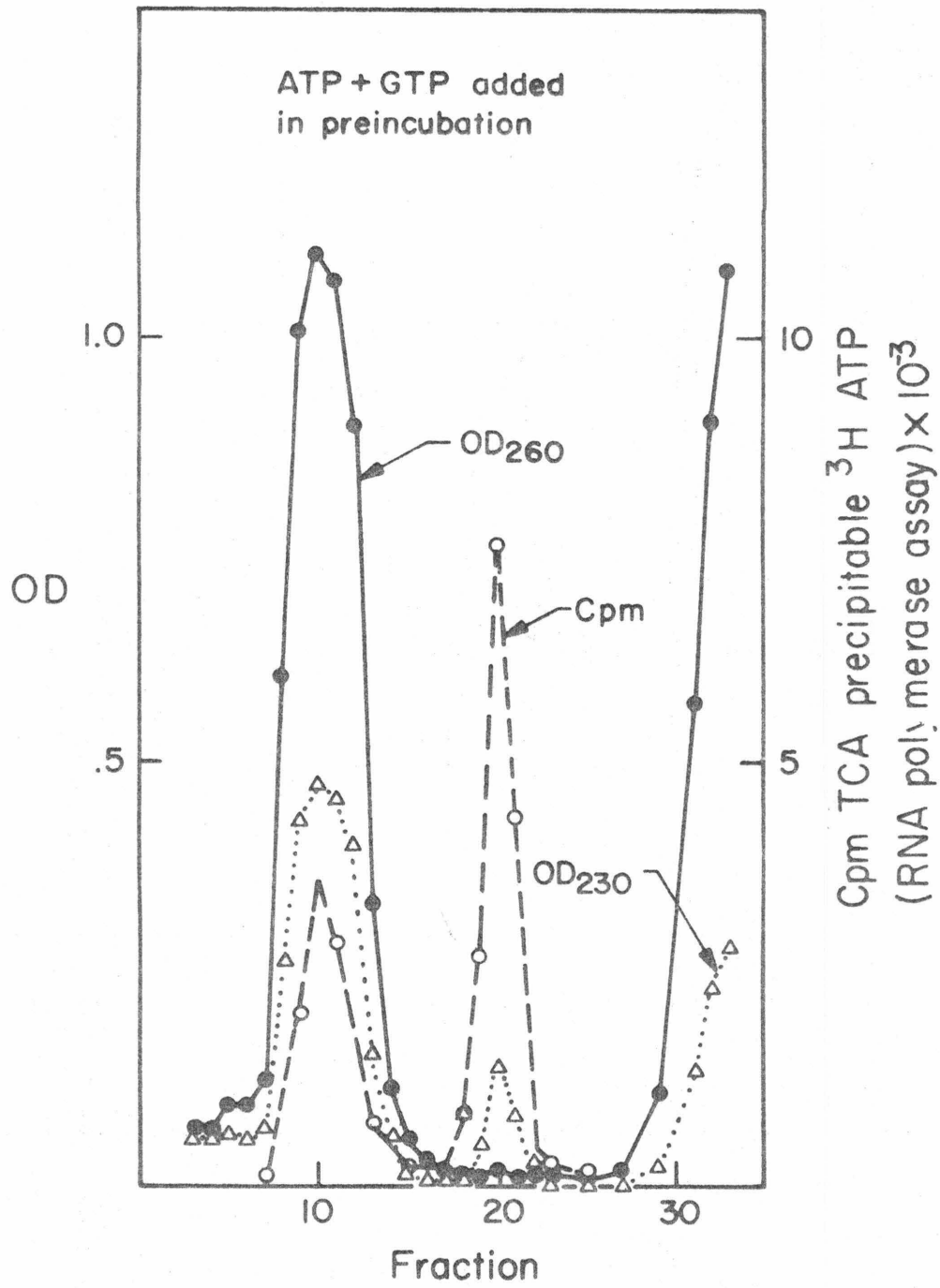
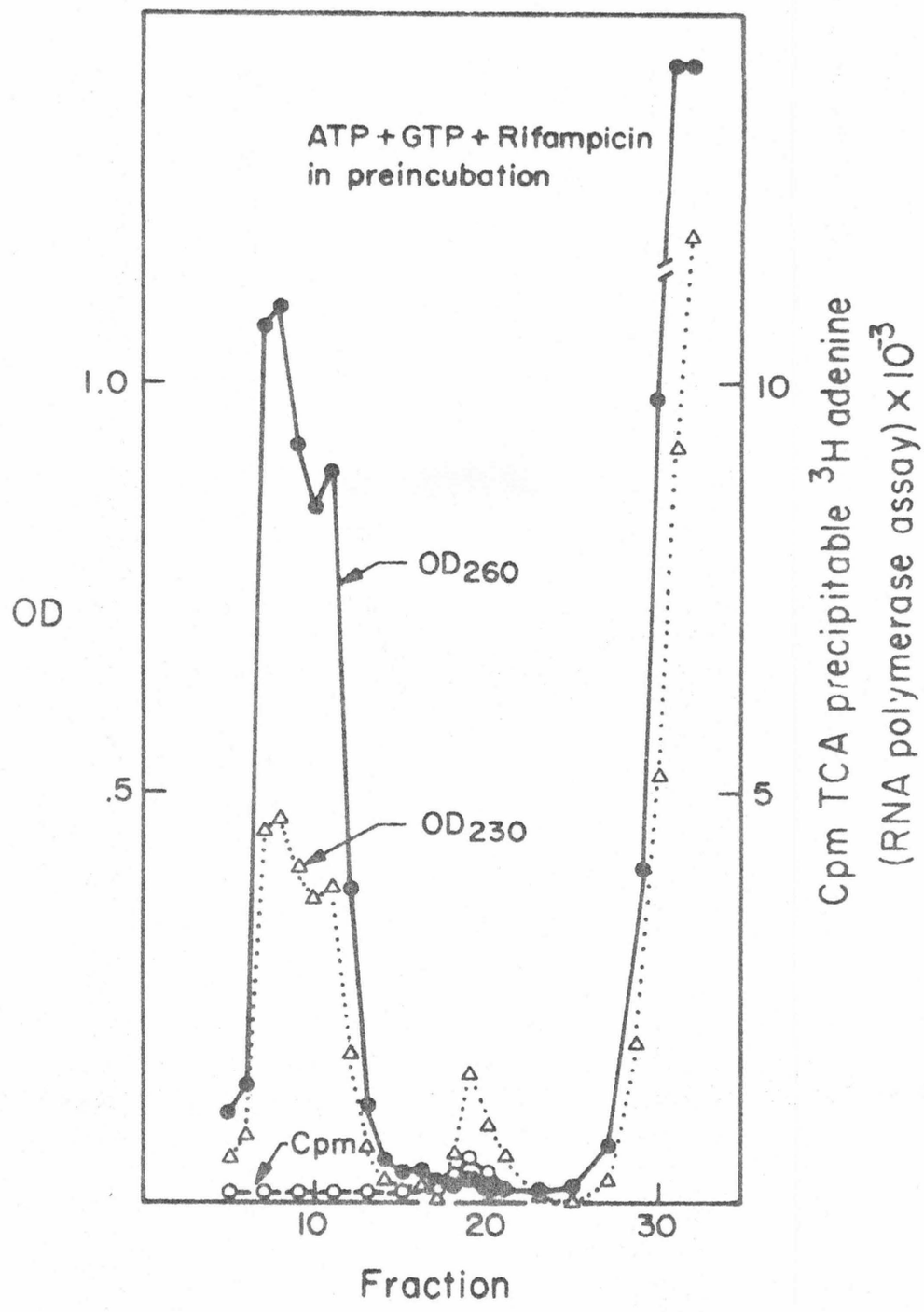

ATP + GTP added
in preincubation
1.0
.5
OD
10
5
OD260
Cpm
OD230
10
20
30
Fraction
Cpm TCA precipitable 3H ATP
(RNA polymerase assay) X 10-3

Figure 4(c)


ATP + GTP + Rifampicin
in preincubation
OD260
OD230
Cpm
OD
1.0
.5
10
5
10
20
30
Fraction
Cpm TCA precipitable ³H adenine
(RNA polymerase assay) × 10⁻³

(40 μg/ml). After incubation they were cooled to 4°C, applied to glycerol gradients and sedimented at 41 K for 17 hr at 4°C. The gradients were composed of a 4 ml shelf of 75% glycerol in .4 M $(NH_4)_2SO_4$, .01 M $MgCl_2$, .01 M tris pH 7.9, .0001 M EDTA, .0001 M dithiothreitol, and 8 ml of 10-20% glycerol in the same buffer (buffer S). The sedimentation coefficient of T7 DNA displays a strong concentration effect (Studier, 1965), so that unless a shelf is present to concentrate the leading edge of the DNA it becomes very dilute. The shelf permits the recovery of the DNA in 2-2.5 ml of solution. The free enzyme remains well separated from the DNA. The fractions were collected, analysed for OD260 and OD 230 and enzyme activity in the presence of excess ascites DNA. The DNA formed a very sharp band centered at fraction 10, while the enzyme represented by a prominent peak of OD 230 was recovered around fraction 20. The enzyme activity in sample 1 coincided with the peak of OD 230, while in sample 2(which contained ATP and GTP) it was equally divided between the DNA peak and the free enzyme peak. Almost no activity was recovered from sample 3. Rifampicin is almost irreversibly bound to E. coli RNA polymerase so that this is as expected provided no enzyme molecules had initiated on the T7 DNA during preincubation and co-sedimented with the DNA.

It is only necessary to add a single nucleoside triphosphate in order to form the salt stable complex (Figure 5).

Figure 5

Co-sedimentation of RNA polymerase and T_7 DNA through high salt glycerol gradients--effect of single nucleotide triphosphates. The conditions of the experiment were exactly as that described in Figure 4 except that 100 µg of enzyme [DC.D], 850 units/mg were used per assay. Four assays contained (i) ATP, (ii) GTP, (iii) CTP, (iv) UTP, each at 1mM. After sedimentation, fractions were assayed for RNA polymerase activity in the presence of 70 µg of ascites DNA, and ^3H ATP [20,000 cpm/mµ mole] as usual. The DNA peak sedimented between fractions 10 and 12 and free enzyme between 20 and 25 from the OD_{260} and OD_{230} profiles. Sedimentation conditions as in Figure 4.

Figure 5(a)

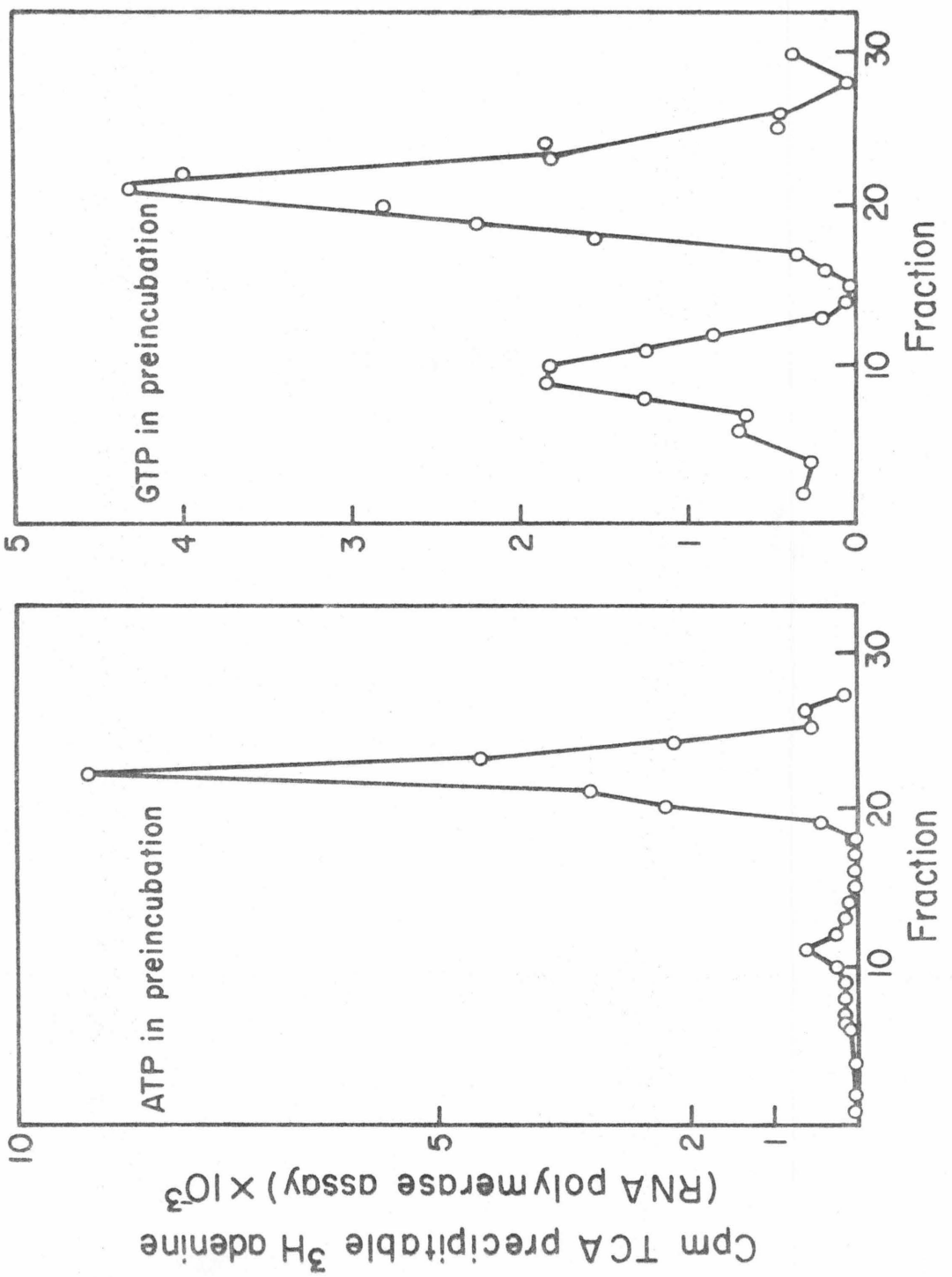
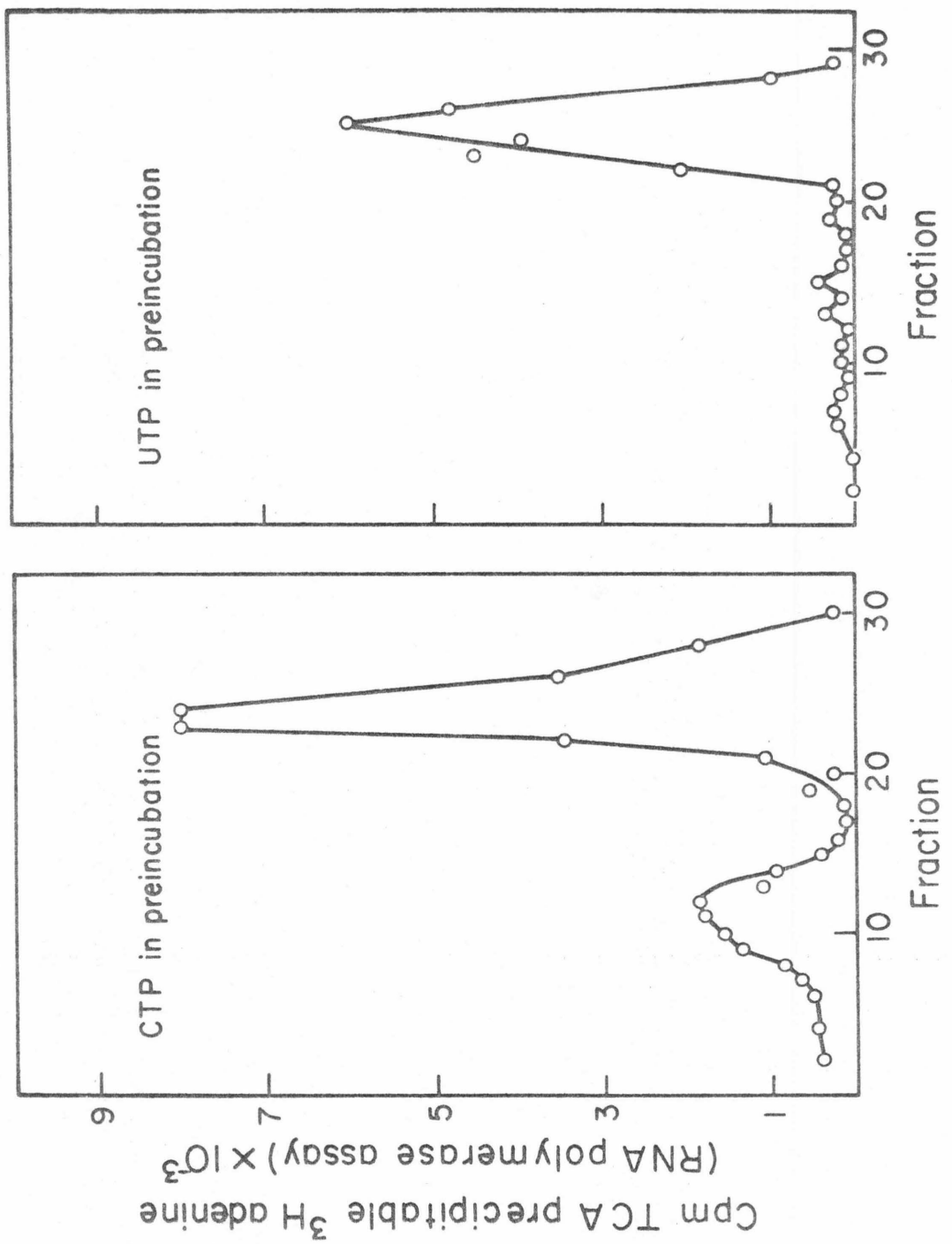

Cpm TCA precipitable ³H adenine
(RNA polymerase assay) × 10³
ATP in preincubation
GTP in preincubation
Fraction
Fraction

Figure 5(b)


Cpm TCA precipitable ^{3}H adenine
(RNA polymerase assay) × 10^{-3}
1
3
5
7
9
CTP in preincubation
UTP in preincubation
10
20
30
Fraction

The conditions were identical to those in the previous experiment except that the shelf in the glycerol gradient was 4 ml of 50% glycerol in buffer S per tube. Either GTP or CTP will cause the formation of a salt stable complex between the enzyme and T7 DNA since only for these cases is there a significant second peak of enzyme activity co-sedimenting with the DNA. Table 3 shows the % enzyme activity in second peak for each nucleotide. This assay is not quantitatively so reliable since it is found that total recovery of enzyme activity from the tubes is not reproducible (Table 3). The values were expressed as percentages to reduce the error caused by this factor.

If RNA polymerase is incubated with T7 DNA in the presence of ^{3}H GTP as the only nucleotide, some ^{3}H GTP cosediments with the enzyme DNA complex (Figure 6). The amount of ^{3}H GTP in the complex is increased if ATP is added, and still further if ^{3}H GTP, UTP and CTP are present. No counts are found in the DNA peak if rifampicin is added to the reaction. As the amount of enzyme added to the reaction is increased the amount of ^{3}H GTP bound to the complex rises (Table 4).

It is evident from these experiments that the formation of the complex depends on the presence of either GTP or CTP and that for the case of GTP it contains the nucleotide in a salt-stable association. The amount of GTP bound is small, in the region of 1 μmole per μmole of DNA.

Table 3

The recovery of RNA polymerase activity from high salt glycerol gradients after preincubation with a single nucleotide. The fractions from the gradients shown in Figure 5(a)(b)(c)(d) were assayed for RNA polymerase activity. The sums of the cpm of TCA precipitable ^{3}H AMP for each assay were calculated for each peak in each tube and tabulated here. The cpm recovered in the DNA peak were expressed as a % of the total recovery.

Table 3. Recovery of RNA polymerase activity from high salt glycerol gradients after preincubation with a single nucleotide

Nucleotide	cpm Total activity recovered	cpm Activity in DNA peak	% of total	Normalized
CTP	43,000	11,850	27.5	93
GTP	30,000	8,800	29.3	100
UTP	28,650	-	-	-
ATP	25,500	1,500	6.0	20.4

Figure 6

Co-sedimentation of ^{3}H GTP and T_7 DNA through high salt glycerol gradients after preincubation with RNA polymerase. RNA polymerase [65 µg DC.D] T_7 DNA [100 µg] and ^{3}H GTP [.2mM, 800,000 cpm/mµ mole] were preincubated in .25 ml .05M tris pH7.9, .15M KC_L, .008M Mg CL_2, .8mM K_2HPO_4, .1mM EDTA, .1mM dithiothreitol for 20 minutes at 37°C before sedimentation. 4 samples contained (a) GTP alone, (b) GTP + ATP, (c) GTP + CTP + UTP, (d)GTP + Rifampicin (160 µg/ml). All nucleotides were .2mM. Sedimentation was for 18 hours at 4.K at 4°C, and the gradients were 8 ml of 10-20% glycerol, 4 ml 75% glycerol shelf all in buffer S (see Figure 4). After sedimentation alternate fractions were read for OD or collected directly into scintillation vials and counted in an NCS/Toluene mix.

Figure 6(a)

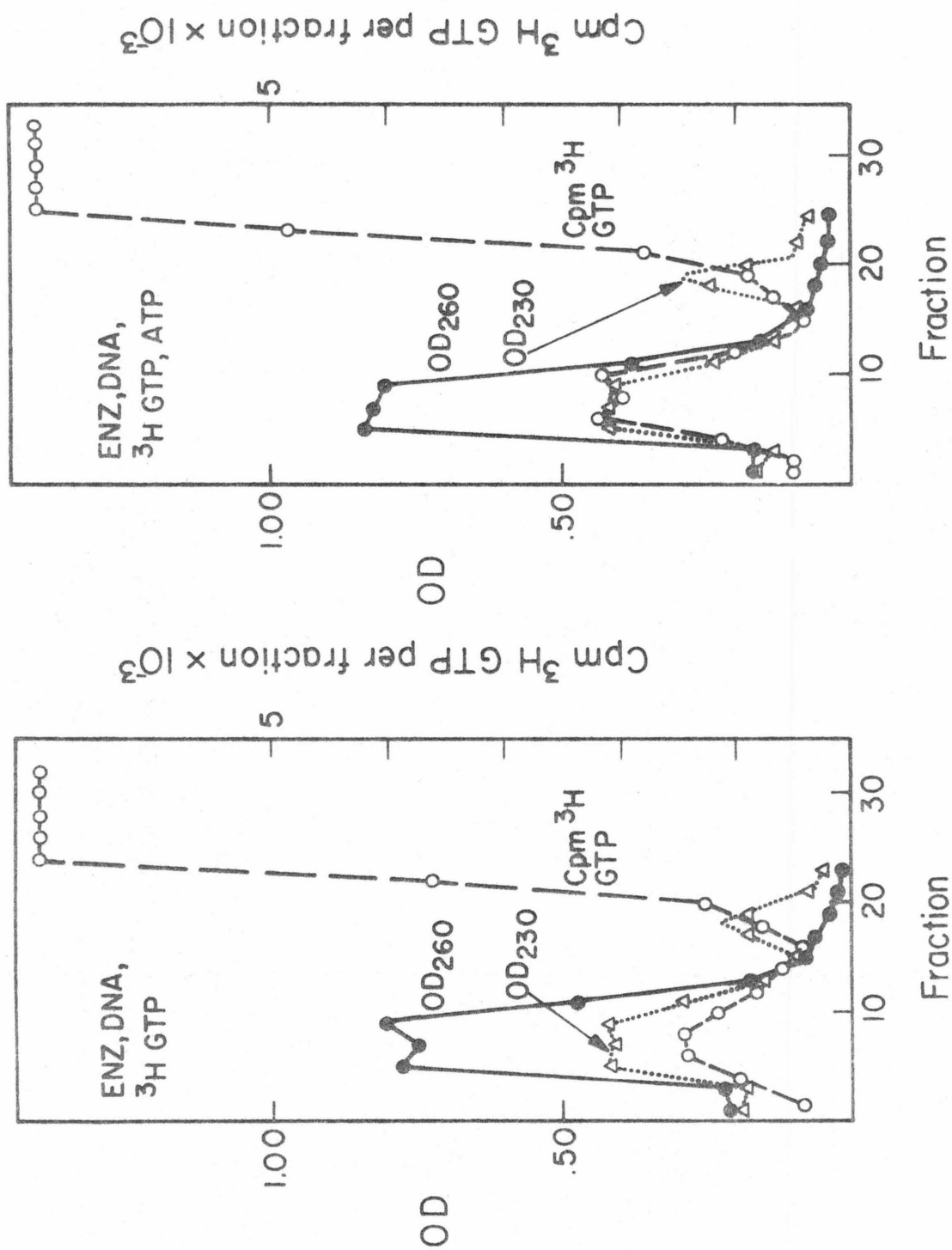

Cpm ³H GTP per fraction x 10³
5
ENZ, DNA, ³H GTP, ATP
Cpm ³H GTP
OD260
OD230
1.00
.50
OD
10
20
30
Fraction
Cpm ³H GTP per fraction x 10³
5
ENZ, DNA, ³H GTP
Cpm ³H GTP
OD260
OD230
1.00
.50
OD
10
20
30
Fraction

Figure 6(b)

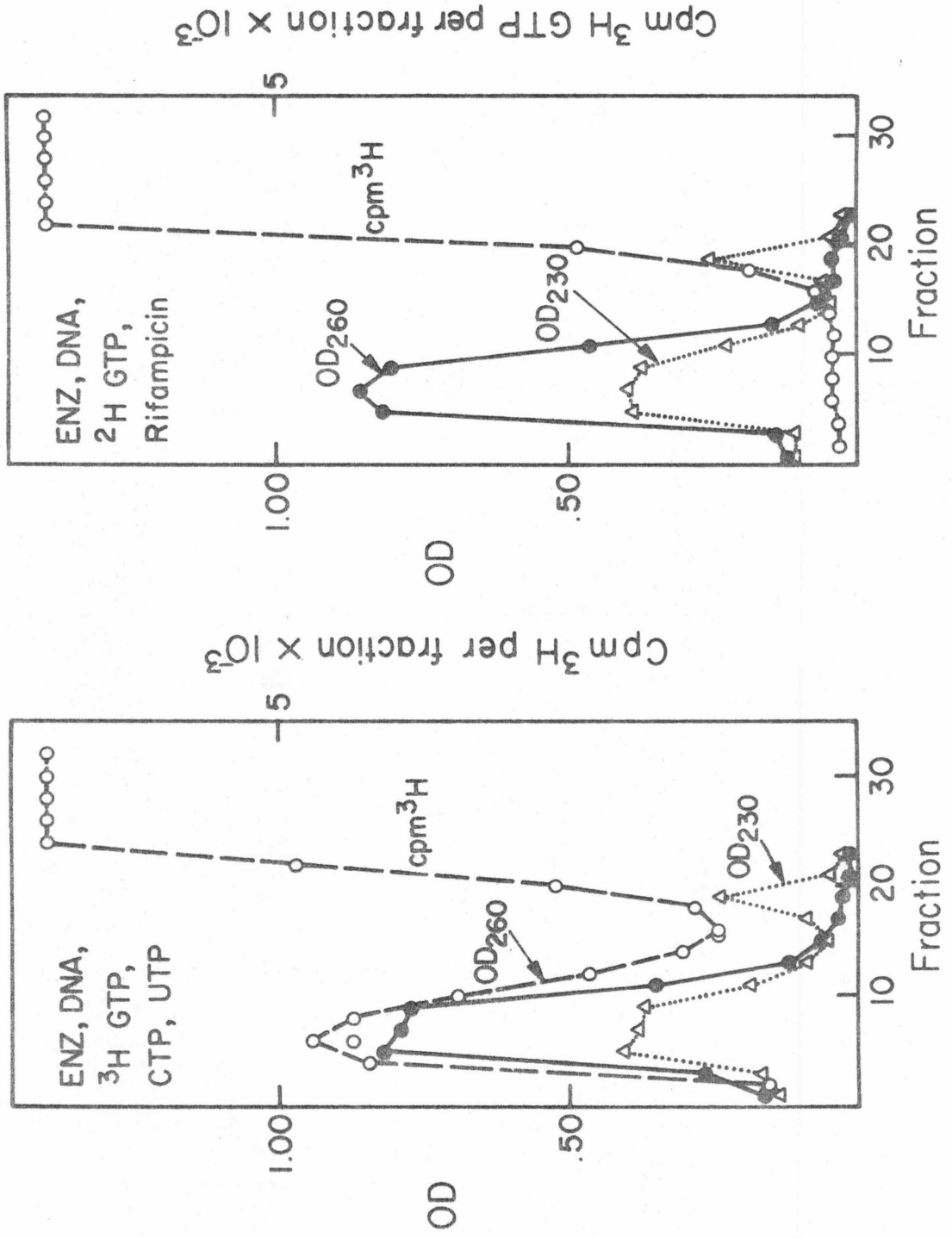

Cpm ³H GTP per fraction x 10³
5
ENZ, DNA,
2H GTP,
Rifampicin
cpm³H
OD260
OD230
1.00
.50
OD
10
20
30
Fraction
Cpm ³H per fraction x 10³
5
ENZ, DNA,
3H GTP,
CTP, UTP
cpm³H
OD260
OD230
1.00
.50
OD
10
20
30
Fraction

Table 4

Co-sedimentation of ^{3}H GTP and T_7 DNA as a function of enzyme added. RNA polymerase, ^{3}H GTP .2mM (3.35×10^{5} cpm/mμ mole) and T_7 DNA (100 μg) were preincubated under the conditions described in Figure 6. Four samples contained increasing amounts of RNA polymerase from 2.1 to 21.0 μg. A fifth sample contained CTP (.2mM) and UTP (.2mM) in addition to ^{3}H GTP. The reactions were carried out in .25 ml at 37°C for 20 minutes, then cooled to 4°C and sedimented through glycerol gradients as described in Figure 6. Fractions were assayed for OD 260, and every second sample counted for ^{3}H GTP. As in Figure 6, cpm of ^{3}H GTP co-sedimented with the T_7 DNA peak. The peak fractions (every second one) were combined, dialysed against 3 changes of 6 litres each of .01 M tris pH 7.9, .1mM EDTA, .1mM dithiothreitol, lyophilised and counted. The table shows the numbers of T_7 DNA and GTP recovered after this procedure.

Table 4. Co-sedimentation of ^{3}H GTP and T_7 DNA as a function of enzyme added

Enzyme added	Nucleotides added	mμ moles of DNA recovered × 10^3	cpm ^{3}H GTP recovered	mμ moles of GTP recovered × 10^3	mμ moles GTP / mμ moles DNA
2.1	^{3}H GTP	1.38	114	.282	.2
4.2	"	1.68	242	.660	.4
8.4	"	1.94	640	1.601	.96
21.0	"	1.47	550	1.582	1.09
8.4	" + CTP + UTP	1.40	1414	3.326	2.97

(iv) The requirements for the formation of a salt-stable complex between E. coli RNA polymerase and T7 DNA assayed by the Millipore filter method.

The most convenient method for assaying the formation of this complex employs Millipore filters as first shown by Jones and Berg (1966), and later developed by Anthony et al., (1966) and Hyman and Davidson (1970). Neither enzyme nor DNA alone will stick to the filters while a complex between them is retained provided the buffer used to wash the filters has a low ionic strength, i.e. $\tau/2 < .1$ (Jones and Berg, 1966). A complex formed in the presence of nucleoside triphosphates is stable at $\tau/2 = 1.2$ (Anthony et al., 1966; Hyman and Davidson, 1970).

The results of such assays are shown in Figure 7 and Tables 5 and 6. Between 10 and 13 µg of ^3H T7 DNA were reacted in the presence of varying amounts of RNA polymerase up to 25 µg in .004 M $MgCl_2$, .05 M tris pH 7.9, .01 M β mercaptoethanol (or .1 mM dithiothreitol) .1 mM EDTA .0008 M K_2HPO_4, .25 ml total volume at 37°C for 20 minutes. The reactions contained various nucleoside triphosphates usually at .2 mM. At 20 minutes further reaction between the enzyme and DNA was stopped by the addition of 2 ml ice cold buffer HSBA (.4 M $(NH_2)_4SO_4$, .05 M tris pH 7.9, .004 M $MgCl_2$, .1 mM dithiothreitol), and filtered through Millipore membranes HAWP, .45 µ per size, 25 mm in diameter and washed with 50 ml of HSBA before counting.

Figure 7(a) shows the effect of adding .15 M KCl to a

Table 5(a)

Retention of T_7 DNA on Millipore filters. T_7 DNA (12.6 µg) was preincubated (37°C for 20 minutes) in the presence of increasing amounts of RNA polymerase (0-25 µg) (GGC). The reactions were carried out in .25 ml of .05M tris pH 7.9, .004M Mg Cl_2, .01M β mercaptoethanol .008M K_2HPO_4, .002mM ADP. They contained various nucleotides (.2mM each) as indicated. After 20 minutes they were made .4M $(NH_4)_2SO_4$, cooled on ice filtered through Millipores, HAWP, 25 mm, .45µ pore size and washed with some of Buffer HSBA (.4M $(NH_4)_2SO_4$, .05M tris pH 7.9, .004M Mg Cl_2, .1mM EDTA, .1mM dithiothreitol). The filters were dried exhaustively (80°C, in vacuo, 10-15 minutes) and counted in a toluene based scintillation fluid. The [B] series of results were obtained after preincubation in the presence of .15M KCL. Figure 7(a) shows a plot of these results.

Table 5(a). Retention of T_7 DNA on Millipore filters

[A]

µg DNA/ reaction	µg RNA polymerase per reaction	.15M KCL	Nucleotides	Inhibitors	% Retention
12.6	0	-	A,U,G	-	1.7
"	.125	-	A,U,G	-	2.9
"	.250	-	A,U,G	-	5.3
"	.500	-	A,U,G	-	13.2
"	1.250	-	A,U,G	-	35.9
"	2.5	-	A,U,G	-	60.3
"	25.0	-	A,U,G	-	98.9
"	2.5	-	A,U,G	Rifampicin	2.2
"	2.5	-	A,U,G	EDTA	2.1
"	2.5	-	G,U	-	27.9
"	2.5	-	A,U	-	11.5
"	2.5	-	U	-	4.5
[B]					
12.8	0	+	A,U,G	-	1.7
"	.125	+	A,U,G	-	3.0
"	.250	+	A,U,G	-	4.7
"	.500	+	A,U,G	-	8.8
"	1.250	+	A,U,G	-	28.0
"	2.5	+	A,U,G	-	58.4
"	25.0	+	A,U,G	-	103.7
"	1.25	+	G,U	-	8.7
"	1.25	+	A,U	-	5.3
"	1.25	+	U	-	3.9

Table 5(b)

The effect of σ on the retention of T_7 DNA on Millipore filters. The preincubations were carried out exactly as described for Table 5(a). KC_L was not present. Two enzyme preparations were tested: firstly core enzyme [PC2.6] which contains only 2.6 mole % σ sub-unit, and secondly a combination of PC2.6 with PC2.2 which has 45.6 weight % σ sub-unit. The results are plotted in Figure 7(b).

Table 5(b). Retention of T_7 DNA on Millipore filters--effect of σ

µg DNA	µg core	µg protein added	µg σ added	nucleotides	% DNA retained
12.5	0	-	-	AUG	1.3
"	.125	-	-	"	1.7
"	.250	-	-	"	3.0
"	.500	-	-	"	6.1
"	1.250	-	-	"	15.3
"	2.500	-	-	"	31.1
"	12.500	-	-	"	88.4
"	.125	.040	.019	"	2.5
"	.250	.080	.038	"	6.0
"	.500	.160	.076	"	14.4
"	1.250	.400	.190	"	42.6
"	2.500	.800	.380	"	64.9
"	12.500	1.100	.440	"	104.6

Table 5(c)

The effect of RNA polymerase or bovine serum albumin, with no added nucleotides on the retention of T_7 DNA by Millipore filters. The reactions were carried out in .25 ml of .05M tris pH 7.9, .15M KCe, .008M $MgCl_2$, .8mM K_2HPO_4, .1mM EDTA, .1mM dithiothreitol for 20 minutes at 37°C. 10 µg ^3H T_7 DNA (2,880 cpm/µg) were present and the amount of RNA polymerase (with or without Rifampicin 80 µg/ml) or bovine serum albumin (fraction V). The reactions were treated and filtered exactly as for Figure 5(a).

Table 5(c). Effect of RNA polymerase or bovine serum albumin with no added nucleotides, on the retention of T_7 DNA by multipore filters

µg DNA	µg protein	Rifampicin	% retention
10	2.1 RNA polymerase	80 µg/ml	3.1
"	4.2 " "	"	3.7
"	8.4 " "	"	4.6
"	21.0 " "	"	4.5
"	5 BSA	-	1.4
"	10 "	-	1.4
"	25 "	-	1.4
"	0 "	-	1.6
"	2.1 RNA polymerase	-	5.3
"	4.0 " "	-	9.7

Table 6(a)

The effect of single nucleotides on retention of T_7 DNA on Millipore filters. The assays were carried out exactly as those described in Figure 7(d).

Table 6(b)

The effect of GTP on the retention of T_7 DNA on Millipore filters. The assays were carried out exactly as those described in Figure 7(d).

Table 6(a). Effect of single nucleotides on retention of T_7 DNA on Millipore filters

µg DNA/ reaction	µg enzyme/ reaction	Nucleotides	% DNA retained		Normalized
10.0	4.0	G (.2mM)	26.0	16.3	100
"	"	A "	18.0	8.3	50.9
"	"	C "	18.4	8.7	53.3
"	"	U "	12.1	2.4	1.5
"	"	- "	9.7	-	-

Table 6(b). Effect of GTP on retention of T_7 DNA on Millipore filters

µg DNA/ reaction	µg enzyme/ reaction	Nucleotides (.2mM)	% DNA retained
10.0	-	G	2.6
"	1.25	G	11.0
"	2.5	G	20.0
"	3.25	G	27.0
"	3.25	G,A	62.0
"	3.25	G,U	65.5
"	3.25	G,C	72.4
"	3.25	G,C,U	100.0

Figure 7(a)

% retention of T_7 DNA on Millipore filters at high salt. The figure is a plot of the results in Table 5(a).

Figure 7(b)

% retention of T_7 DNA on Millipore filters at high salt--the effect of σ . This figure is a plot of the results in Table 5(b).

Figure 7(a)

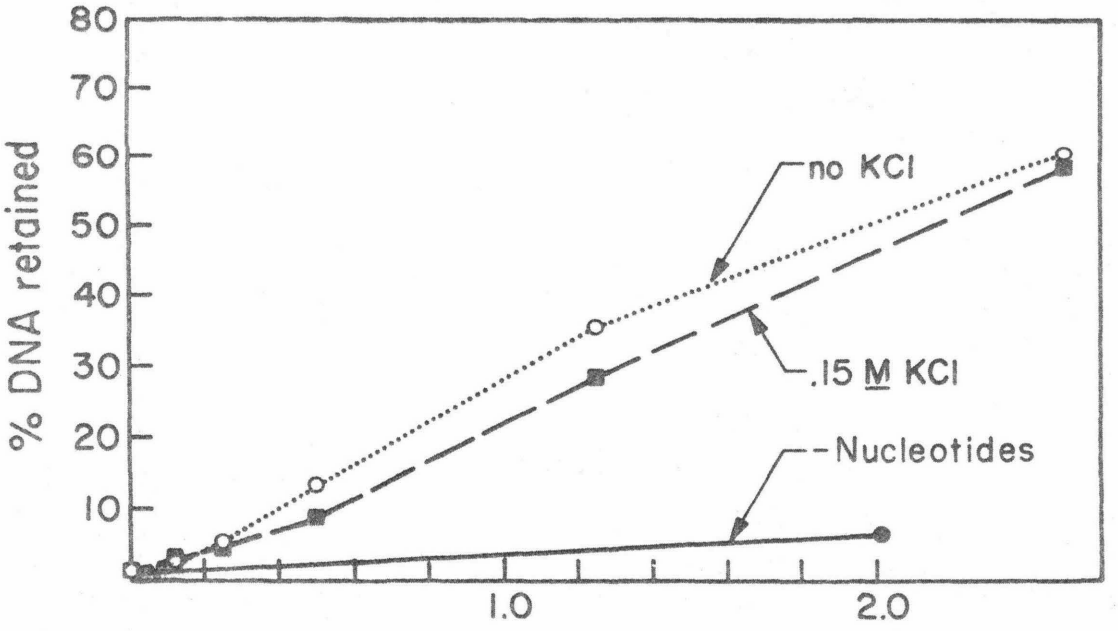


Figure 7(b)

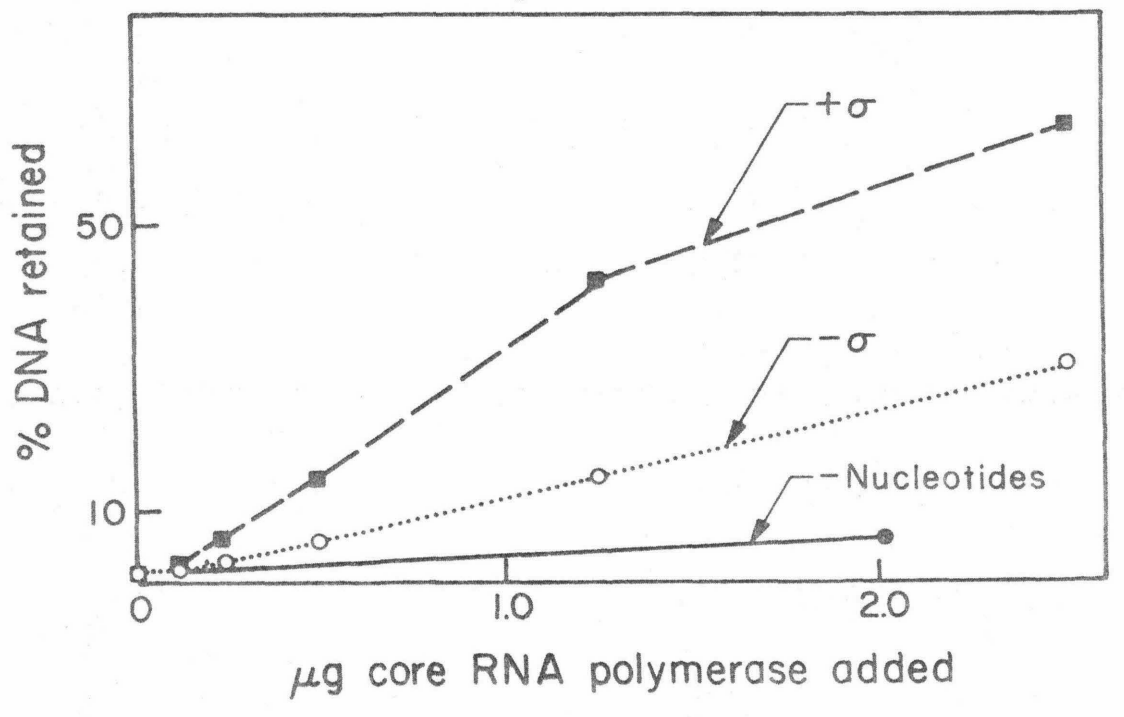


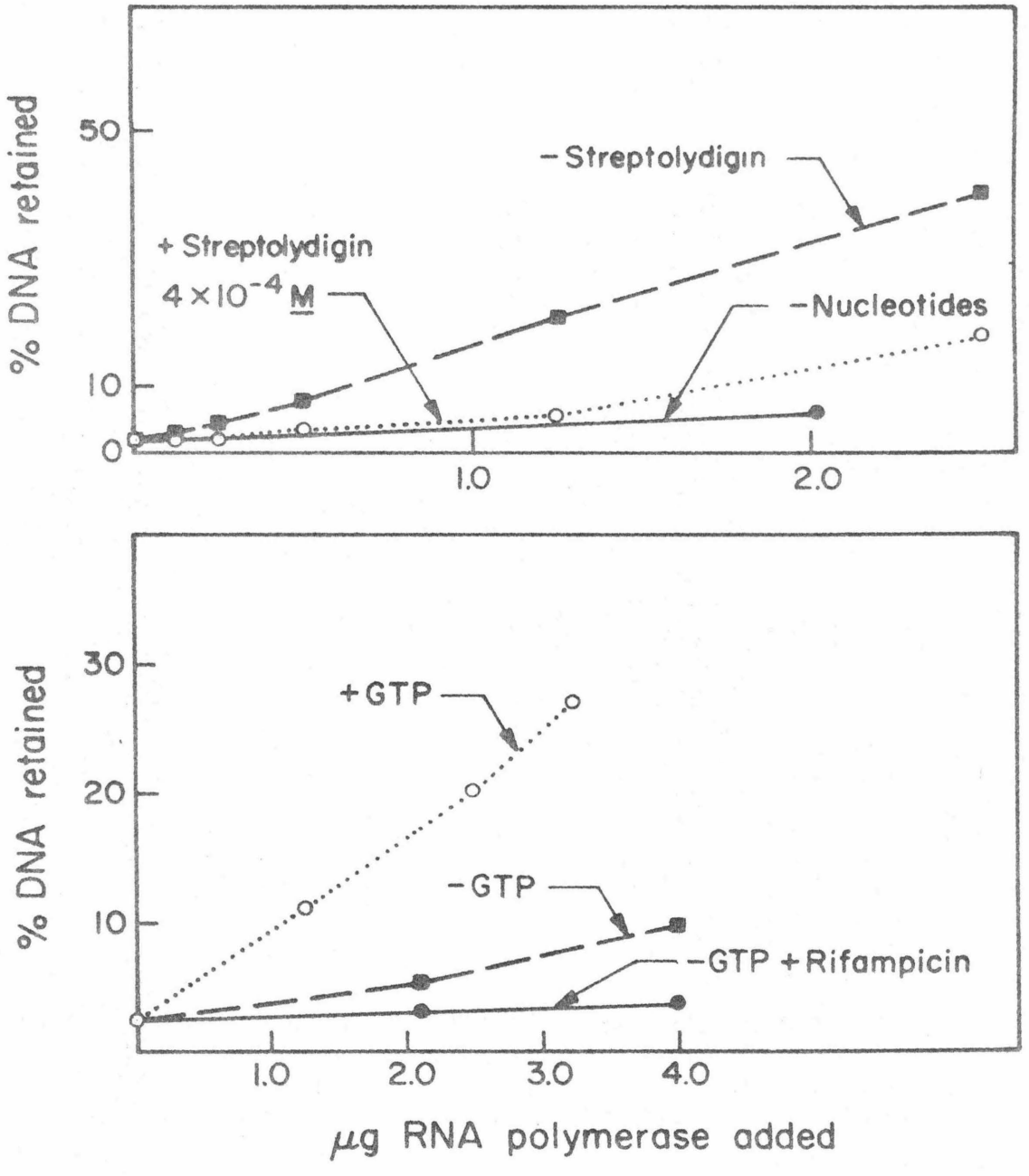
Figure 7(c)

The effect of streptolydigin on the retention of T_7 DNA on Millipore filters. Increasing amounts of RNA polymerase [0-2.5 µg GGC] were added to reactions containing 10 µg of ^3H T_7 DNA (8500 cpm), ATP, GTP, UTP each .2mM, under the conditions as described in Table 5(a). The samples were treated exactly as in Table 5(a) and the graph plots retention on the Millipore filter versus enzyme added when the pre-incubation medium contained (i) no streptolydigin or (ii) 4×10^{-4}M streptolydigin. A plot of retention in the absence of any nucleotides is also shown.

Figure 7(d)

The effect of GTP on the retention of T_7 DNA on Millipore filters. Increasing amounts of RNA polymerase [0-4.0 µg DC.D] were added to 10 µg ^3H T_7 DNA (12,000 cpm) in the presence of GTP alone (.2mM), or no GTP, or no GTP + Rifampicin (80 µg/ml). The reactions were carried out in .25 ml .05M tris pH7.9, .15M KC_L, .008M Mg Cl_2, .8mM K_2HPO_4, .1mM EDTA, .1mM dithiothreitol for 20 minutes at 37°C before raising the salt concentration to .4M $(NH_4)_2SO_4$ and filtering through Millipores as usual.

Figure c and d



reaction mix containing ATP, GTP and UTP. This limits binding of enzyme to DNA (Pettijohn and Kamiya, 1967), presumably increasing the specificity of binding. As the amount of enzyme increases so does the retention of the DNA. The efficiency of complex formation was not altered by .15 M KCl suggesting that only specific initiation complexes are stable under the assay conditions. In the absence of nucleotides a much lower amount of DNA is retained, about 10% of the amount retained in the presence of ATP + GTP + UTP (Tables 5a and 5c).

The presence of σ has a considerable effect. In Figure 7 (b) it is seen that core enzyme ($<$ 3 mole per cent σ subunit) can form a complex in the presence of ATP, UTP and GTP (Table 5 b). This is consistent with its reported ability to transcribe DNA (Burgess et al., 1969; Summers and Siegel, 1969). In the presence of σ the efficiency of formation is more than doubled, although the total protein added to the reaction is only 50% of the amount of core, so that it is not a direct function of total protein.

The reaction is inhibited strongly by rifampicin (Table 5a), and by streptolydigin (Figure 7 c) both specific inhibitors of RNA polymerase. Rifampicin blocks initiation and streptolydigin propagation (Figure 3). Magnesium is required for complex formation (Table 5a).

The effect of different nucleotides is shown in Table 6(a) GTP is the most efficient substrate for the reaction,

while ATP and CTP are approximately equal. UTP is a poor substrate causing only a small increase above background. Retention is greatly increased if other nucleotides are present in addition to GTP (Tables 5 , 6b). Furthermore the increases are greater than additive. For example (Table 5a) the amount retained by 2.5 ug of RNA polymerase in the presence of AUG (60%) is greater than the combined amounts of DNA retained by GU (28%) AU (11.5%) and U alone (4.5%).

Figure 7 (d) is a plot of % retention in the presence of GTP as a function an increased amount of enzyme. If GTP is not present the amount retained is significantly above the background (1.6% where no enzyme is added). Since the presence of rifampicin reduces this still further it is concluded that specific binding occurs at low efficiency in the absence of nucleotides which is stable to high salt for short periods of time. Even rifampicin at high concentrations (80 µg/ml) does not completely eliminate binding, since up to 3.7% of the DNA is retained when 4 µg of enzyme are added, which greater than background (1.6%). As shown in Figure 2 and Figure 8 , and Figure 9 high concentrations of rifampicin do not completely eliminate RNA synthesis on T7 DNA. The effect of RNA polymerase, RNA polymerase + rifampicin, and bovine serum albumin (BSA) on retention in the absence of nucleotides is shown in Table 5(c). It is apparent that at least some complex formed with RNA polymerase at .15 M KCl without any nucleotide is stable to

.4 M $(NH_4)_2SO_4$. The amount is reduced by rifampicin by approximately 50%, while BSA does not cause any retention in excess of a blank containing no added protein (1.3 - 1.7%).

(v) The formation of a rifampicin resistant complex between RNA polymerase and T7 DNA.

Since rifampicin is a potent and specific inhibitor of initiation by RNA polymerase, any process which can overcome this inhibition must be one which cause formation of a tightly bound enzyme-DNA complex which has already completed the steps of the reaction prior to propagation. Figure 8 shows the results of an experiment in which RNA polymerase (20 µg) was preincubated with T7 DNA (100 µg), ^3H GTP (.2 mM) and rifampicin (160 µg per ml) in all possible combinations for 20 minutes at 37°C. The samples were placed on ice while the remaining nucleotides and the other missing components were added in the order DNA, rifampicin, GTP and other nucleotides, then returned to 37°C, and aliquots taken for TCA precipitation to follow the time course of synthesis. The only difference between the samples was the condition of preincubation. Sample 2 in which enzyme, DNA and GTP had been preincubated together displayed rifampicin-resistant synthesis which was significantly higher (9x) than the control (sample 7) in which enzyme was preincubated with rifampicin alone. Synthesis was linear for 30 minutes. Table 7(a) shows the comparison of the amount of

Figure 8

The formation of rifampicin-resistant complex between RNA polymerase and T_7 DNA. RNA polymerase [21 µg, DC.D, 850 units per mg] was preincubated for 20 minutes at 37°C in .25 mµ .05M tris pH7.9, .008M Mg CL_2, .8mM K_2HPO_4, .15M KC_L, .1mM EDTA, .1mM dithiothreitol. 7 samples contained the following additional components:

1. T_7 DNA (100 µg), GTP (.2mM), rifampicin (160 µg/ml)
2. T_7 DNA, GTP
3. T_7 DNA, rifampicin
4. GTP, rifampicin
5. T_7 DNA
6. GTP
7. Rifampicin

After 20 minutes at 37°C, the reactions were cooled and an additional .25 ml volume was added to each containing (1) the same concentrations of salts as above, (2) the components which were not present in the preincubation, and (3) CTP, UTP and ^3H ATP to give final concentrations of .1 mM, .1mM and .01mM, respectively. The additions were made in the order: DNA, rifampicin, GTP, and [salts + XTP].

The reactions were then returned to 37°C and a time course of synthesis was obtained by measuring the TCA precipitable counts in 100λ aliquots taken at time intervals as shown.

The control reactions 8 and 9 (Figure 8(c)) contained no rifampicin.

The graphs show TCA precipitable cpm plotted versus time. The specific activity of the ^3H ATP was 372,000 cpm/mµ mole.

Figure 8(a)

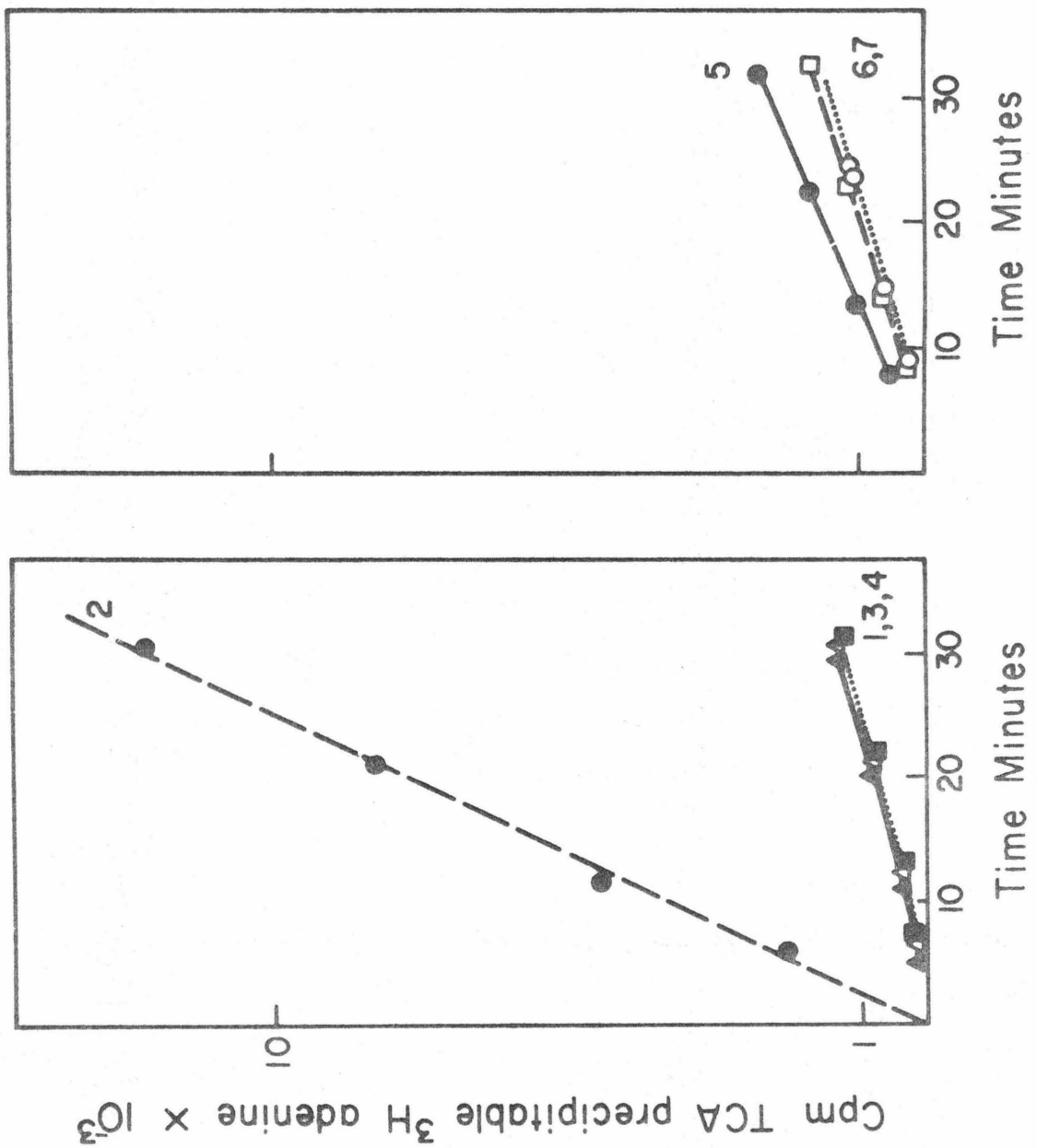
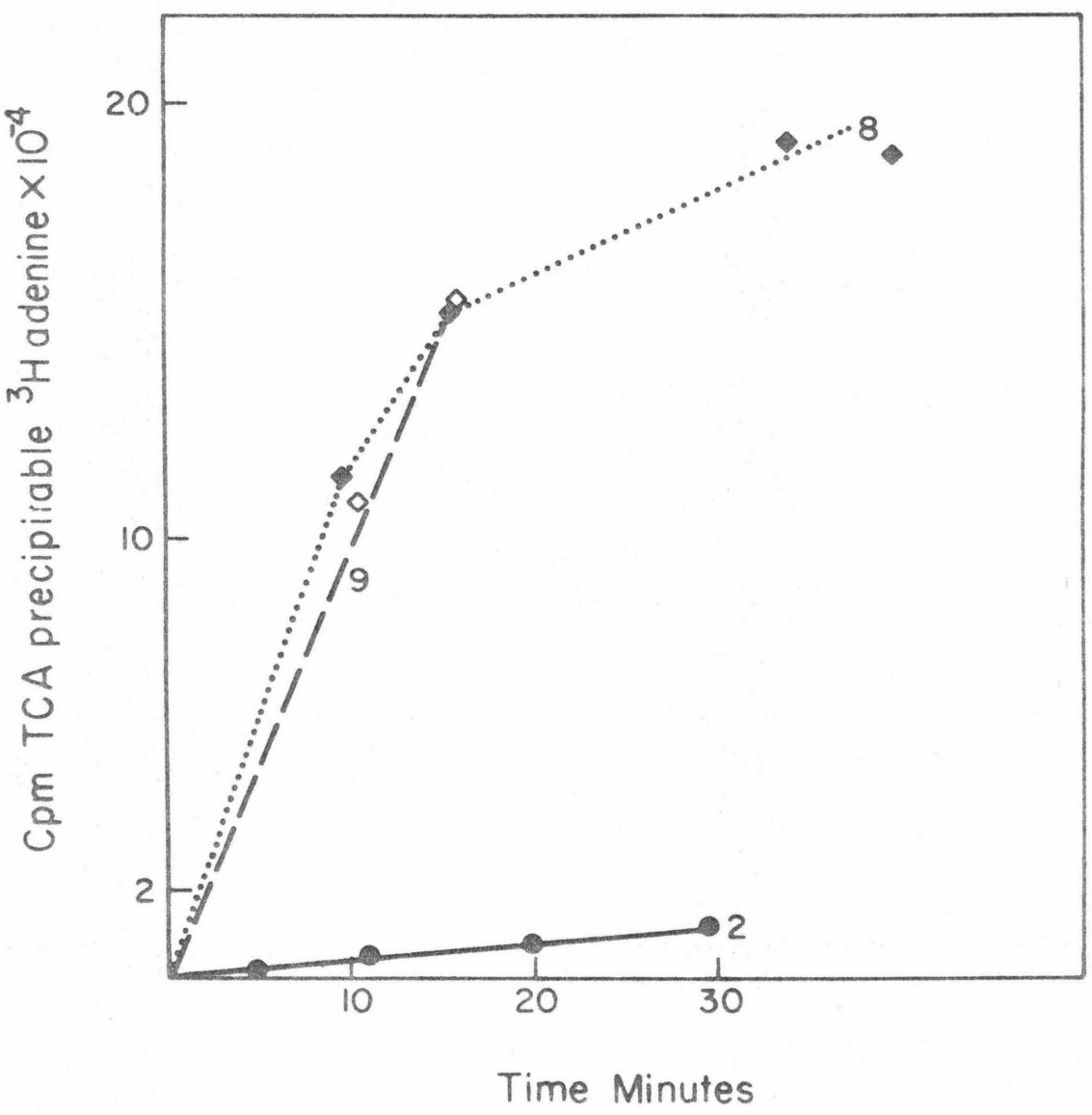

Cpm TCA precipitable ^3H adenine x 10^3
10
1
2
1,3,4
10
20
30
Time Minutes
5
6,7
10
20
30
Time Minutes

Figure 8(b)


Cpm TCA precipirable ^{3}H adenine $\times 10^{-4}$
20
10
2
8
9
2
10
20
30
Time Minutes

Tables 7(a)(b)

Preincubation with GTP and resistance towards Rifampicin. The results shown in Figure 8(a)(b)(c) and Figure 9 for the Rifampicin resistant synthesis at 30 minutes were extracted, tabulated and normalized.

Table 7(a). Preincubation and resistance towards Rifampicin

	Preincubation components	Incorporation at 30 minutes cpm TCA precipitable/50λ	Normalized
1	ENZ, DNA, GTP, RIF	1,400	11.7
2	ENZ, DNA, GTP	12,000	100.0
3	ENZ, DNA, RIF	1,250	10.4
4	ENZ, GTP, RIF	1,200	10.0
5	ENZ, DNA	2,300	19.1
6	ENZ, GTP	1,550	12.9
7	ENZ, RIF	1,400	11.7
8	- no rifampicin added	180,000	1500.0

Table 7(b)

	Preincubation components	Incorporation at 30* minutes cpm TCA precipitable/50λ	Incorporation Sample 5	%
1	ENZ, DNA, GTP	77,000	65,500	100.
2	ENZ, DNA, CTP	25,000	13,500	20.6
3	ENZ, DNA, UTP	14,000	2,500	3.8
4	ENZ, DNA, ATP	16,500	5,000	7.6
5	ENZ, DNA,	11,500	-	
6	ENZ, DNA, ATP, GTP	124,000	122,500	187.0

incorporation at 30 minutes for each sample. Sample 5, in which enzyme and DNA were preincubated was slighltly higher (1.6 x) than the control. The reaction was carried out at .15 M KCl, and in the presence of .0008 M phosphate to inhibit polynucleotide phosphorylase. The results suggest that in .15 M KCl the formation of a rifampicin resistant complex requires a nucleotide, DNA and enzyme.

The effect of different nucleotides was tested and the results are shown in figure 9. It was found that the order of effectiveness in forming the rifampicin resistant complex was GTP > CTP > ATP > UTP > none (Table 7b). Since CTP has not been described as a common initiating nucleotide for T7 DNA (Stevens, 1969) it was not expected that it would behave differently from UTP, but similar results were obtained using the Millipore filter and co-sedimentation assays.

If both A and G are present during preincubation, the amount of rifampicin resistant synthesis is more than the combined amounts obtained when each is added separately.

(vi) The nature of the ternary complex (ENZ-DNA-nucleotide).

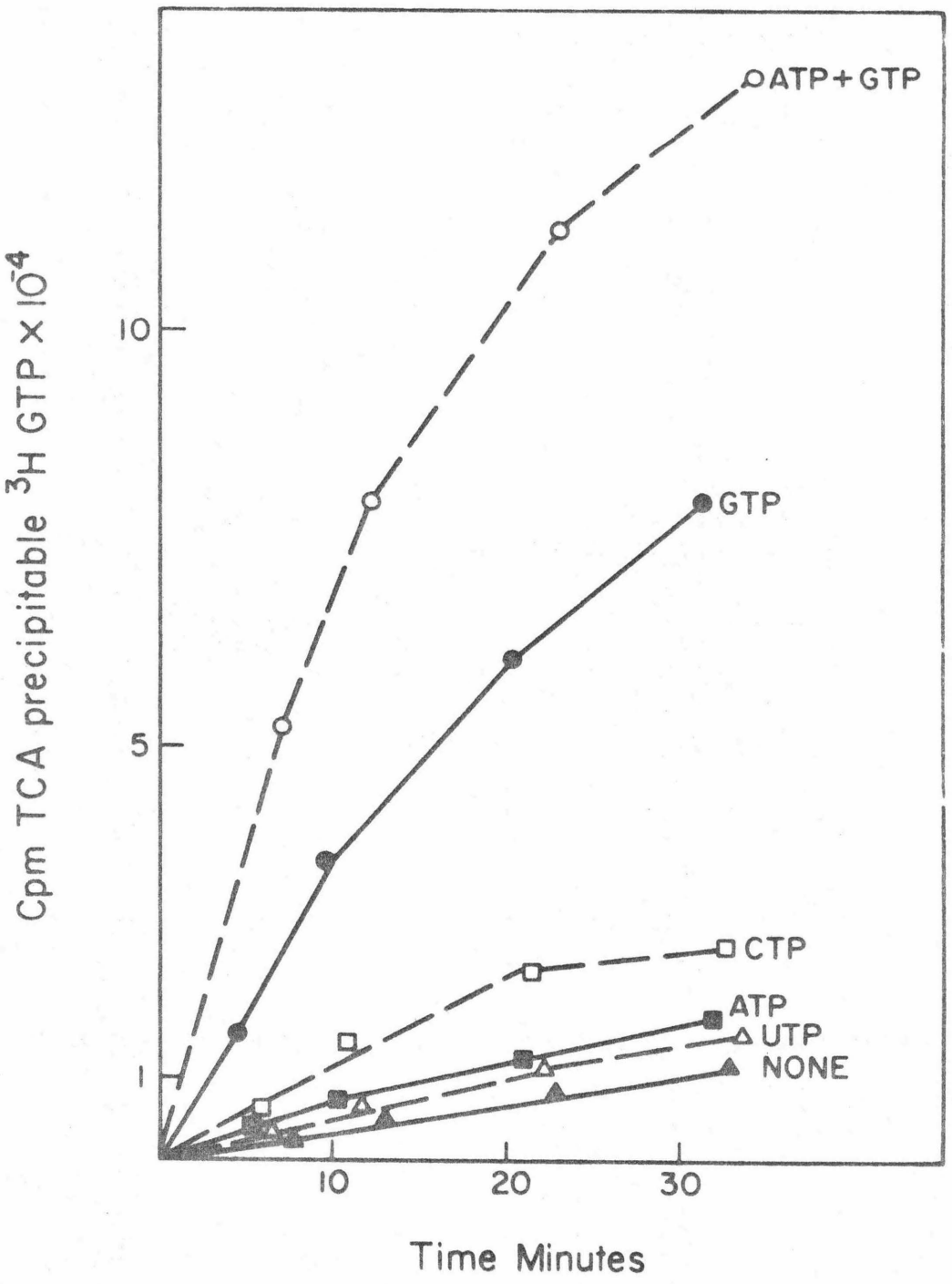
The results obtained from the co-sedimentation experiments in which RNA polymerase and T7 DNA had been incubated with ^{3}H GTP (7.5×10^{5} cpm/mμmole) showed that the complex contained a small amount of ^{3}H, that the amount was increased as more enzyme was added and that the number average approached 1 mμmole of GTP per mμmole of DNA. The same

Figure 9

The effect of GTP, CTP, UTP and ATP on the formation of a rifampicin-resistant complex. The scheme for this reaction is similar to that described in Figure 8, except that 50 μg of T_7 DNA and 10 μg of RNA polymerase [DC.D] were used, and the volume of the preincubation assay was .125 ml. Five reactions contained the following nucleotides in the preincubation medium with the T_7 DNA and RNA polymerase

1. GTP (.2mM)
2. CTP (.2mM)
3. UTP (.2mM)
4. ATP (.2mM)
5. ATP + GTP (each .2mM)
6. None

After 20 minutes at 37°C the missing nucleotides, etc. were added and rifampicin at 160 μg/ml. to give a final volume of .25 ml and the reactions returned to 37°C. The final nucleotide concentration was .1mM. ^{3}H GTP (5×10^5 cpm per mμ mole) was used to measure TCA precipitable material in 50λ aliquots taken at the times shown. The values are given for TCA precipitable cpm per 50λ aliquot.


ATP+GTP
GTP
CTP
ATP
UTP
NONE
10
5
1
10
20
30
Time Minutes
Cpm TCA precipitable ^{3}H GTP $\times 10^{-4}$

experiment was repeated using $\gamma\,^{32}P$ GTP to establish the number of 5' phosphates per DNA. Although the specific activity was exceedingly high (10^6 cpm ^{32}P per mμmole) so that co-sedimentation of equimolar quantites of DNA and γ phosphate would have yielded cpm per fraction greater than 600 it was impossible to identify a peak of counts in the DNA region. Analysis was complicated by the high background due to some rapidly sedimenting labelled material which was not associated with the DNA. This background approached 2000 cpm, but a peak of several tubes of 600 counts above this would have been detected if present. If each fraction of the DNA peak had 60 cpm above the high background this would not have been detected.

This result suggested that the counts associated with the DNA represented oligonucleotides. This was confirmed by showing that a high proportion of these counts are precipitable by 10% TCA (Table 8). T7 DNA (100 μg), RNA polymerase (65 μg) and .2 m$\underline{M}$ ^3H GTP (7.5×10^5 cpm/mμmole) were incubated in a high salt (.15 $\underline{M}$ KCl) reaction mixture containing .8 m$\underline{M}$ K_2HPO_4 to inhibit polynucleotide phosphoylase, at 37°C for 20 minutes. Four samples contained (i) no additional nucleotides (ii) ,2 m$\underline{M}$ ATP (iii) .2 m$\underline{M}$ CTP and .2 m$\underline{M}$ UTP amd (iv) rifampicin. The reaction volumes were layered on a high salt glycerol gradient (10-20%) with a 75% glycerol shelf, and centrifuged at 41 K for 18 hr at 4°C. The sedimentation profiles and ^3H GTP distribution are shown in Figure 6. The fractions which contained the DNA were

Table 8

TCA precipitation of cpm ^{3}H GTP cosedimenting with the enzyme/DNA complex. Every second fraction from the DNA peaks shown in Figure 6(a)(b)(c)(d) was combined for each sample. The volumes were adjusted to 5.5 ml and 1 ml aliquots were either counted directly in NCS/Toluene, or precipitated with 10% TCA and the precipitates collected, filtered, and counted, or made 4.5M NaC_L to dissociate the complex and then TCA precipitated. The counts were adjusted for the relative counting efficiencies for ^{3}H in NCS/Toluene or on filters in Toluene and tabulated.

Table 8. TCA precipitation of cpm ^{3}H GTP cosedimenting with the enzyme/DNA complex.

Counting Method	Vol. Sample	Treatment	Sample cpm ^{3}H GTP 1 GTP	2 G+A	3 G+C+U	4 G+RIF
NCS/Toluene	1.0 ml	-	469	642	1284	60
Filter/Toluene	1.0 ml	TCA precipitation	558	723	1206	48
Filter/Toluene	1.0 ml	NACe dissociation + TCA precipitation	391	488	950	37

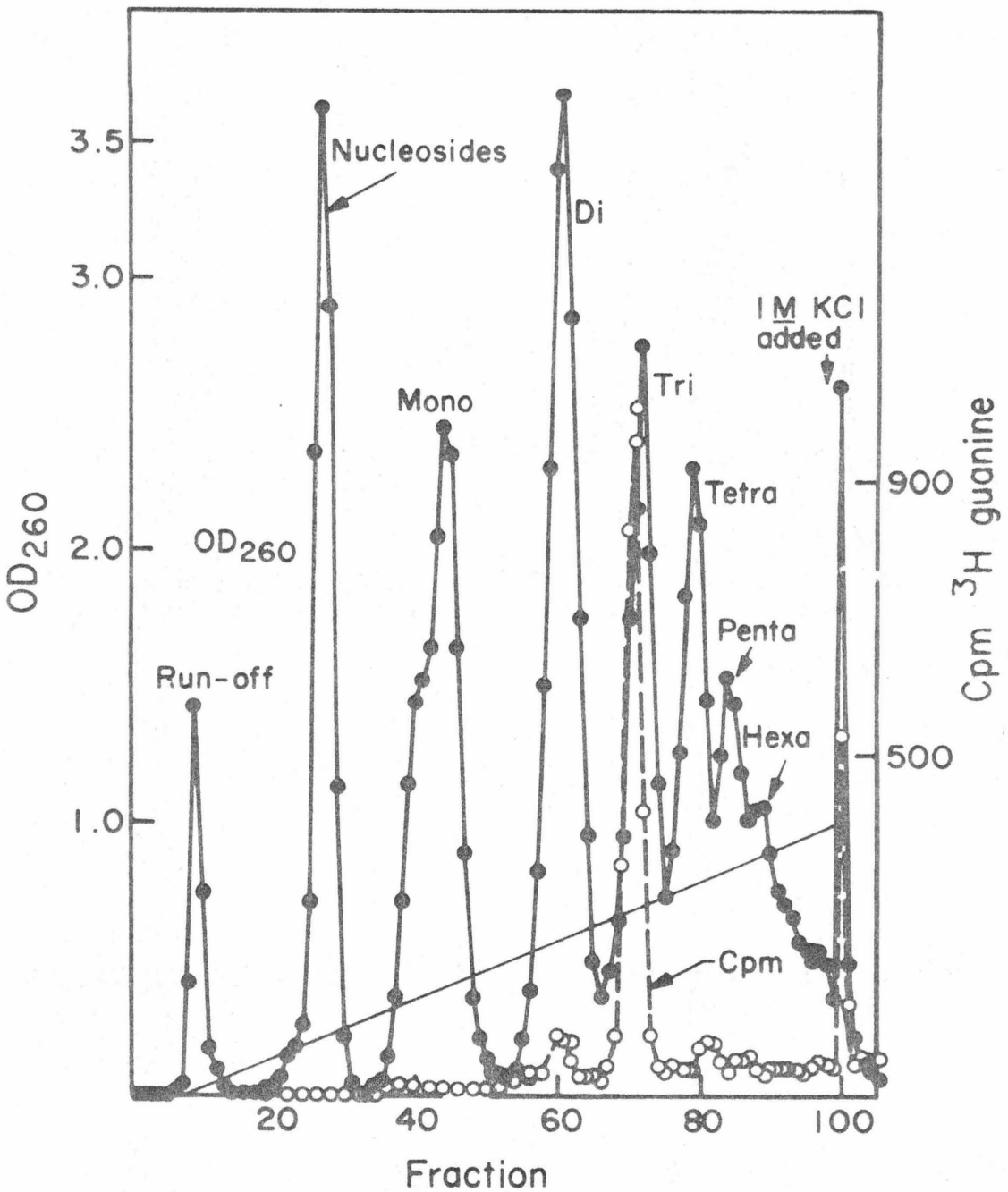
combined, and aliquots were counted before and after TCA preceipitation. One aliquot was made 4.5 M NaCl before precipitation to dissociate the ternary complex. The results in Table 8 show that all of the counts are TCA precipitable unless the complex is dissociated by NaCl when 30-40% of the counts are not. 10% TCA is expected to precipitate oligonucleotides greater than decanucleotides.

Chromatography on DEAE Sephadex (Figure 10) was used as a second method to detect the presence of oligonucleotides in the ternary complex.(Hatlen et al., 1969). The complex was prepared as above except that 100 µg of enzyme were used. After sedimentation the fractions from the DNA peak were combined and dialysed against .01 M tris pH 7.6 to remove the ammonium sulphate. The solution was made 7 M urea and 10 mg of cold RNA predigested with pancreatic ribonuclease were added before chromatography on a DEAE Sephadex A-25 column (.7 x 60 cm) eluting with a linear gradient of 0-.4 M KCl in .02 M tris pH 7.6, 7 M urea. The column was washed with 1 M KCl, .02 M tris pH 7.6, 7 M urea after the gradient. Of the counts applied (33,800 cpm) 60% or 20,000 counts were recovered from the column (Figure 10). Of these 9,800 eluted just before the trinucleotide peak in the position characteristic of GTP (Hatlen et al., 1969). A very small amount eluted as GDP while the remainder came off at higher salt concentrations as expected if the sample contained oligonucleotides. The recovery from the column was not good (60%), which would be explained if some oligonucleotides

Figure 10

Oligonucleotide analysis of the cpm of ^{3}H GTP which cosediment with T_7 DNA. RNA polymerase (200 µg), T_7 DNA (200 µg) and ^{3}H GTP (.2mM, 175,000 cpm/mµ mole) were preincubated in .5 ml of .15M KC_L, .05M tris pH7.9, .008M Mg CL_2, .8mM K_2HPO_4, .1mM EDTA, .1mM dithiothreitol, at 37°C for 20 minutes.

The reaction volume was then sedimented at 41K for 17 hours at 4°C through a standard high salt [buffer S] glycerol gradient. The DNA peak was located by reading OD_{260} and the peak fractions were combined and dialysis against .01M tris pH7.9 to remove the $(NH_4)_2SO_4$. An aliquot was counted and 33,800 cpm were applied to a DEAE Sephadex A-25 column (.7 × 60 cm) in 7M urea .02M tris pH7.6. 10 mg of yeast soluble RNA predigested with pancreatic ribonuclease was added as carrier and the column eluted with 300 ml of a linear gradient from zero to .4M KC_L in 7M urea, .02M tris pH7.6. The column was then washed with 1M KC_L, 7M urea, .02M tris pH7.6. The fractions (3 ml) were read at 260 mµ and aliquots counted in NCS/Toluene.


3.5
3.0
2.0
1.0
OD260
OD260
Nucleosides
Di
Mono
Tri
1 M KCl
added
Tetra
Penta
Hexa
Run-off
Cpm
900
500
Cpm 3H guanine
20
40
60
80
100
Fraction

were very long, and therefore not eluted from the column.

(vii) Purification of nucleotides before the formation of the ternary complex.

Samples of ATP and GTP were purified on Dowex -1x8 as described in Materials and Methods. Two completely separate but identical systems were set up to minimise cross-contamination. Figure 11 shows the results of two chromatograms, one for GTP and the other for ATP. Most GTP preparations did not have as much contamination with GDP. The peak fractions were pooled, the ammonium formate was removed on Dowex 50 H+ columns and the nucleotides recovered after lyophilisation. They had excellent spectra with the predicted ratios of OD 250/OD260 and OD 280/OD 260. To check that the high concentrations of formic acid and ammonium fornate had not caused degradation the purified GTP was tested by a dilution with ^{3}H GTP and gave a perfect dilution curve. The ATP was compared with new ATP in its ability to serve as a substrate for RNA synthesis and was equally good.

The purified nucleotides were tested for their ability to act as substrates for complex formation. For both ATP and GTP the amount of complex was considerably reduced when assayed by the Millipore filter method (Table 9). The reductions were 57% for GTP and 56% for ATP.

The value for purified ATP (9.4% of DNA retained) is slightly below the background observed if 4 µg of enzyme are incubated with DNA in the absence of nucleotides (9.7%),

Figure 11

Purification of GTP and ATP by chromatography on Dowex 1 × 8. Approximately 100 mg of GTP or ATP were applied to separate Dowex 1 × 8 columns (2.2 × 21.5 cm) and eluted with linear gradients (1500 ml) from 1.5M formic acid to 2.0M formic acid 1M ammonium formate. The fractions from the triphosphate peak were combined and applied to a Dowex $50H^+$ column (3 × 67 cm) to remove the ammonium ion, and then lyophilised to remove the HCOOH.

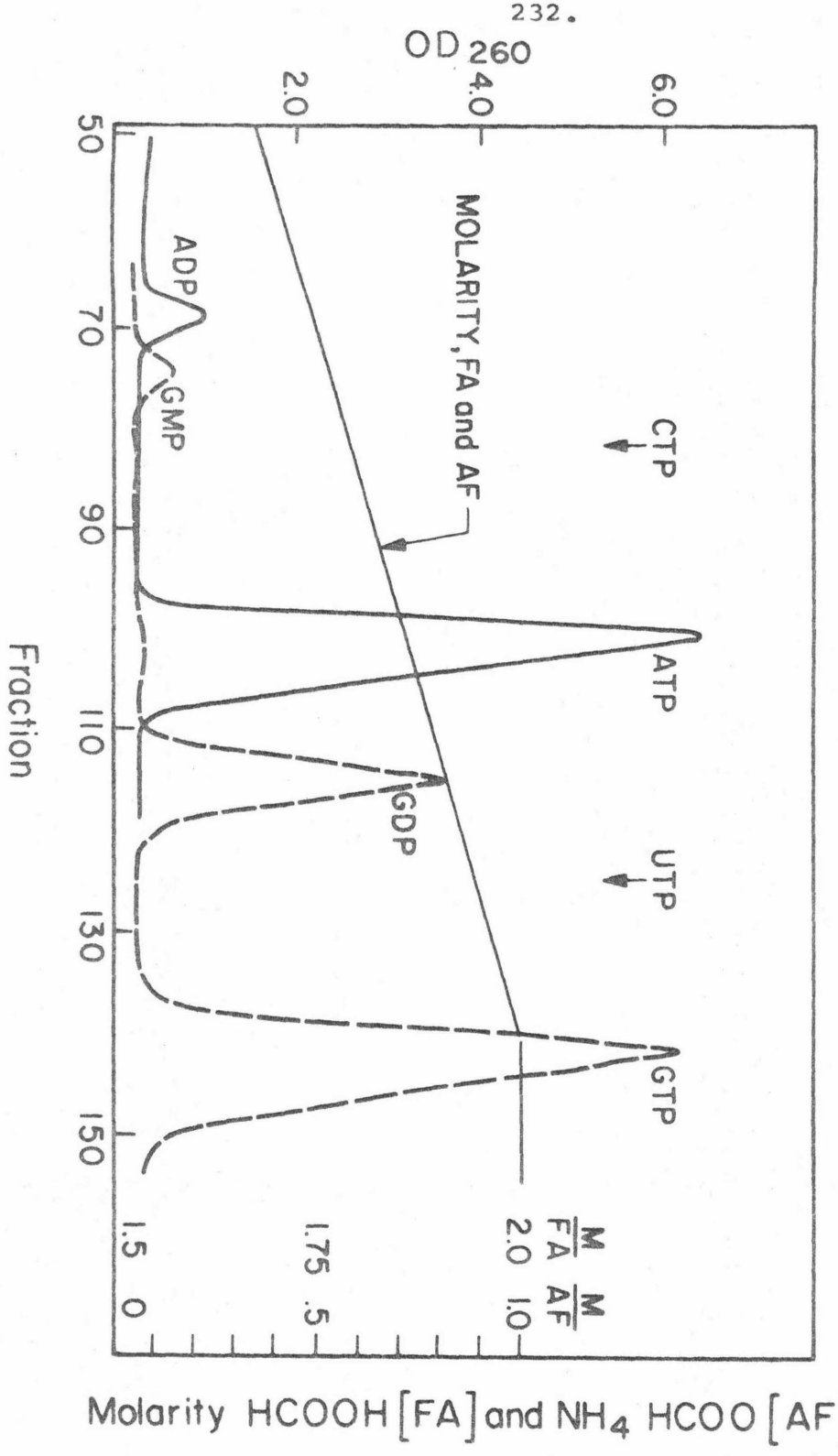

OD 260
2.0
4.0
6.0
50
70
90
110
130
150
Fraction
MOLARITY, FA and AF
ADP
GMP
CTP
ATP
GDP
UTP
GTP
M/FA
M/AF
1.5
0
1.75
.5
2.0
1.0
Molarity HCOOH [FA] and NH4 HCOO [AF]

Table 9

The effect of purified nucleotides on the retention of T_7 DNA on Millipore filters. Experiment 1: Commercial preparations of ATP and GTP were purified as shown in Figure 11. The purified XTPs were then compared for their ability to act as substrate for complex formation with commercial XTP in assays identical to those described in Table 5(c). The amounts of enzyme, DNA and nucleotides present are shown.

Experiment 2: The values for the amount of DNA retained at .2mM of the designated XTP were extracted from Figure 12 and tabulated.

Table 9. The effect of purified nucleotides on the retention of T_7 DNA on Millipore filters

Experiment 1

µg DNA/reaction	µg Enzyme/ reaction	Nucleotides	% DNA retained
10.0	8.4	G (.2mM)	38.4
10.0	"	G (.2mM)	37.6
10.0	"	G(purified)(.2mM)	16.0
10.0	"	G(")(.2mM)	17.3
10.0	"	A (.15mM)	25.3
10.0	"	A (.15mM)	25.0
10.0	"	A(purified)(.15mM)	11.2
10.0	"	A(")(.15mM)	10.6
10.0	-	-	1.6
10.0	-	-	1.6
*10.0	4.0	-	9.7

*See Table 5(c)

Experiment 2

10.0	8.4	G (.2mM)	30.6
10.0	"	G (.2mM)	31.6
10.0	"	G(purified)(.2mM)	13.7
10.0	"	G(purified)(.2mM)	13.8
10.0	"	A (.2mM)	19.2
10.0	"	A (.2mM)	19.2
10.0	"	A(purified)(.2mM)	8.0
10.0	"	A(")(.2mM)	8.0

while the value for purified GTP (15.2%) is significantly higher. Furthermore purified ATP and GTP behave differently if added at higher concentrations (Figure 12). As the concentration of purified GTP is raised the efficiency of complex formation increases linearly, while no response is observed if the concentration of purified ATP is raised. In contrast for non-purified ATP a linear increase is observed.

It is concluded that commercial preparations of nucleotides contain impurities which contribute to the efficiency of complex formation. If these impurities are reduced in the case of ATP, it no longer increases the rate of complex formation when compared against an enzyme blank. After a similar purification procedure, GTP can still still stimulate complex formation although at a reduced (~ 50%) rate. If the GTP is absolutely pure this may represent homopolymer synthesis, although the rate is only 1.5 times background, low enough to suggest that impurities are still present.

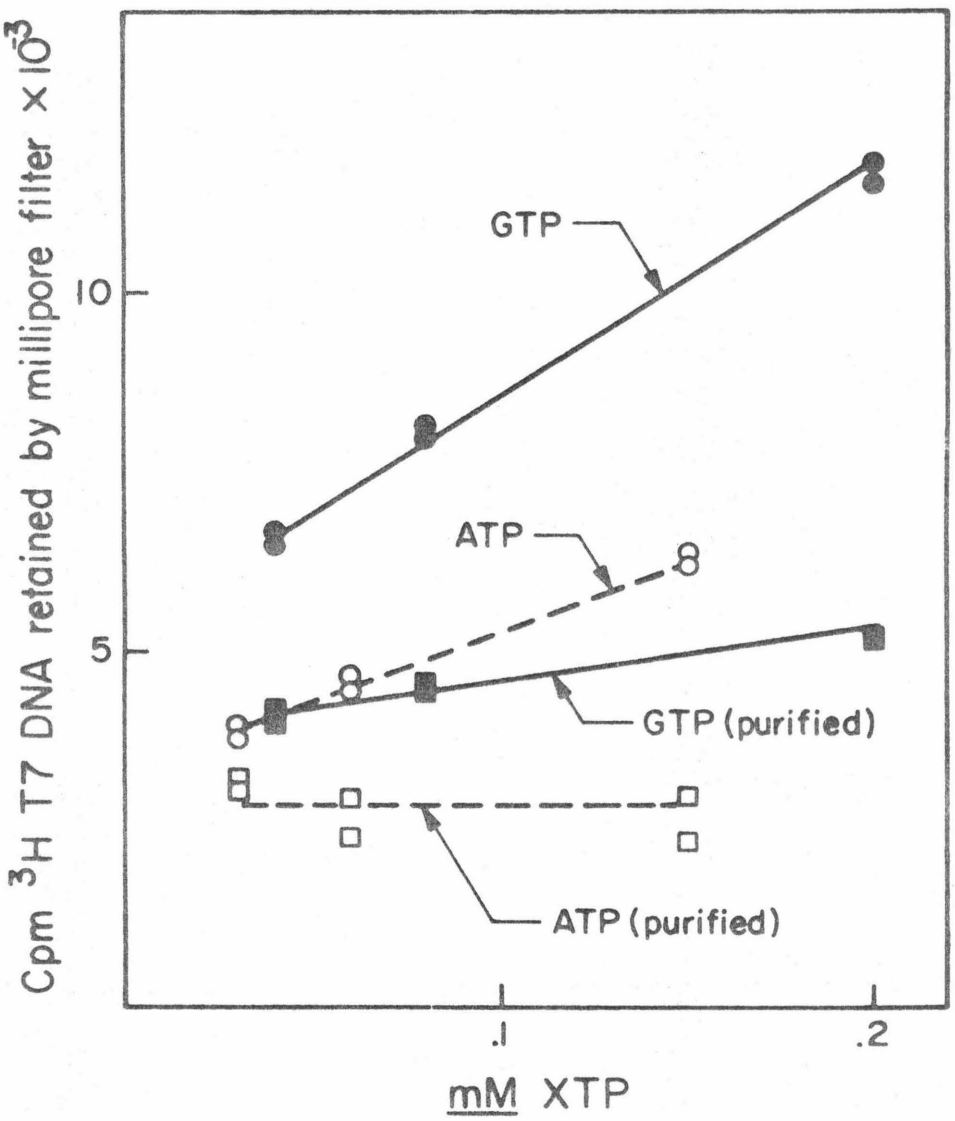
Since ATP when purified does not stimulate the formation of this complex, but is known to initiate RNA synthesis on T7 DNA being incorporated at the 5' terminal position (see section (viii)), it is concluded that a complex of enzyme, DNA , and terminal nucleotide is not stable.

(viii) <u>The 5' nucleotides of RNA synthesised on T7 DNA by RNA polymerase.</u>

Initiation by $\gamma\ ^{32}P$ ATP and $\gamma\ ^{32}P$ GTP at both high and low salt was investigated using a highly purified RNA

Figure 12

Retention of T_7 DNA-RNA polymerase complex on Millipore membranes for commercial and purified ATP and GTP. The reaction was carried out under the same conditions as described for Figure 7(d). 10 µg of ^3H T_7 DNA (37,500 cpm) and 8.4 µg of RNA polymerase (DC.D, 850 units/mg) were present in each assay. The reactions contained either commercial GTP, purified GTP, commercial ATP or purified ATP at the concentrations shown. The amount of ^3H T_7 DNA in the complex was measured after 20 minutes at 37°C by raising the salt concentration and filtering on Millipore filters HAWP as usual. The background level for assays containing no nucleotides was 9-10%.


Cpm ^{3}H T7 DNA retained by millipore filter $\times 10^{-3}$
10
5
GTP
ATP
GTP (purified)
ATP (purified)
.1
.2
mM XTP

polymerase preparation of specific activity 850 (DC.D). RNA polymerase (4.2 μg) and T7 DNA (10 μg) were incubated under the conditions described in Figure 13 with either $\gamma\ ^{32}P$ GTP or $\gamma\ ^{32}P$ ATP. Aliquots were taken at times up to 65 minutes and precipitated with 10% TCA, the precipitates collected and counted as usual. The high salt assay contained .15 M KCl and .028 M $MgCl_2$, while the low salt assay was .06 M KCl and .008 M $MgCl_2$. Phosphate (.8mM) and ADP (.02 mM) were added to inhibit polynucleotide phosphorylase and polyphosphate kinase respectively. Figure 13 represents the time course of the reaction. Both $\gamma\ ^{32}P$ GTP and $\gamma\ ^{32}P$ ATP were incorporated at both high and low salt. The rate of initiation with ATP was about twice that with GTP (Figure 13 a). Propagation at high salt as measured by the incorporation of ^{3}H GTP showed a typical lag phase but continued almost linearly up to 60 minutes (Figure 13b). In contrast at low salt there was no lag and propagation had reached a plateau by 60 minutes. The rate of initiation did not increase at high salt because the concentration of nucleotides was dropping below the Km of the reaction. The rate of propagation at high salt between 15 and 25 minutes was 5-10 nucleotides per enzyme per sec assuming, (1) that only A and G initiated synthesis, and (2) that no chains which had started propagation between zero and 25 minutes had terminated. This may be a low estimate, the first assumption is good (Stevens, 1969) while the second is

Figure 13

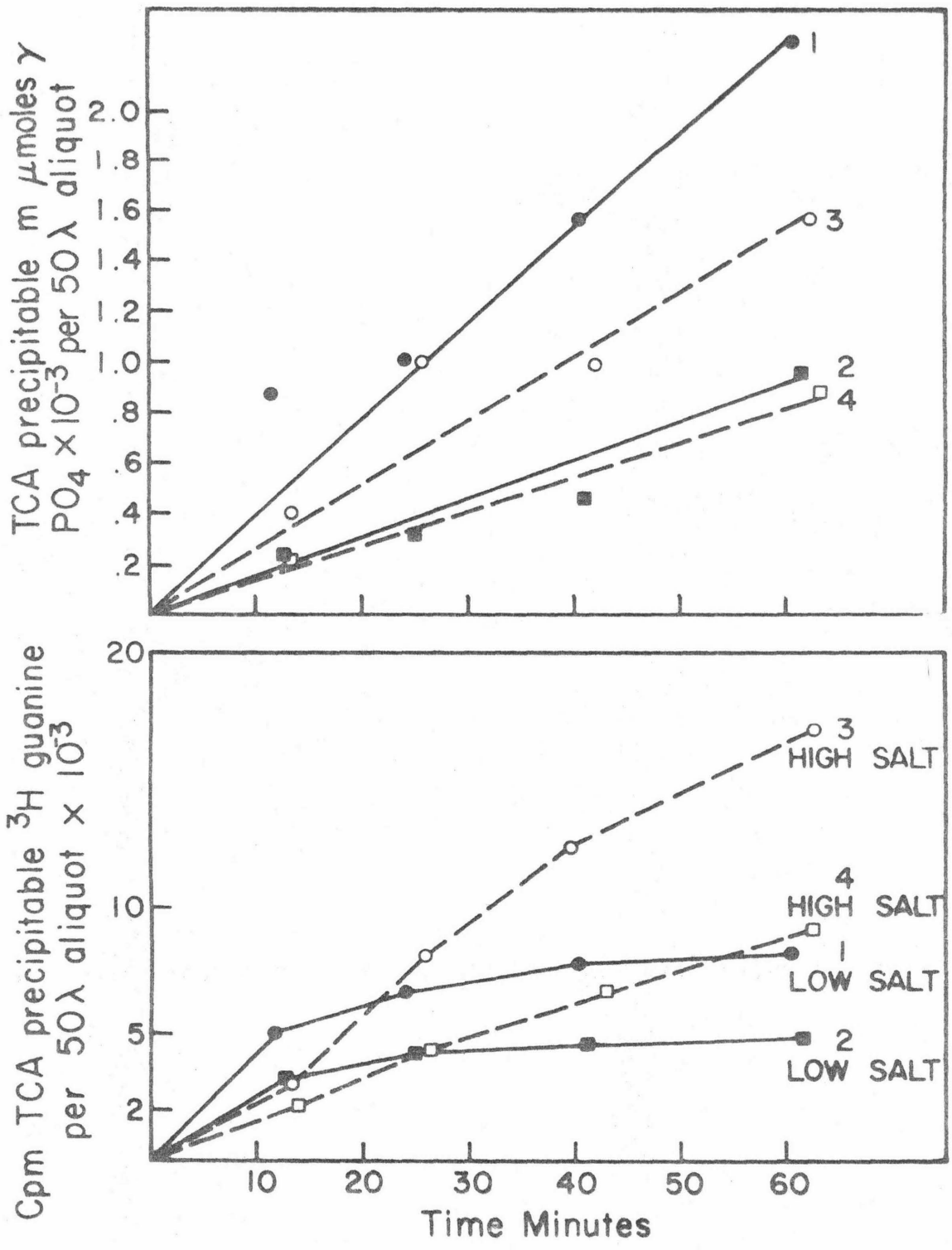
Initiation of RNA synthesis on T_7 DNA. γ^{32}P ATP (.2mM, 4.25×10^5 cpm/mμ mole) and γ^{32}P GTP (.2mM, 2.8×10^5 cpm/mμ mole) were used to follow initiation of RNA synthesis at high salt [.15M KC_L and .028M Mg CL_2] or low salt [.06M KC_L and .008M Mg CL_2] in the presence of 10 μg of T_7 DNA, 4.2 μg of RNA polymerase [DC.D, 850 units/mg] , .05M tris pH7.9, .8mM K_2HPO_4, .1mM EDTA, .1mM dithiothreitol, .2mM CTP, .2mM UTP (.25 ml total volume). Propagation was followed using ^{3}H GTP [4000 cpm/mμ mole in assays containing γ^{32}P ATP and 2000 cpm/mμ mole in assays containing γ^{32}P GTP] . Reactions were incubated at 37°C. Aliquots of 50λ were precipitated with TCA, and the precipitates collected, washed and counted as usual. Reactions (6) were set up as follows:

1. γ^{32}P ATP (low salt)
2. γ^{32}P ATP (high salt)
3. γ^{32}P GTP (low salt)
4. γ^{32}P GTP (high salt)
5. γ^{32}P ATP (low salt) - no enzyme
6. γ^{32}P GTP (low salt) - no enzyme

Background counts were 220 cpm γ^{32}P ATP in reaction 5 and 180 cpm γ^{32}P GTP in reaction 6.

Figure 13(a) shows a time course of total synthesis cpm TCA precipitable ^{3}H GTP. Figure 13(b) is a plot of initiation.

Reactions contained .02 mM ADP to inhibit polyphosphate kinase.


TCA precipitable m μmoles γ PO4 × 10-3 per 50 λ aliquot
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
1
3
2
4
Cpm TCA precipitable 3H guanine per 50 λ aliquot × 10-3
2
5
10
20
3 HIGH SALT
4 HIGH SALT
1 LOW SALT
2 LOW SALT
10
20
30
40
50
60
Time Minutes

is probably not (Millette and Trotter, 1970). The DNA used in this experiment was in good condition 70% of the single strands being unknicked. The RNA polymerase had a small amount of DNAase activity, enough to produce a calcaulated .001 knicks per T7 DNA molecule per 20 minutes. The number of initiations observed was of the order of 10 starts per DNA molecule per 20 minutes. The rate of initiation is half as rapid as that observed by Davis and Hyman (1970), who used twice the nucleotide concentration.

DISCUSSION

(i) The role of σ.

We have shown that the σ sub-unit is required for initiation on T7 DNA as has already been shown for the transcription of ϕ E DNA (Losick and Sonenshein, 1969) and the early genes of ϕ T4 DNA (Travers, 1970). The rate of synthesis on T7 DNA is stimulated up to 8 times by the addition of σ to an enzyme preparation which has only 2.6 mole per cent σ. The plot of stimulation versus σ added shows no signs of levelling off, but the highest amount added (.48 μg σ per μg β) did not exceed the saturation value of Travers and Burgess (1969) of .57 μg σ per μg β for the complete enzyme. Summers and Siegel (1969) have shown that (core + σ) enzyme transcribes T7 DNA assymmetrically and that only early genes are transcribed.

In contrast σ was found to be very ineffective at stimulating the transcription of E. coli DNA. The highest stimulation observed was 40%, much less than that for T7 DNA (800%). When T7 and E. coli DNAs are compared directly for their template activity in the presence of σ T7 is at least 700% more efficient. Both templates were of high molecular weight (greater than 20 x 10^6) and had been prepared by standard phenol extractions. It is hard to quantitate the degree of knicking of a heterogeneous DNA preparation such as that from E. coli, but there can be little doubt that it contains more knicks than T7 DNA. Its ability to prime

poly A synthesis which reflects the degree of single-strandedness is 25 times that of T7 DNA (Part II, Chapter I of this thesis). Single-stranded regions are expected to prime the initation of RNA synthesis as has been shown for knicks (Vogt, 1969), so that if anything the relative template activity of the E. coli DNA is an upper estimate. It is therefore concluded that the frequency of promoters specific for σ is lower on E. coli DNA than on T7.

For the case of T7 the number of σ-specific promoters is probably less than four and some workers have observed only one (Davis and Hyman, 1970) for a molecular weight of 23×10^6. Assuming that T7 DNA has 2 σ-specific promoters, that the molecular weight of E. coli DNA is 1.8×10^9 (Cairns, 1963), and that % stimulation of synthesis by σ on a particular template is a direct measure of the number of σ-specific promoters, then one molecule of E. coli DNA has 8 such promoters.

Similar results have been reported for T4 DNA using different techniques (Su et al., 1970). They showed that if RNA polymerase was added to a mixture containing a 25:1 ratio of E. coli DNA to T4 DNA that 80% of the RNA synthesised hybridized to T4 DNA. If the DNA samples were denatured before the RNA was synthesized then 80% of the RNA hybridised to E. coli DNA.

The in vitro experiments on T4 and T7 can be interpreted if we postulate that the early phage genes carry promoters

with high affinity for the "complete" E. coli RNA polymerase (core + σ). The preference for T4 DNA displayed in vitro is sufficient to account for the selective transcription in vivo (Su, et al., 1970). By measurements on E. coli DNA we have shown that it contains a small number of σ-specific promoters.

Other mechanisms have been proposed to explain the preferential transcription of ø DNA, for example specific degradation of the host DNA. Hausmann and Gomez (1967) have found for T7 that this requires transcription of T7 gene 1, while Summers (1969 b) reported that the kinetics of the shut-down of host RNA synthesis are the same whether or not gene 1 is active. It is therefore concluded that the ø DNA can monopolise the bacterial RNA polymerase without degradation of the host DNA.

E. coli RNA polymerase has been isolated by a great variety of techniques, and with one exception these methods have not been shown to remove σ (Burgess, 1969). Chromatography on DEAE cellulose, DNA cellulose, hydroxyapatite, sedimentation at high and low salt concentration, precipitation with ammonium sulphate, protamine, and streptomycin, phase separation and other techniques yield RNA polymerase which contains σ. The one exception is the use of phosphocellulose, which, it is proposed, mimics the surface properties of the DNA (Burgess, 1969), so dissociating σ and core which is known to occur when RNA synthesis is initiated

(Travers and Burgess, 1969). Unless initiation, or something rather similar takes place, it is likely that σ binds strongly to the core enzyme. It is therefore expected that much of the core enzyme in the cell will have σ attached and will initiate on T7 DNA at a high frequency, as occurs in vitro.

In contrast to the recovery of σ sub-unit with the core enzyme, other control factors have not been identified in the usual RNA preparations. Recently, such a control factor ψ has been identified in extracts of E. coli after infection with $\phi\beta$ (Kamen, 1970; Travers and Kamen, 1970). A polypeptide coded by the E. coli genome, was isolated as a sub-unit of the $\phi\beta$ RNA polymerase. When added to the "complete" E. coli RNA polymerase (core + σ) it stimulated the transcription of the E. coli ribosomal RNA genes to the extent that 20% of the RNA made was ribosomal. Since ψ is not isolated with the core enzyme, it may be postulated that it has a low affinity for it, perhaps interacting directly with the DNA, or that it is present only in low concentrations. Alternatively, one of the steps common to all isolation procedures for RNA polymerase such as ammonium sulphate precipitation may separate core from ψ .

The T7 gene 1 promoter is transcribed very efficiently by (core + σ), whereas the E. coli ribosomal genes are only transcribed at high frequency when both σ and ψ are added to core. It is implied that the ribosomal gene promoter has one region in common with the T7 gene 1 promoter, at which

σ interacts, and another region absent from the gene 1 promoter, at which ψ interacts. Therefore the ribosomal gene promoter may be considered bipartite and the T7 gene 1 promoter as a single unit. Since the E. coli genome has probably less than 8 promoters at which (core + σ) initiates frequently, it is implied that most E. coli promoters are bipartite and require the presence of a 2° control factor such as ψ for efficient transcription. The facts that σ binds tightly to core enzyme, so that most RNA polymerase molecules probably exist in vivo as (core + σ), and that the phage early promoters do not require 2° control factors in order to bind RNA polymerase effectively are sufficient to account for preferential transcription of ø genes.

It has been observed by several groups that higher organism and animal virus DNAs are transcribed assymmetrically by E. coli RNA polymerase in vitro, and that the RNA synthesised is competed out by in vivo RNA in hybridisation experiments (Paul and Gilmour, 1966; Bekhor et al., 1969; Tan and Miyagi, 1970; Reeder and Brown, 1970; Westphal, 1970). These results are simply explained if it is postulated that the strand selection sequence has been conserved. We have observed that the presence of σ stimulates the transcription of ascites tumour DNA by 300%. Burgess et al., (1969) observed a stimulation of 260% on calf thymus DNA. It is apparent that higher organism DNAs contain σ-specific promoters. Taken together, the assymmetric transcription and the presence

of σ-specific promoters in higher organism DNA suggest that σ may be responsible for strand selection on this DNA. It is known that in the absence of σ symmetric transcription occurs on ϕ fd and T4 (Suguira et al., 1970; Bautz, et al.,1969).

These observations on bacterial, phage, higher organism and animal viral DNAs can be explained by the following postulates:

1. The σ sub-unit of E. coli RNA polymerase may be a strand selector.
2. A sequence at which strand selection can be made by σ may have been conserved.
3. A 2^{o} control factor may be required in addition to σ in order to transcribe most E. coli genes.
4. Such additional factors may not be required to transcribe the early genes of T7 and T4.

(ii) Initiation of synthesis on T7 DNA by E. coli RNA polymerase.

We have found that both GTP and ATP are incorporated as the 5' terminal nucleotide of RNA made in vitro on T7 DNA. Stevens (1969) and Bautz and Bautz (1970) have reported similar results. We have used highly purified RNA polymerase of specific activity 850 units per mg, and T7 DNA of which 70% of the single-strands were unknicked. The RNA polymerase has a very low amount of DNAase activity, measured by an assay which detects single breaks in double-stranded SV40 closed-circular DNA. Under the conditions of the assay, by 20

minutes there was sufficient DNAase to produce .001 knicks per T7 DNA molecule, while an average of 10 RNA chains had been initiated per DNA in that time. It cannot be ruled out that some of these knicks contributed to the initiations. 60% of the T7 molecules had a knick at zero time, amounting to 23 % of potential initiator sites if there are two "early"promoters per molecule. By 20 minutes later the DNAase activity would have increased the number of knicks by a negligible amount. Electron micrographs of RNA polymerase which had initiated on T7 DNA showed that more than 95% of the enzyme molecules were located in the left half of the DNA molecule (Chou, 1970) where a high frequency promoter has been physically mapped (Davis and Hyman, 1970). This suggests that if initiation occurs at knicks, which should be distributed randomly, then it happens at low frequency.

Polyphosphate kinase is a common contaminant of RNA polymerase preparations (This thesis, Part II, Chapter I). It incorporates the γ phosphate from ATP into polyphosphate which is acid precipitable in the presence of protein (Kornberg et al., 1956). If present and active the apparent incorporation of γ phosphate from ATP is artifactually high, so that precautions must be taken to control its activity. The reaction was therefore carried out in the presence of .02 mM ADP which completely inhibits this enzyme (This thesis, Part II, Chapter I).

The γ phosphate of both ATP and GTP was incorporated to

a significant extent, increasing with time in a linear fashion, at both high and low salt. Starts with ATP are 2.5 times more frequent than those with GTP at low salt, but only 1.8 times as frequent at high salt. In a high salt assay, Stevens (1969) found that ATP was 1.5 times more frequent as GTP. Bautz and Bautz (1970) observed about equal incorporation of 5' terminal A and G, but their assay measured initiation resistant to rifampicin, and so may not be comparable. Stevens (1969) has reported that only 2% of the RNA molecules initiate with C or U.

The observation that both G and A can initiate synthesis is consistent with the presence of at least two promoters. Stevens (1969) has analysed the 5' terminal dinucleotides, of which 6 were found: pppAU (25%), pppAC (34%), pppGU (9%), pppGA (16%), pppGG (4%) and pppGC (10%). Davis and Hyman (1970) identified only one initiation site by electron microscopy, but, in the presence of ρ (Roberts, 1969) they found two species of RNA of molecular weights $.94 \times 10^6$ and $.45 \times 10^6$ daltons. Siegel and Summers (1970) identified 4 species of RNA of molecular weights 1.1×10^6, $.45 \times 10^6$, $.29 \times 10^6$ and $.20 \times 10^6$ daltons which were synthesised in vivo in the presence of chloramphenicol. Davis and Hyman (1970) may not have detected the 2 low molecular weight species by their technique.

Davis and Hyman (1970) proposed that there may only be a single promoter at .013 map units from the left end of the

molecule, that four cistrons are read sequentially and that when $\int$ is present, four separate RNA molecules are released at four successive termination sites. The RNA polymerase is released at the fourth site.

Since both A and G initiate synthesis on T7, and since six 5' terminal dinucleotide sequences have been observed it is necessary to make additional assumptions to account for all of the initiations. There may be only one promoter at which the enzyme binds, but several adjacent nucleotide sequences on which it can initiate transcription. If so, these sequences do not start with T-T or T-C (Stevens, 1969). Alternatively, a 5' triphosphate is used to start each new RNA chain after termination by $\int$, so that $\int$ functions as a release agent, not as an endonuclease. This would account for 4 of the 6 observed 5' terminal dinucleotides. The others, one of which occurs at low frequency (pppGG is 4%) may be due to variation in one of the initiation sequences. Stevens (1969) provides no information on the integrity of the template, or the DNAase activity of the RNA polymerase which she used.

(iii) <u>The formation of a salt and rifampicin stable complex between T7 DNA and E. coli RNA polymerase</u>.

Three assays have been used to detect the formation of a stable complex between RNA polymerase and T7 DNA; (1) co-sedimentation of enzyme and DNA through high salt ($\tau/2 > 1.2$) glycerol gradients; (ii) retention of enzyme and DNA on Millipore membranes in the presence of high salt ($\tau/2 > 1.2$) and (iii) the formation of a rifampicin-resistant

complex. All of these assays measure initiation since in each case little or no complex is detected if RNA polymerase and rifampicin are added simultaneously. For each assay we have shown that the amount of complex is considerably increased if enzyme and DNA are preincubated with one or more nucleoside triphosphates. Qualitatively each assay provided the same result, namely that when tested alone the four ribonucleoside triphosphates (commercial preparations) stimulated the formation of complex in the order of efficiency GTP $\succ$ CTP $>$ ATP $>$ UTP $\geq$ none. The results are summarised in Table 10. There are considerable quantitative differences between the 3 assays. The co-sedimentation assay was affected by two variables not associated with either of the others, firstly the non-reproducible recovery of enzyme activity from the gradients, and secondly, the much greater length of time (18-24 hr) during which the complex had to remain stable in order to be detected. Davis and Hyman (1970) have observed that 50% of RNA-DNA-enzyme complexes dissociate in 24 hr. This experiment has only been carried out once, so no quantitative conclusions may be drawn from it. The other assays have been repeated at least twice each. Some variation was observed in the Millipore assay which depended on the length of time allowed between raising the salt concentration and filtering. The values given are for an experiment in which five minutes at $0^{\circ}C$ elapsed before filtering. Considerable differences are found for the relative effectiveness of CTP and ATP in

Table 10

Summary. Effect of nucleotides on complex formation assayed by three methods. The values are abstracted from Tables 3, 6a, and 7b and measure the effectiveness of each nucleotide in forming the complex in the three assays relative to the effect of GTP[=100].

Table 10. Summary. Effect of nucleotides on complex formation assayed by three methods

	Sedimentation analysis	Millipore assay	Rifampicin resistance
G	100	100	100
C	93	51	21
A	20.4	53	8
U	-	1.5	4

the Millipore and rifampicin assays, which may reflect intrinsic properties of the dissociating agents. GTP is consistently the best substrate, and UTP the worst in both assays.

Several pieces of evidence show that complex formation involves propagation in addition to initiation. Firstly, 50% of the counts which co-sediment with enzyme in the T7 DNA peak were recovered as oligonucleotides after DEAE Sephadex chromatography. The fact that 40% of the counts were not eluted by 1M NaCl is consistent with their being large RNA molecules. In a different experiment between 60 and 100% of the counts in the DNA peak were found to be TCA precipitable. Furthermore while ^{3}H GTP counts were readily observed co-sedimenting as a clear peak with the T7 DNA, no $\gamma^{32}P$ GTP was found in the same peak, although it would have been detected if the ratio of 5' terminal to internal nucleotides had been more than .2.

Secondly, the efficiencies of complex formation (assayed by Millipore filters or by rifampicin resistance) for different combinations of nucleotides are greater than additive. The synergism can be explained if propagation is required, since one nucleotide would then contribute to initiation at its own "specific" site and to propagation away from sites at which the 5' terminal is a different nucleotide. Freeman and Jones (1967) have presented evidence that more than one enzyme must be bound per DNA for it to be retained on the

Millipore filter.

Thirdly, streptolydigin, an inhibitor of propagation inhibits complex formation . This should be interpreted cautiously since it has not been shown that it does not block initiation (the synthesis of the first phosphodiester bond).

All of the experiments summarised above were carried out with commercial preparations of ribonucleotides. Further evidence that propagation is required to obtain a stable complex, comes from the fact that where ATP or GTP is purified by a single passage through Dowex-1x8, the rate of complex formation is reduced, to background for ATP and to 1.5 times background for GTP, from 2.3 times and 4 times respectively. Commercial preparations of UTP have low activity in the assays without additional purification, and it seems likely that the efficiency of CTP and GTP would be reduced to background levels on further purification.

From the results using purified ATP,the concept that a salt- or rifampicin-stable complex can form in the presence of a known 5' nucleotide,RNA polymerase and DNA is shown not to be correct. Propagation is required, involving the synthesis of at least one phosphodiester bond. The results of Anthony et al., (1966) and di Mauro et al., (1969) must be reconsidered since they did not report any purification of their nucleoside triphosphates. If their nucleotides were pure then homopolymer synthesis is implied perhaps at

abnormal initiation sites.

Our results have not ruled out the possibility that the efficient formation of complex by GTP and CTP may occur even after extensive purification by homopolymer synthesis. Stretches of poly dG.dC do occur on T7 DNA. Summers and Szybalski (1968 b) suggested that 30-75 poly UG binding sites, thought to contain a high proportion of C, were present on the r strand of T7 DNA. They estimated that these stretches were 15-40 nucleotides long. However, it has recently been established that there are no stretches of poly dC (or poly dG) more than 6 units long (Mushynski and Spencer, 1970 a) and there is only 1 of these per T7 molecule on the r strand. These authors have found in addition that there are 2 stretches of hexathymidine both on the l strand. If homopolymer synthesis were to occur on the hexa (dG.dC) in the formation of complex it would require additional assumptions to explain why it does not occur on the stretches of hexa (dA.dT).

Mushynski and Spencer (1970 a,b) have obtained a frequency distribution of isostichs for both T7 and λ, revealing a more complicated picture than first envisaged by Szybalski et al., (1966). They analysed whole DNA, and the left and right strands in an effort to correlate the presence of a particular isostich with those strands which are transcribed. The case of T7 is simplest since only the r strand is transcribed (Summers and Szybalski, 1968 a). For this DNA 10 isostiches

of 11, 12, or 13 nucleotides each, containing together on an average about equal amounts of C and T, were found to be unique to the r strand. Their work did not rule out the possibility that long stretches of C or T are present separated at intervals (of less than seven) by either A or G.

REFERENCES

Anthony, D.D., Zeszotek, E., and Goldthwait, D.A., (1966). Proc. Natl. Acad. Sci., Wash., 56, 1026.

Anthony, D.D., Zeszotek, E., and Goldthwait, D.A. (1969)a Biochem. Biophys. Acta, 174, 458.

Anthony, D.D., Wu, C.W., and Goldthwait, D.A., (1969)b Biochemistry 8, 246.

Bautz, E.K.F., and Bautz, F.A., (1970). Nature, 226, 1219.

Bautz, E.K.F., Bautz, F.A., and Dunn, J.J., (1969). Nature 223, 1022.

Bekhor, I., Kung, G.M., and Bonner, J., (1969). J. Mol. Biol. 39, 351.

Berg,P., Kornberg, R.D., Fancher, H., and Dieckmann, M., (1965). Biochem. Biophys. Res. Comm. 18, 932.

Burgess, R.R. (1969). J. Biol. Chem. 244, 6160.

Burgess, R.R., Travers, A.A., Dunn, J.J., and Bautz, E.K.F., (1969). Nature, 221, 43.

Cairns, J., (1963). J. Mol. Biol. 6, 208.

Chamberlin, M., McGrath, J., and Waskell, L. (1970). Personal communication.

Chou, L., (1970). Personal Communication.

Davis, R.W., and Hyman, R.W. (1970). Personal Communication.

de St. Groth, S.F., Webster, R.G., and Datyner, A., (1963). Biochim. Biophys. Acta, 71, 377.

di Mauro, E., Snyder, L., Marino, P., Lamberti, A., Coppo,A., and Tocchini-Valentini, G.P. (1969) Nature, 222, 533.

Elgin, S. (1970) Personal communication.

Fox, C.F., Gumport, R.I., and Weiss, S.B., (1965). J. Biol. Chem., 240, 2101.

Freeman, E.J., and Jones, O.W., (1967). Biochem. Biophys. Research Comm. 29,45.

Fuchs, E., Millette, R.L., Zillig, W., Walter, G., (1967). Europ. J. Bioch. 3, 183.

Geiduschek, E.P., Wilson, D.L., and Gage, L.P., (1969). J. Cell Physiol., 74, Sup.1, 81.

Gilbert, W., and Muller-Hill, B., (1966). Proc. Natl. Acad. Sci., Wash., 56, 1891.

Gray, H.B., and Hearst, J.E. (1968). J. Mol. Biol., 35,111.

Green, M., Parsons, J.T., Pina, M., Fujinaga, K., Caffier , H., and Landgraf-Lewis, I. (1970) Cold Spring Harbour Symp. for Quantitative Biology. Abstracts.

Hatlen, L.E., Amaldi, F., and Attardi, G., (1969). Biochemistry, 8, 4989.

Hausmann, R., Gomez, B., (1967). J. Virol. 1, 779.

Hurlbert, R.R., Schmitz, H., Brumm, A.F., and Potter, V.R., (1954). J. Biol. Chem. 209, 23.

Hyman, R.W., and Davidson, N., (1970). J. Mol. Biol., 50, 421.

Jones, O.W., and Berg, P., (1966). J. Mol. Biol. 22, 199.

Kadoya, M., Mitsui, H., Takegi, Y., Otaka, E. Suzuki, H., and Osawa, S., (1964). Biochim. Biophys. Acta, 91,36.

Kamen, R., (1970), Cold Spring Harbour Phage Meeting, Abstract.

Kameyama, T., Matsukage, A., and Tanaka, Y., (1969) J. Cell. Physiol. 75, Sup. 1, 219.

Kates, J., and Beeson, J., (1970). J. Mol. Biol. 50, 19.

Kornberg, A., Kornberg, S.R., and Simms, E.S. (1956). Biochim. Biophys. Acta., 20, 215.

Labaw, L.W., (1951). J. Bacteriol. 62, 169.

Labaw, L.W., (1953). J. Bacteriol., 66, 429.

Losick, R., and Sonenshein, A.L. (1969). Nature, 224, 35.

Lunan, K.D., and Sinsheimer, R.L., (1956). Virology, 2, 455.

Maitra, U., and Hurwitz, J., (1965). Proc. Natl. Acad. Sci. Wash., 54, 815.

Martin, R.G., (1969). Ann. Rev. Genetics, 3, 181.

Matsukage, A., Murakami, S., and Kameyama, T., (1969), Biochim. Biophys. Acta, 179, 145.

Millette, R.L., and Trotter, C.D., (1970). Proc. Nat. Acad. Sci., Wash., 66,701.

Mushynski, W.E., and Spencer, J.H., (1970) a. J. Mol. Biol., 52, 91.

Mushynski, W.E., and Spencer, J.H., (1970) b. J. Mol. Biol., 52, 107.

Naono, S., and Tokuyama, K., (1970). Cold Spring Harbour Symp. Quant. Biol. Abstract.

Paul, J., and Gilmour, R.S., (1966). J. Mol. Biol. 15, 160.

Pettijohn, D., and Kamiya, T., (1967). J. Mol. Biol., 29, 275.

Ptashne, M., (1967). Proc. Nat. Acad. Sci., Wash., 57, 306.

Putnam, F.W., Miller, D., Palm, L., and Evans, E.A., Jr., (1952) J. Biol. Chem., 199, 177.

Reeder, R.H., and Brown, D.D., (1970) J. Mol. Biol. 51, 361.

Richardson, C.C., Inman, R.B., and Kornberg, A., (1964) J. Mol. Biol., 9, 46.

Richardson, J.P. (1966)a J. Mol. Biol., 21, 115.

Richardson, J.P., (1966)b, Proc. Natl. Acad. Sci., Wash., 55, 1616.

Ritchie, D.A., Thomas C.A.,Jr.,MacHattie,L.A., and Wensinck,P. C., (1967) J. Mol. Biol. 32, 365.

Roberts, J.W., (1969). Nature, 223, 680.

Rubenstein, I., (1968). Virology, 36, 356.

Schleif, R., (1969). Nature, 223, 1068.

Schmid, C.W., and Hearst, J.E. (1969). J. Mol. Biol., 44, 143.

Sentenac, A., Simon, E.J., and Fromageot, P., (1968). Biochim. Biophys. Acta., 161, 299.

Siegel, R.B., and Summers, W.C., (1970). J. Mol. Biol., 49, 115.

Silverstone, A.E., Magasanik, B., Reznikoff, W.S., Miller, J.H., and Beckwith, J.R., (1969). Nature, 221, 1012.

Sippel, A., and Hartmann, G., (1968) Biochim. Biophys. Acta, 157, 218.

Stead, N.W., and Jones, O.W., (1967) Biochim. Biophys. Acta, 145, 679.

Stevens, A., (1969). J. Cell. Physiol., 74, sup.1, 205.

Straat, P.A., and T'so, P.O.P., (1970). Biochemistry, 9, 926.

Su, S., Weinberg, F., So, A.D., and Davie, E.W., (1970) Nature, 225, 62.

Sugiura, M., Okamoto, T., and Takanami, M., (1970). Nature 225, 598.

Summers, W.C., (1969)a Virology, 39,175.

Summers, W.C., (1969)b. Lepetit Colloquia on Biology and Medicine. 1. RNA polymerase and Transcription (ed. Silvestri, L.,) p,110.

Summers W.C., and Siegel, R.B., (1969). Nature, 223, 1111.

Summers, W.C., and Szybalski, W., (1968)a. Virology 34,9.

Summers, W.C., and Szybalski, W., (1968) b. Biochim. Biophys. Acta, 166, 371.

Studier, F.W., (1965). J. Mol. Biol., 11, 373.

Studier, F.W., (1969). Virology, 39, 562.

Studier, F.W., and Maizel, J.V., Jr., (1969). Virology 39,575.

Szybalski, W., (1969)a, J.Cell. Physiol., 74, Sup. 1, 33.

Szybalski, W., (1969)b. Canadian Cancer Conference (National Cancer Institute of Canada). Pergamon Press, Oxford, Vol. 8., 183.

Szybalski, W., Kubinski, H., and Sheldrick, P., (1966). Cold Spring Harbour Symp. Quant. Biol., 31, 123.

Tan, C.H., and Miyagi, M., (1970). J. Mol. Biol. 50, 641.

Taylor, K., Hradecna, Z., and Szybalski, W., (1967). Proc. Natl. Acad. Sci., Wash., 57, 1618.

Travers, A.A., (1970). Nature 225, 1009.

Travers, A.A. and Burgess, R.R., (1969). Nature 222, 537.

Travers, A.A., and Kamen, R., (1970). Cold Spring Harbour Phage Meeting, Abstract.

Vogt., V., (1969). Nature, 223, 854.

Westphal, H., (1970). J. Mol. Biol., 50, 407.

Wilson, D.L., and Geiduschek, E.P., (1969). Proc. Natl. Acad. Sci., Wash., 62, 514.

Wu, C.W., and Goldthwait, D.A. (1969)a. Biochemistry, 8, 4450.

Wu, C.W., and Goldthwait, D.A., (1969)b. Biochemistry 8, 4458.

Yarus, M., (1969). Ann. Rev. Bioch., 38, 841.