PURIFICATION AND CHARACTERIZATION OF BACTERIOPHAGE gh - 1 - INDUCED DEOXYRIBONUCLEIC ACID - DEPENDENT RIBONUCLEIC ACID POLYMERASE FROM PSEUDOMONAS PUTIDA

Dissertation for the Degree of Ph. D.
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PURIFICATION AND CHARACTERIZATION OF BACTERIOPHAGE
gh-1-INDUCED DEOXYRIBONUCLEIC ACID-DEPENDENT
RIBONUCLEIC ACID POLYMERASE FROM
PSEUDOMONAS PUTIDA

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Howard Colgate Towle

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Major professor

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ABSTRACT

PURIFICATION AND CHARACTERIZATION OF BACTERIOPHAGE gh-1-INDUCED DEOXYRIBONUCLEIC ACID-DEPENDENT RIBONUCLEIC ACID POLYMERASE FROM *PSEUDOMONAS PUTIDA*

By

Howard Colgate Towle

This research was divided into three distinct sections, each involving the enzymology of nucleic acid synthesis in a different system. The first and major portion of this research involved a study of the induction of a novel DNA-dependent RNA polymerase after the infection of *Pseudomonas putida* by the virulent bacteriophage gh-1. The second section involved research on the DNA-dependent RNA polymerases of Novikoff hepatoma cells and the inhibition of these RNA polymerases by the structural analogs of ATP, 3'-deoxyadenosine 5'-triphosphate and 3'-*O*-methyladenosine 5'-triphosphate. Finally, the characterization of a novel DNA polymerase induced after Marek's disease herpesvirus infection of duck embryo fibroblasts constituted the third section of this research.

The infection of a bacterial cell with a virulent bacteriophage provides a useful model system for studying the control of genetic expression. The bacteriophage gh-1 is a small, virulent phage of *P.*

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not consistent with the action of 3'-dA as a selective inhibitor of ribosomal RNA synthesis in whole cells. Inhibition of *in vitro* RNA synthesis by RNA polymerases I and II by 3'-*O*-methyladenosine 5'-triphosphate was also tested. The apparent Ki values for this analog of ATP were 5- to 6-times higher than those for 3'-dATP for both enzymes.

Infection of duck embryo fibroblasts by Marek's disease herpesvirus (MDHV) led to the induction of a novel DNA polymerase. The MDHV-induced DNA polymerase could be distinguished from the DNA polymerases of uninfected duck embryo fibroblasts by its chromatographic behavior on phosphocellulose, by its sedimentation coefficient, and by its catalytic properties. The MDHV-induced DNA polymerase eluted from phosphocellulose at 0.2 *M* KCl, ahead of the DNA polymerases of uninfected duck embryo fibroblasts. The sedimentation coefficient of the viral-induced DNA polymerase, as determined by sucrose density gradient centrifugation at 0.25 *M* KCl, was 5.9S. The sedimentation coefficient of DNA polymerases from uninfected duck embryo fibroblasts were 3.1, 7.3, and 8.0S. MDHV-induced DNA polymerase could not effectively utilize either poly(dA)•oligo(dT) or poly(dC)•oligo(dG) as template-primers. The DNA polymerases from uninfected duck embryo fibroblasts could use these synthetic template-primers. The MDHV-induced DNA polymerase was shown not to be a polymerase of the type R-DNA polymerase, a reverse transcriptase, or a terminal nucleotidyl transferase.

putida with a linear double-stranded DNA having a molecular weight of 23 X 10^6. The infection of _P. putida_ by the phage gh-1 induced the synthesis of a novel DNA-dependent RNA polymerase. This gh-1-induced RNA polymerase was purified to near homogeneity by chromatography on DEAE-cellulose, phosphocellulose, and Bio-Gel P-200, followed by sedimentation velocity centrifugation in a glycerol gradient. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified gh-1 polymerase revealed that it was composed of a single polypeptide with a molecular weight of approximately 98,000. This value is consistent with the molecular weight of gh-1 polymerase calculated from the experimentally determined values for its sedimentation coefficient of 6.1S and its molecular Stokes radius of 38 angstroms. In contrast, the host _P. putida_ RNA polymerase is composed of five subunits ($\alpha_2\beta\beta'\sigma$) having a combined molecular weight of 506,000.

The gh-1 polymerase was also distinct from the host _P. putida_ RNA polymerase with respect to many of its catalytic properties. The gh-1 polymerase would only utilize Mg^{2+} to satisfy its divalent cation requirement, whereas the host polymerase could utilize Mg^{2+} or Mn^{2+}. The activity of the gh-1 polymerase was inhibited markedly by the addition of monovalent ions to the _in vitro_ RNA synthesis reaction mixture at concentrations which did not affect the host polymerase activity. The bacterial RNA polymerase inhibitors, rifampicin and streptolydigin, were not inhibitors of the gh-1 polymerase activity. The gh-1 polymerase showed a highly specific template requirement for DNA from the homologous gh-1 phage. Low levels of gh-1 polymerase activity were observed when the pyrimidine-containing synthetic

polymers were used as templates. The host RNA polymerase would utilize efficiently as a template every DNA with which it was tested. Finally, the gh-1 polymerase activity was very sensitive to inhibition by the ATP analog, 3'-deoxyadenosine 5'-triphosphate (3'-dATP), compared to the host RNA polymerase activity and RNA polymerases from a eukaryotic source. The structure and catalytic properties of the gh-1-induced RNA polymerase were very similar to those reported for RNA polymerases induced in _Escherichia_ _coli_ after infection by the bacteriophages T3 or T7.

3'-Deoxyadenosine (3'-dA) has been found to inhibit the synthesis of ribosomal precursor RNA in certain eukaryotic cells when present at concentrations which do not affect heterogenous nuclear RNA synthesis. A reasonable hypothesis on how this selective inhibition occurs is that the enzyme responsible for ribosomal RNA synthesis (RNA polymerase I) is more sensitive to the triphosphate derivative of 3'-dA than the enzyme responsible for heterogenous nuclear RNA synthesis (RNA polymerase II). To test this hypothesis, RNA polymerases were partially purified from Novikoff hepatoma cells. These RNA polymerases were classified as I and II on the basis of their order of elution from DEAE-Sephadex and the response of their enzymatic activities to changes in ionic strength, Mn^{2+} or Mg^{2+} as divalent metal ions, and the fungal toxin, α-amanitin. The inhibition of both RNA polymerases I and II by 3'-dATP was competitive with ATP. The apparent Ki values for this ATP analog were 1.4×10^{-5} _M_ for RNA polymerase I and 7×10^{-6} _M_ for RNA polymerase II. Thus, the relative sensitivities to 3'-dATP of _in_ _vitro_ RNA synthesis by the two RNA polymerases are

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By

Howard Colgate Towle

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DEDICATION

To My Parents

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LIST OF ABBREVIATIONS

SDS	sodium dodecyl sulfate
3'-dATP	3'-deoxyadenosine 5'-triphosphate
3'-AmTP	3'-O-methyladenosine 5'-triphosphate
3'-dA	3'-deoxyadenosine
MDHV	Marek's disease herpesvirus
DEF	duck embryo fibroblasts

GENERAL INTRODUCTION

The cellular regulation of the expression of genetic information is one of the most important and intriguing problems of modern molecular biology. The control of genetic expression is the basis of such fundamental processes as cellular development, cellular response to certain external stimuli (e.g., hormones), and possibly many human diseases (e.g., cancer). Although there are numerous sites at which genetic expression can be controlled, one of the prime targets would have to be the enzymes responsible for the biosynthesis of the informational DNA and RNA molecules, the DNA-dependent DNA polymerases and DNA-dependent RNA polymerases. This thesis involves research on the nucleic acid-synthesizing enzymes of three different biological systems: the RNA polymerases present after the infection of a bacterial cell, *Pseudomonas putida*, by the virulent bacteriophage gh-1; the RNA polymerases involved in the synthesis of different classes of RNA in Novikoff hepatoma cells; and the DNA polymerases present after the infection of duck embryo fibroblasts with the Marek's disease herpesvirus.

The first section of this thesis involves the infection of *P. putida* with the bacteriophage gh-1. In bacteria, RNA synthesis is thought to involve only a single, albeit complex, DNA-dependent RNA polymerase for the synthesis of all classes of cellular RNA. When a bacterial cell becomes infected by a virulent bacteriophage, a dramatic shift occurs in the type of RNA being synthesized from entirely bacterial RNA to largely phage-specific RNA. The different types of mechanisms of the regulation of genetic expression seen in a variety of bacteriophage infections are discussed in the literature survey. Following the literature survey, the results of work on the phage gh-1 infection of *P. putida* are presented. The isolation and biochemical properties of a novel gh-1-induced DNA-dependent RNA polymerase are described. A large portion of the contents of this section has been submitted to *Biochemistry* for publication. A preliminary report of some of the data on the gh-1-induced RNA polymerase was presented at the 57th Annual Meeting of the Federation of American Societies for Experimental Biology (Towle, H. C.; Jolly, J. F.; and Boezi, J. A. [1973], *Federation Proceedings* *32*, 645).

During the course of study on the gh-1-induced RNA polymerase, it was observed that the ATP analog, 3'-deoxyadenosine 5'-triphosphate (3'-dATP), could inhibit the gh-1 polymerase activity at concentrations which had no affect on the host *P. putida* RNA polymerase activity. This observation stimulated interest on the effects of 3'-dATP on the RNA polymerases of eukaryotic cells. It was known that the administration of the antibiotic, 3'-deoxyadenosine (3'-dA), to HeLa cells resulted in the selective inhibition of ribosomal RNA production. Since distinct RNA polymerases are responsible for the synthesis of ribosomal RNA and other classes of RNA, it was reasonable to postulate that the differential inhibition by 3'-dA might be due to the greater sensitivity of the nucleolar RNA polymerase to the triphosphate derivative of this drug. Consequently, studies were initiated in collaboration with Ron Desrosiers and Dr. Fritz Rottman of this department on the RNA polymerases of Novikoff hepatoma cells. The RNA polymerases of Novikoff hepatoma cells were separated, partially purified, and characterized with respect to many of their catalytic properties. These RNA polymerases were then tested to determine the effect of 3'-dATP on *in vitro* RNA synthesis by the enzymes. The results of these experiments are presented in the form of a scientific paper in the second section of this thesis.

The final section of this thesis involves a study of the DNA polymerases of uninfected and Marek's disease herpesvirus (MDHV)-infected duck embryo fibroblasts. These studies were performed with Dr. Lucy Lee of the USDA Regional Poultry Research Laboratory, East Lansing, Michigan, and Mark Koenig, Robert Blakesley, and Dr. John Boezi of this laboratory. Marek's disease is a highly contagious, malignant lymphoma of chickens whose etiological agent is a herpesvirus. In the acute form of the disease, lymphoid tumors develop in the abdominal organs, especially the gonads, liver, kidneys, and lungs. Marek's disease herpesvirus can be propagated in culture in duck or chicken embryo fibroblasts. Viral infectivity is cell associated and free virus particles are not produced. The DNA polymerases of uninfected and MDHV-infected duck embryo fibroblasts were examined. A novel DNA polymerase was found in the MDHV-infected duck embryo fibroblasts. The results of these studies are presented as a summary of a paper which has been submitted for publication.

SECTION I

PURIFICATION AND CHARACTERIZATION OF BACTERIOPHAGE gh-1-INDUCED DNA-DEPENDENT RNA POLYMERASE FROM *PSEUDOMONAS PUTIDA*

LITERATURE SURVEY

Introduction

The development of a bacteriophage in a susceptible host cell provides a very useful model system for the study of the regulation of genetic expression. The genomic content of bacteriophages is relatively small compared to that of bacteria and eukaryotic organisms. All bacteriophages, however, regulate the temporal order with which phage-specific proteins appear during the lytic cycle. A wide range of biochemical and genetic studies indicates that most regulation takes place at the level of DNA transcription (Calendar, 1970). Thus, the sequential appearance and disappearance of different classes of viral proteins is a direct consequence of turning-on and turning-off of the transcription of the appropriate classes of viral mRNA. These facts, taken together with the relative ease with which bacteriophages can be studied both biochemically and genetically, have led to a great amount of research using bacteriophage systems to study the regulation of gene control.

The purpose of this survey is to examine the molecular mechanisms involved in the control of the expression of the phage genome. First, the patterns of *in vivo* transcription of several bacteriophage infections will be examined to learn what type of changes occur during bacteriophage development. Next, the *in vitro* transcription of bacteriophage DNA using the isolated components of the transcription machinery will be studied. Finally, attempts will be made to correlate the *in vivo* and *in vitro* patterns of transcription in terms of the molecular mechanisms operating to control gene expression.

In hypothetical terms, there are two general sites at which control can be exerted on transcriptional activity. The first is the DNA molecule, itself. By physical modification of the structure of the DNA or by binding of a specific protein at given sites in the DNA, the ability of the DNA molecule to act as a template for RNA polymerase could be altered. The second site at which transcriptional activity may be controlled is the DNA-dependent RNA polymerase. Such mechanisms could involve the alteration of the specificity of the pre-existing RNA polymerase by chemical modification or binding of transcriptional factors. Alternatively, the *de novo* synthesis of a new RNA polymerase with a different transcriptional specificity than the pre-existing enzyme could result in changing the pattern of transcription. In this survey, examples of mechanisms exerting control at both these potential sites will be discussed.

This survey will be divided into sections on the basis of the various bacteriophages to be examined. Discussion will be limited to the double-stranded DNA-containing bacteriophages. (For review of control in RNA-containing and single-stranded DNA-containing bacteriophages, see Calendar, 1970.) Emphasis will be placed on the well-characterized and studied bacteriophages of *Escherichia* coli: T4, T7 and λ. Examples have been selected to illustrate as many different molecular mechanisms of regulation as possible.

Host Transcriptional Machinery

Before beginning the discussion of mechanisms of gene control in bacteriophage development, it is probably important to mention briefly some pertinent aspects of the host transcriptional machinery which the phage must cope with after infection. A single enzyme--the DNA-dependent RNA polymerase--is thought to be responsible for the transcription of all classes of cellular RNA. This enzyme has been the subject of a tremendous amount of research on its structure, properties, and mechanism of action in the past ten years. Several excellent reviews of this prodigious literature are available and served as resource information for the following short discussion (Burgess, 1971; Travers, 1971; Chamberlin, 1970).

RNA polymerase has been isolated and purified to homogeneity from a number of bacterial sources including Escherichia coli, Bacillus subtilus, Azotobacter vindlandii, and Pseudomonas putida. The bacterial RNA polymerase is structurally a very complex enzyme, being composed of four types of subunits: α,β,β', and σ. The molecular weight of these subunits from a number of different sources are: α, 39,000 to 44,000; β, 145,000 to 155,000; β' 150,000 to 165,000; and σ, 62,000 to 98,000. The stoichiometry of the holoenzyme form of RNA polymerase is $\alpha_2\beta\beta'\sigma$.

Chromatography of holoenzyme from E. coli on phosphocellulose causes its dissociation into the σ subunit and the core polymerase,

$\alpha_2\beta\beta'$. The core polymerase by itself is capable of catalyzing the basic transcription process: the template-directed polymerization of nucleoside triphosphates into RNA. The templates, poly [d(A-T)] and calf thymus DNA, are utilized as efficiently by core polymerase as by holoenzyme to support RNA synthesis. With DNA from certain bacteriophages, such as T4 and T7, however, the holoenzyme was much more efficient than the core enzyme. Furthermore, the holoenzyme transcribed the bacteriophage DNA assymmetrically on the biologically correct strand, whereas the core enzyme reads symmetrically from both DNA strands. Consequently, σ factor is thought to be required for the efficient initiation of RNA synthesis at the correct sequences on the DNA molecule.

The σ factor was found to act catalytically in the initiation of RNA chains (Travers and Burgess, 1969). Soon after the initiation of RNA synthesis, the σ subunit is physically released from the DNA-enzyme complex (Gerard et al., 1972) and the core polymerase completes the synthesis of the RNA molecule. The σ subunit can then reunite with another molecule of core polymerase to promote further correct initiation. It has not yet been demonstrated conclusively whether the actual recognition of the proper initiation sequences is carried out by σ factor, core polymerase, or a combination of the two. σ factor has been shown to cause the stabilization of RNA polymerase-DNA complexes formed at proper initiation sites.

The presence of several protein factors which alter transcriptional activity of the E. coli RNA polymerase have been reported. Perhaps the best understood of these is the catabolite gene-activating protein (CAP) which is required for the expression of genes subject to catabolite repression. In a purified system consisting of lactose-operon containing DNA and core polymerase, the synthesis of lactose-specific mRNA is dependent on the addition of CAP, cyclic AMP, and σ factor. Conversely, the addition of purified *lac* repressor to the complete system results in the blockage of *lac*-mRNA transcription. Both cAMP and CAP have been shown to be required for the binding of RNA polymerase holoenzyme to the *lac* promoter. This protein factor, therefore, alters the initiation specificity of holoenzyme to make it recognize initiation sequences it would not ordinarily utilize.

Other protein factors whose exact roles in transcription have not been elucidated are known. M factor is a protein isolated from a high salt wash of *E. coli* ribosomes which stimulates transcription of DNA by holoenzyme seventeen-fold. A factor termed ψ was originally thought to promote the transcription of ribosomal genes by *E. coli* RNA polymerase (Travers *et al.*, 1970). The role of these two protein factors is currently unknown.

The termination factor, ρ, has been purified from *E. coli* and found to be a tetramer of 200,000 molecular weight. Using DNA from several bacteriophages, ρ factor has been shown to cause the specific termination and release of RNA chains during *in vitro* synthesis. Since it is known that specific termination of RNA synthesis can also occur under certain assay conditions, the extent to which ρ-mediated terminations occur *in vivo* is unknown. Elucidation of the role of ρ factor would be greatly facilitated by the isolation of conditional-lethal mutants of its structural gene.

Rifampicin is an antibiotic which specifically inhibits RNA synthesis by the bacterial RNA polymerases *in vivo* and *in vitro*. The drug acts at a step in the RNA synthetic process before the formation of the first phosphodiester bond; RNA polymerase molecules which have initiated RNA synthesis are not affected by rifampicin. Complexes of RNA polymerase and DNA formed at specific initiation sites were found to be insensitive to inhibition by rifampicin. By analysis of rifampicin-resistant mutants of *E. coli*, the β subunit was identified as the site of rifampicin action. Because of the high specificity of this drug and its mode of action, rifampicin has proven to be a very useful tool in the study of transcription.

The Coliphage T4

The *Escherichia coli* bacteriophage T4 is a very large, complex phage. The molecular weight of phage T4 DNA is 1.3×10^8, or approximately 1/20 the size of *E. coli* DNA. Due to the large coding capacity of its DNA, the phage T4 is more highly independent of host functions necessary for its development than most other bacteriophages. Adding to the complexity of phage T4 is the fact that its DNA contains the unusual base, hydroxymethylcytosine, in place of the normal cytosine. This unusual base is glycosylated.

The phage T4 is one of the most virulent phages known. All host macromolecular syntheses are shut off by two to three minutes after infection. The shutoff of host DNA, RNA, and protein each seems to be controlled by a unique phage-specific mechanism. At later times in the infectious cycle, the host chromosome is broken down by the action of T4-coded nucleases to the level of mononucleotides. The unusual base, hydroxymethylcytosine, of T4 DNA is thought to function, in part, by protecting T4 DNA from this degradation. The rapid shutoff of host-directed syntheses greatly facilitates the analyses of phage-specific products.

In the early stages of T4 development, phage proteins necessary for T4-specific DNA metabolism are synthesized. These proteins include the enzymes responsible for the synthesis of hydroxymethyldeoxycytidine 5'-triphosphate, as well as a T4-specific DNA polymerase. After T4 DNA replication has commenced, the pattern of protein synthesis shifts to the manufacture of structural components of the viral particle and products necessary for cell lysis. These include the major protein of the phage head, which constitutes 50% of late T4 protein synthesis.

In Vivo Transcription of T4 DNA

The pattern of phage protein synthesis after T4 infection is a direct consequence of changes in the pattern of T4 DNA transcription. There appear to be at least four distinct classes of phage-specific RNA transcribed in T4-infected cells (for review see Calendar, 1970). These classes can be defined by the temporal order with which their synthesis occurs after infection. The identification of the different classes of T4 RNA has been achieved largely by RNA-DNA hybridization-competition techniques (Salser et al., 1970). The first class of T4-specific RNA appears immediately after infection and, thus, has been termed "immediate early" RNA. The synthesis of this class of RNA can occur when T4 infection takes place in the presence of protein synthesis inhibitors such as chloramphenicol. The synthesis of immediate early RNA, therefore, as opposed to all later classes of RNA, is not thought to require phage-specific protein synthesis. Approximately two minutes after infection, a second class of T4-specific RNA, called "delayed early" appears. The syntheses of both immediate early and delayed early RNA are turned-off approximately five minutes after infection. A third class of T4 RNA, "quasi-late" RNA, is synthesized starting at about two minutes after infection, but rather than turning-off, its production is increased at later times of infection. Finally, the last class of T4 RNA to appear is the true "late" RNA. The synthesis of late RNA is absolutely dependent on phage DNA replication, which occurs starting at eight to ten minutes after infection. In addition, the gene products of the two maturation genes, 33 and 55, are required for the synthesis of T4 late RNA (Bolle _et al._, 1968).

The classes of T4 RNA not only are synthesized at different times after infection, but are also distinct in that they are coded for by different regions of the T4 DNA. Virtually all early T4 RNA is transcribed from one strand (the l-strand) and in one region of the T4 DNA molecule (Guha and Szybalski, 1968; Travers, 1970b). On the other hand, the late T4 RNA is largely (about 80%) transcribed from the opposite strand (the r-strand) and in a different region of the T4 genome.

Modifications of the Host RNA Polymerase after T4 Infection

The sequential appearance and disappearance of the classes of T4 RNA, as well as the shutoff of host RNA synthesis (Hayward and Green, 1965; Nomura *et al.*, 1966), are all thought to be programmed by phage-specific regulatory proteins (Calendar, 1970). One likely target for such regulatory proteins is the host RNA polymerase after T4 infection will be outlined.

Studies with the bacterial RNA polymerase inhibitor rifampicin demonstrated that T4-specific RNA synthesis was sensitive to the drug throughout the infectious cycle (Haselkorn *et al.*, 1969; diMauro *et al.*, 1969). In mutants of *E. coli* containing a rifampicin-resistant RNA polymerase, however, the T4 RNA synthesis was no longer sensitive to this inhibitor. While other interpretations are possible, these results strongly indicate that the host RNA polymerase, or a significant portion of it, is utilized for transcription of all T4 genes. This conclusion is substantiated by radioactive labeling experiments which have shown that the α, β and β' subunits of host RNA polymerase are conserved throughout infection (Stevens, 1972). Thus, any changes which occur in RNA polymerase after T4 infection must be the result of modification of the pre-existing host enzyme.

The fate of the host RNA polymerase in T4-infected cells has become quite confused by the claims of discovery of several putative T4-specific sigma factors, with several reported T4-induced chemical modifications of the RNA polymerase subunits, and with the finding of several newly-synthesized polypeptides associated with the RNA polymerase from infected cells. Attempts to correlate these changes in the host RNA polymerase with in vitro changes in the transcription of T4 DNA have been largely unsuccessful.

RNA polymerase isolated immediately after infection with T4 was found to be inefficient at utilizing T4 DNA as a template, compared to normal RNA polymerase (Seifert et al., 1969). The α subunit of this RNA polymerase was observed to have a different overall-charge than normal α subunit by cellulose acetate electrophoresis (Seifert et al., 1971; Walter et al., 1968). These changes in the RNA polymerase were not thought to require phage protein synthesis, since they occurred in the presence of chloramphenicol or even, to some extent, when T4 ghosts were used to infect (Seifert et al., 1969).

When T4 phage protein synthesis is allowed to occur, other chemical modifications of the host RNA polymerase subunits were observed (Seifert et al., 1969). The α subunit underwent a further increase in overall negative charge and a slight concommitant increase in molecular weight (Seifert et al., 1969; Bautz and Dunn, 1969). Growth of T4-infected cells in a ^{32}P-containing medium indicated that these changes might be due to the incorporation of a 5'-mononucleotide (probably AMP) into the α subunit (Seifert et al., 1971; Goff and Weber, 1970). Analysis of the separated RNA polymerase subunits by tryptic fingerprint maps revealed other changes in their chemical structure (Zillig et al., 1970; Schachner and Zillig, 1971). The α and β' subunits both contained additional spots in the tryptic fingerprint maps, while the pattern of the β subunit was significantly different than the subunit from uninfected cells. The significance

of these various chemical modifications in the host RNA polymerase is still unclear. No mutants which failed to modify the host enzyme have been obtained; nor experiments with *in vitro* T4 DNA transcription have been performed to clarify the role of these modifications.

In addition to these changes in the subunits of the core polymerase, Travers has reported that the σ factor of uninfected cells is replaced by T4-specific σ factors after infection which direct the core polymerase to transcribe different regions of the T4 genome (Travers, 1969; Travers, 1970b). The interpretation of these results is now questionable, however, due to the finding that RNA polymerase purified from T4-infected cells is lacking any type of σ factor (Bautz and Dunn, 1969). The normal σ factor can be retrieved from T4-infected cells by purified core polymerase from uninfected cells (Stevens, 1972). Thus, the binding of σ factor to host RNA polymerase appears to be reduced after T4 infection. This finding probably accounts for the decrease in the activity of RNA polymerase from T4-infected cells on T4 DNA (Seifert *et al.*, 1969). The lower binding affinity of σ factor and core polymerase could be due to one of the aforementioned modifications of core polymerase, or perhaps a modification of the σ factor itself. Evidence that the σ factor of T4-infected cells might be modified was suggested when it was found that σ factor isolated from infected cells was less efficient than normal σ in stimulating uninfected core polymerase to read T4 DNA (Stevens, 1974). The key question, however, of whether σ factor in T4-infected cells, despite its lower binding affinity, still functions in RNA synthesis remains unresolved.

Recently, Stevens has demonstrated that RNA polymerase isolated from T4-infected cells is associated with three, or possibly four, newly-synthesized polypeptides (Stevens, 1972). These polypeptides had molecular weights of 22,000; 15,000; 12,000; and 10,000, as determined by SDS-polyacrylamide gel electrophoresis. The kinetics of labeling of these polypeptides, as well as their appearance in DNA-negative mutants, suggested that these polypeptides are translated from early messengers. Interestingly, RNA polymerase isolated from a T4 mutant in either genes 33 or 55, neither of which synthesize late T4 RNA, did not contain the polypeptide with a molecular weight of 12,000 or 22,000, respectively. These results were substantiated by Horvitz who was able to show that the polypeptide of 12,000 daltons was the product of gene 33, and not due to an indirect effect of that gene product (Horvitz, 1973). The obvious conclusion to these studies is that the products of gene 33 and 55 both act by binding to the RNA polymerase, and, thus, alter its transcriptional specificity. Verification of this hypothesis will require an efficient *in vitro* system for the transcription of T4 late genes.

Positive Regulation of Delayed Early RNA Synthesis

By examination of the *in vitro* transcription of T4 DNA by *E. coli* RNA polymerase and comparison to the pattern of T4 RNA synthesis *in vivo*, some hypotheses on the control mechanisms involved in T4 gene expression can be made. The turning-on of delayed early genes at about two minutes after infection is the regulatory event which is perhaps best understood in T4 development. The turning-on of the synthesis of delayed early RNA is thought to be directed by a phage-specific protein, since it does not occur in the presence of chloramphenicol. This conclusion must be taken with some caution, however, for it is known that chloramphenicol can have secondary effects on cell metabolism. Furthermore, no specific T4 genes necessary for the production of delayed early RNA have yet been identified.

There are two general mechanisms which have been proposed to explain the positive regulation of delayed early genes. The first mechanism evolved from the following observations: When T4 DNA was transcribed by purified RNA polymerase holoenzyme from uninfected *E. coli* for short periods of time, only immediate early RNA was synthesized (Milanesi *et al.*, 1969; Bautz *et al.*, 1969). Incubation for longer times, however, caused the appearance of delayed early RNA (Milanesi *et al.*, 1970; Bautz *et al.*, 1969). The delayed early RNA sequences were found on the promoter-distal portion of RNA molecules containing immediate early RNA sequences at their promoter-proximal termini (Milanesi *et al.*, 1970). Thus, the two classes of genes are interspersed on the T4 DNA and the delayed early genes are transcribed from promoters for the immediate early genes in this *in vitro* system. If the termination factor, ρ, was added to the *in vitro* system, RNA synthesis was restricted to immediate early sequences only, even at longer times of incubation (Travers, 1970a). Thus, one possible mechanism for turning-on delayed early genes is the "read-through" of host RNA polymerase molecules which had initiated at a set of immediate early promoters into the adjacent delayed early genes. In this model, a phage-specific protein would act as an "anti-terminator" to allow RNA polymerase to proceed past sequences at the end of the immediate early genes where termination normally occurs. This putative "anti-terminator" protein could act either by binding to the T4 DNA at the normal termination site or modifying the RNA polymerase to alter its behavior. The finding that the first delayed early RNA sequences detected *in vivo* occur on mRNA molecules too long to have been initiated at two minutes after infection offered evidence that this mechanism might also occur *in vivo* (Brody *et al.*, 1970).

The second mechanism proposed for the positive control of delayed early RNA synthesis involves the presence of a second class of promoters (delayed early promoters) which are activated approximately two minutes after infection by the action of a phage-specific protein. Travers has found that the addition of a partially purified fraction from T4-infected cells to an *in vitro* transcription system containing T4 DNA and RNA polymerase isolated from T4-infected cells caused a shift from the transcription of only immediate early RNA to the synthesis of both immediate early and delayed early RNA (Travers, 1970b). While the designation of this fraction as a T4-specific σ factor may have been premature, it did demonstrate that RNA polymerase could acquire a new initiation specificity *in vitro*. Evidence for the *in vivo* utilization of specific promoters for certain delayed early genes has been obtained by two groups of workers using different methods (Schmidt *et al.*, 1970; Hercules and Sauerbier, 1973). The existence of *in vitro* and *in vivo* evidence for both mechanisms of delayed early RNA positive regulation led to the hypothesis that both mechanisms may be occurring simultaneously in T4-infected cells (Schmidt *et al.*, 1970). Recently, Hercules and Sauerbier have found evidence indicating that for certain delayed early genes a switch occurs during infection from transcription starting at immediate early promoters to delayed early promoters (Hercules and Sauerbier, 1974). The extent to which each of the mechanisms contributes to the overall production of delayed early RNA *in vivo* is unknown.

Positive Regulation of Late RNA Synthesis

The switch from the synthesis of early RNA species to true late RNA species is experimentally difficult to study, due to the requirement of T4 DNA replication for late T4 RNA synthesis. Temperature shift experiments with a temperature-sensitive mutant of T4 DNA polymerase indicated that T4 DNA replication is continuously required for late transcription to proceed (Riva *et al.*, 1970a). Thus, to study the *in vitro* transcription of late genes, the *in vitro* replication of T4 DNA must be simultaneously occurring. Riva *et al.* (1970b) have been able to observe late T4 transcription in the absence of T4 DNA replication in *E. coli* infected with T4 containing mutants in T4 DNA polymerase and DNA ligase. It was postulated that such mutants would contain DNA with single-stranded breaks or gaps, suggesting that some structural feature of the replicating DNA is necessary for late gene transcription.

In addition to concomitant DNA replication, the products of the muturation genes 33 and 55 are required for late T4 transcription in vivo. Using a crude lysate from T4-infected cells, Snyder and Geiduschek were able to demonstrate that some RNA synthesized in vitro by a RNA polymerase-vegatative DNA complex was late RNA (Snyder and Geiduschek, 1968). When the transcription complex was identically isolated from a mutant in gene 55 grown at non-permissive conditions, however, no late RNA synthesis was seen. Addition of the supernatant fraction from wild type T4-infected cells, supposedly containing gene 55 product, resulted in the production of late RNA. These experiments suggested that gene 55 product is necessary for in vitro late transcription, but did little to elucidate the mechanism. The recent demonstration that the products of genes 33 and 55 are associated with the RNA polymerase in T4-infected cells leads to the hypothesis that they may be specificity determinants (Stevens, 1972; Horvitz, 1973). Verification of this hypothesis, however, will require an in vitro system capable of efficiently transcribing late T4 RNA.

In conclusion, it can be seen that there is much more experimentation necessary before the control mechanisms for T4 RNA synthesis will be completely understood. Little is known, for instance, on the negative control mechanisms involved in turning-off the synthesis of either host RNA or T4 early RNA. The possibility exists that a mechanism which turns-on the transcription of a given set of genes by altering the RNA polymerase specificity would simultaneously cause the turning-off of another set of genes. The study of T4 has given some information about control mechanisms. This information will be summarized at the end of this survey.

Bacillus subtilus Bacteriophages SP01 and SP82

Infection of Bacillus subtilus by the closely related bacteriophages SP01 or SP82 has several similarities to T4-infection of *E. coli*. Like T4, both SP01 and SP82 are very large phages containing double-stranded DNA with a molecular weight of 1 to 1.3 X 10^8. The DNA of these phages contains an unusual base, hydroxymethyluracil, in place of the normal thymine. The viral-specific transcription pattern of SP01- or SP82-infected cells is relatively complex with several different temporal classes of RNA. Finally, the development of phage remains sensitive to inhibition by rifampicin throughout the infectious cycle, suggesting the host RNA polymerase is utilized for all classes of phage DNA transcription (Geiduschek and Sklar, 1969). Although SP01 and SP82 have not been studied as extensively as T4, it appears they may have some advantages for studying the control of genetic expression during bacteriophage development.

For both SP01 and SP82 infections, six distinct classes of RNA, as defined by the time of their appearance and disappearance in infected cells, have been demonstrated (Gage and Geiduschek, 1971; Spiegelman and Whiteley, 1974b). The first class of RNA to appear, which will be called "immediate early" in this discussion, was synthesized even when infection occurred in the presence of chloramphenicol. The synthesis of this class, thus, did not require phage-specific protein synthesis. About four to five minutes after infection, later classes of RNA, dependent on phage-protein synthesis, appeared. Also about this time, the synthesis of a portion of the immediate early RNA ceased (Gage and Geiduschek, 1967). Mutants of SP01 which cannot transcribe late RNA, but do replicate viral DNA, have been isolated (Fujita et al., 1971). A mutant of SP01 which only synthesizes immediate early RNA has also been found (Fujita et al., 1971). These mutants are thought to be defective in the synthesis of certain transcriptional control elements necessary for normal phage development.

The in vitro transcription of either SP01 or SP82 DNA by purified E. coli or B. subtilus RNA polymerase resulted in the assymmetric production of only immediate early RNA sequences (Grau et al., 1970; Spiegelman and Whiteley, 1974b). Wilson and Geiduschek (1969) have isolated a factor from SP01-infected cells, which would specifically inhibit the in vitro transcription of SP01 DNA. The factor, which was termed TF1, would not block transcription of denatured SP01 or B. subtilus DNA. TF1 was not found in cells infected in the presence of chloramphenicol. It was thought to be the agent responsible for turning-off the transcription of certain immediate early genes. TF1 has subsequently been purified to homogeneity and found to be a basic protein of 24,000 molecular weight (Johnson and Geiduschek, 1972). It is thought to act by binding to SP01 DNA and interfering with the initiation of RNA synthesis. No genetic evidence exists, however, showing that TF1 actually functions in vivo as a negative control element for immediate early SP01 RNA synthesis.

When RNA polymerase was isolated from SP01-infected B. subtilus, it was found to have a different transcriptional specificity than the enzyme from uninfected B. subtilus (Grau et al., 1970). A partially purified preparation of this RNA polymerase activity from infected cells synthesized only small quantities (10-20%) of immediate early

RNA (Duffy and Geiduschek, 1973). Instead, the majority of RNA synthesized was of a class termed "middle" (RNA synthesized beginning at four to five minutes after infection). This activity from infected cells was also found to be much more resistant to the *in vitro* repressor TF1 than the uninfected *B. subtilus* enzyme. Mutants restricted to the synthesis of immediate early RNA contained an RNA polymerase activity identical to the uninfected cell enzyme in transcriptional specificity and response to TF1.

The RNA polymerase with altered transcriptional specificity has been partially purified from SP01-infected cells (Duffy and Geiduschek, 1973) and purified to near homogeneity from SP82-infected cells (Spiegelman and Whiteley, 1974a). These enzymes were found to contain little, if any, of the σ subunit. Since these RNA polymerases from infected cells did, however, show the correct transcriptional specificity, the presence of σ factor may not be necessary for correct initiation in these phage-infected cells. In addition to the normal RNA polymerase subunits α, β, and β', small polypeptides were found associated with the RNA polymerase. For the enzyme from SP82-infected cells, polypeptides of 21,000; 19,000; and 16,000 molecular weight were found with the RNA polymerase even after extensive purification. Whether one or more of these polypeptides is the cause of the altered transcriptional activity remains to be demonstrated. The presence of an RNA polymerase activity from infected cells whose properties correspond with the *in vivo* expectations in terms of transcriptional specificity and repression by a specific inhibitor is definitely encouraging. No such RNA polymerase activity has yet been demonstrated in T4- or λ-infected *E. coli*.

The Coliphage T7

The basic regulatory features controlling the development of the virulent bacteriophage T7 of *E. coli* are probably better understood than those of any other phage. This is in large part due to the small size of the DNA molecule, 2.5×10^7 daltons, and the ability to separate and identify almost all phage-specific proteins synthesized during the infectious cycle (about 25 to 30). The T7 DNA molecule is terminally redundant for approximately 0.7% of its total length and does not contain any unusual bases. Over 80% of the nucleotides found in the mature T7 phage DNA were present in the host chromosome at the time of infection. The host chromosome is degraded, beginning approximately six minutes after infection, by the action of two essential phage gene products, an endonuclease and an exonuclease (Sadowski and Kerr, 1970). Using the separated strands of T7 DNA, it was found that essentially all phage-specific RNA synthesized during T7 development was transcribed from only one strand (r-strand) of the DNA (Summers and Szybalski, 1968).

Temporal Appearance of Gene Products after T7 Infection

Due largely to the work of Studier, much is known about the phage-specific proteins synthesized in T7-infected cells (for review see Studier, 1972). Studier was able to separate essentially all of the T7-specific proteins synthesized after infection according to molecular weight by the use of SDS-polyacrylamide gel electrophoresis in gels of varying porosity (Studier and Maizel, 1969). By analysis of several hundred conditional-lethal mutations, T7 was found to contain 19 essential genes and the polypeptide corresponding to each gene was identified in the SDS-polyacrylamide gel electrophoresis patterns (Studier, 1969; Studier and Maizel, 1969). These essential genes were mapped and numbered consecutively from left to right on the T7 DNA molecule. The protein products for 17 of the 19 essential genes, which account for 70% of the coding capacity of the T7 DNA, have been identified. In addition, several non-essential genes of T7 have been identified by analysis of deletion mutations. These genes are numbered by fractions according to which essential genes they map between.

The proteins of T7 phage infection can be divided into three groups by their time of appearance and disappearance in the infected cell. Class I proteins are synthesized from four to eight minutes after infection and contain one essential gene, gene 1, and several non-essential genes. Class II proteins (genes 2-6) are synthesized from six to fifteen minutes after infection. Mutants of the genes in this class do not replicate viral DNA or break down the host chromosome. Although mutants of Class II proteins do not replicate DNA, they do make Class III proteins (Siegel and Summers, 1970). Therefore, unlike phage T4, late gene transcription in T7 infection can occur independent of replication. Class III proteins (genes 7-19) are synthesized from six minutes after infection to the time of lysis (about 25 to 30 minutes). These gene products include the proteins of the phage particle, as well as proteins necessary for maturation of the phage DNA. The fact that the appearance of gene products corresponds directly with the order of the genes on the T7 DNA molecule indicates that transcription occurs sequentially from left to right along the T7 genome.

Mutants of gene 1 were the only mutants found which affected the synthesis of more than one protein (Studier and Maizel, 1969). In mutants of any gene of T7 except gene 1, only the particular gene product of the mutated gene was lost from the SDS-polyacrylamide gel electrophoresis pattern. When a mutation occurred in gene 1, however, only the non-essential Class I proteins were formed; none of the Class II or III proteins appeared. Thus, gene 1 is a positive regulatory element for appearance of all later proteins.

Summers has shown that thirteen phage-specific mRNA species (of size greater than 2 X 10^5 molecular weight) are synthesized in T7-infected _E_. _coli_ (Summers, 1969). Hybrid molecules formed between these _in vivo_ T7 mRNA species and the r-strand of T7 DNA were viewed directly by electron microscopy (Hyman, 1971). No silent regions greater than 0.5% of the length of the T7 genome were found, excluding the terminal redundancies. Thus, essentially all available genetic material of T7 DNA is transcribed during the infective cycle.

When T7 infection occurred in the presence of chloramphenicol or with gene 1 mutants at non-permissive conditions, only three or four of the thirteen T7 mRNA species were found (Siegel and Summers, 1970). These mRNA species code for the Class I proteins and have been termed early or phage-function independent RNA. The mRNA species for

Class II and III proteins, which only appear if gene 1 is expressed, are called "late" or phage-function dependent RNA. The early T7 mRNA species, synthesized in the presence of chloramphenicol, were found to hybridize to approximately the first 20% of the length of the T7 DNA by electron microscopy (Hyman, 1971). The control in switching from the synthesis of Class I to Class II and III proteins occurs at the level of transcription by means of positive regulation by gene 1 product.

Control of Genetic Expression by the Gene 1 Product

The elucidation of the mechanism of the positive switch from early to late transcription was provided by Chamberlin *et al.* who found that the product of gene 1 is a new DNA-dependent RNA polymerase (Chamberlin *et al.*, 1970). This new RNA polymerase was not found when infection occurred in the presence of chloramphenicol or with mutants of gene 1 grown at non-permissive conditions. The RNA made *in vitro* by the T7 RNA polymerase using T7 DNA was found to be complementary to only the r-strand of T7 DNA and could be completely competed for by late *in vivo* T7 RNA in competition-hybridization experiments (Summers and Siegel, 1970; Chamberlin *et al.*, 1970). Therefore, this new enzyme appeared to be able to select the biologically correct strand and region of that strand to transcribe.

The T7 RNA polymerase, as well as a similar enzyme found after infection by the closely-related bacteriophage T3, has been purified to homogeneity. Both T3 and T7 polymerases are composed of single polypeptides of 108,000 to 110,000 molecular weight (Dunn *et al.*, 1970; Chakraborty et al., 1973; Chamberlin et al., 1970). These polymerases are much simpler physically than the complex structure of *E. coli* RNA polymerase.

One of the most striking characteristics of the phage polymerases is their stringent template specificity. Each enzyme is most highly active with DNA from the homologous phage. T7 polymerase would utilize T3 DNA about 50% as efficiently as T7 DNA, whereas T3 polymerase would utilize T7 DNA about 10% as well as T3 DNA (Dunn et al., 1970; Maitra, 1971; Chamberlin and Ring, 1973). No other naturally-occurring DNA from either bacteriophage or bacterial sources were found to support RNA synthesis. Nearly all denatured and single-stranded DNA tested, however, would support RNA synthesis at rates of 4 to 35% of the homologous native phage DNA (Salvo et al., 1973; Chamberlin and Ring, 1973). Therefore, the high degree of specificity with native DNA from various sources seen with the T3 and T7 RNA polymerase is lost when single-stranded templates are used.

Both T3 and T7 RNA polymerases are highly resistant to the bacterial RNA polymerase inhibitors, rifampicin and streptolydigin, as well as antisera to purified host RNA polymerase (Dunn et al., 1970; Maitra, 1971; Chamberlin et al., 1970). This resistance to rifampicin explains why the development of T7 becomes insensitive to inhibition by this drug approximately four minutes after infection (Summers and Siegel, 1969). The coliphage polymerases are inhibited by concentrations of monovalent ions greater than 50 m*M*. By contrast, *E. coli* RNA polymerase activity is optimum at KCl concentrations between 100 and 200 m*M*.

Shutoff of Host RNA Synthesis

The appearance of a new phage-induced RNA polymerase with a unique initiation specificity for late regions of T7 DNA provides an efficient mechanism for the positive switch from early to late RNA synthesis. Negative control mechanisms must also be present for turning-off transcription of early T7 genes, as well as the shutoff of host RNA synthesis. The shutoff of host transcription, which is essentially complete by five minutes after T7 infection (Brunovskis and Summers, 1971), does not occur in the presence of chloramphenicol (Summers and Szybalski, 1968). Host RNA shutoff does occur in mutants of gene 1 (Brunovskis and Summer, 1971). Therefore, an early gene function other than the T7 polymerase must be responsible for this shutoff. Recently, Brunovskis and Summers have isolated a deletion mutant of the early non-essential gene 0.7, which does not shut off the host RNA synthesis (Brunovskis and Summers, 1972). Interestingly, this mutant also failed to turn off early T7 RNA synthesis. While the mechanism of this negative control remains unknown, it is very tempting to speculate that shutoff occurs by inactivation of the host RNA polymerase. Such a mechanism would account for the shutoff of both host and early T7 RNA synthesis, since both are synthesized by the host polymerase.

Transcription of T7 Early Genes in vivo and in vitro

After the bacteriophage T7 DNA enters the cell, the first 20% or so of its genome is transcribed by the host RNA polymerase. The transcription of this region both *in vivo* and *in vitro* has been the subject of much work in the past few years and led to several interesting findings on the molecular mechanisms of transcription. By the use of polyacrylamide gel electrophoresis to resolve RNA species and deletion mutants of various non-essential early genes to map them (Studier, 1973), the *in vivo* transcription pattern of early T7 genes has been well-characterized. Five major transcripts with molecular weights of 2.1; 6.0; 9.8; 2.1; and 4.0 X 10^5, in order of appearance from left to right on the T7 DNA, have been found (Summers *et al.*, 1973; Simon and Studier, 1973; Minkley, 1974). These five early mRNA species map contiguously from 1.8 to 20.2% of the length of T7 DNA (Simon and Studier, 1973). The largest transcript must code for the T7 RNA polymerase, due to its relative size and the absence of any deletion mutants of this gene (Hyman and Summers, 1972). The only other gene product whose activity is definitely known is the 4.0 X 10^5 molecular weight transcript (gene 1.3 product), which codes for a DNA ligase. The RNA transcripts from the region to the left of gene 1 code for proteins with molecular weights of 9,000 (gene 0.3 product) and 40,000 (gene 0.7 product). Since both of these transcripts could potentially code for proteins 1 1/2 to 2 times larger than these, it is possible that some processing of protein products occurs (Simon and Studier, 1973). It has been found that the five major transcripts are not present in equimolar quantities (Summers *et al.*, 1973; Minkley, 1974). Most notably the gene 0.3 transcript is present in 10-fold molar excess over the gene 1 transcript. The differences in molar quantities of the early transcripts must be accounted for in any model for transcription of this region.

A number of smaller RNA transcripts ranging in size from 0.2 to 0.64 X 10^5 daltons are also seen in early _in vivo_ transcription (Simon and Studier, 1973; Minkley, 1974). These species are present in lower quantities than the five major transcripts and no protein products of these smaller RNA species have been detected (Simon and Studier, 1973). Several of these smaller RNA species have been demonstrated to arise from the region to the left of the gene 0.3 (Simon and Studier, 1973).

Purified _in vitro_ transcriptional systems using T7 DNA and _E. coli_ RNA polymerase have been studied to attempt to learn what _in vitro_ conditions are necessary for accurate transcription of the early genes. The presence of σ factor is required for assymmetric transcription of the biologically correct strand of T7 DNA (Goff and Minkley, 1970).

One model proposed for the transcription of early genes was that T7 transcripts arise from independent transcription units of the early region, each with its own promoter and terminator signal for _E. coli_ RNA polymerase. Promoters of varying efficiencies would account for any differences in molar ratios of RNA transcripts. This model was based on experiments with γ- ^{32}P-labeled nucleoside triphosphates (Takeya and Fujisawa, 1973) and specific dinucleotides which caused selective initiation (Minkley and Pribnow, 1973), both of which suggested the presence of several promoters for RNA polymerase in the early region. When the size of the _in vitro_ transcription products of T7 DNA by _E. coli_ RNA polymerase were examined, however, it was found that a single transcript of 2.2 to 2.7 X 10^6 molecular weight was made (Dunn and Studier, 1973a; Millette _et al._, 1970; Brautigam and Sauerbier, 1973; Minkley, 1974). The size of this RNA is consistent with a transcript of the entire early region of T7 DNA, suggesting a single promoter site. Mapping of the _in vitro_ RNA polymerase-T7 DNA-nascent RNA complex by electron microscopy also indicated only one site on the T7 DNA where RNA initiation occurred (Davis and Hyman, 1970). These observations led to a second model for early transcription in which all transcription proceeds from a single promoter near the left end of the T7 DNA and the correct-sized transcripts are generated by the action of the termination factor, ρ. According to this model, RNA polymerase could terminate RNA synthesis and release an RNA molecule, and then reinitiate RNA synthesis without releasing itself. Several studies have been performed in which the ρ factor was added to the normal _in vitro_ transcription system. While the presence of ρ did cause the production of several discrete RNA species (Dunn _et al._, 1972; Takeya and Fujisawa, 1973; Davis and Hyman, 1970; Goff and Minkley, 1970), no faithful reproduction of the _in vivo_ RNA transcripts was produced.

The inability of ρ factor to generate the proper-sized RNA transcripts led Dunn and Studier to search in _E. coli_ for some other factor which would have this activity (Dunn and Studier, 1973a). A protein factor was purified which would cause the _in vitro_ production of RNA transcripts of the same size as the five major early _in vivo_ transcripts. This "sizing factor" isolated by Dunn and Studier has subsequently been shown to be RNase III, an RNase which is active on double-stranded regions of RNA. In _E. coli_ mutants of RNase III infected with T7, the *in vivo* early RNA transcribed was a single large species similar in size to that seen *in vitro* in the absence of ρ factor (Dunn and Studier, 1973b). When this large RNA molecule was treated with purified RNase III, it was cleaved to give an RNA pattern indistinguishable from the normal early *in vivo* transcripts. Interestingly, the RNase III-negative mutants also did not process the precursor to ribosomal RNA to form the normal 16S and 23S rRNA. Thus, this RNase may play an essential role in the post-transcriptional modification of many RNA species in uninfected *E. coli*.

If the early T7 RNA species are formed by post-transcriptional cleavage of a large precursor molecule, only one of the RNA transcripts should contain a γ-phosphate group at its 5'-terminus. Labeling with $[\gamma\text{-}^{32}P]$ ATP or $[\gamma\text{-}^{32}P]$ GTP, however, revealed that none of the five major transcription products contained a 5'-γ-phosphate group (Dunn and Studier, 1973a). Instead, the $\gamma\text{-}^{32}P$ label was found in three small RNA molecules. These RNA molecules were found to arise from three tightly spaced promoters between the end of the terminal redundancy of T7 DNA (about 0.7%) and the start of gene 0.3 (about 1.8%) and contained overlapping sequences. Therefore, the leftmost cleavage point of the RNase III must be at the 5'-terminal end of the 0.3 gene transcript. These results help explain some earlier unresolved questions. First, several minor RNA bands were found *in vivo* which mapped to the left of the gene 0.3 and had no apparent function (Simon and Studier, 1973). Second, several initiation sites for *E. coli* RNA polymerase had been suggested by studies with initiating dinucleotides. The model which is most consistent with *in vivo* transcription patterns currently is as follows: *E. coli* RNA polymerase can initiate RNA synthesis at any of three tightly spaced promoters to the left of gene 0.3. The RNA molecules transcribed from all three promoters extends to a ρ-independent termination site at about 20.2% of the length of the T7 DNA. After transcription, the large RNA precursor is cleaved at a minimum of five sites to produce the five major T7 early transcripts plus three smaller RNA species from the 5'-end of the precursor molecule. The only major problem which this model does not explain is the higher molar quantities of certain RNA transcripts. The cleavage of a single precursor molecule would predict that all major transcription products would be present in equimolar quantities. No reasonable explanation of how higher quantities of certain transcripts could be generated from this model has yet been made.

The existence of a large precursor RNA molecule for the entire early T7 region raised the question of whether cleavage of the precursor was a necessary prerequisite for translation of the RNA. Hercules *et al.* have recently shown that the early T7 precursor RNA isolated from RNase III mutants was a poor template for translation of several early phage proteins as compared to normal early T7 mRNA species in a cell-free protein synthesizing system prepared from *E. coli* RNase III mutants (Hercules *et al.*, 1974). Ribosomes from normal *E. coli* contain RNase III activity and, thus, can cleave the early T7 RNA precursor and translate it efficiently. If these ribosomes were washed with NH_4Cl to remove RNase III, they also lost their ability to translate the precursor molecule. Thus, it appears that cleavage may be necessary for the formation of efficient messenger for protein synthesis.

Transcription of Late Genes

Compared to the transcription of the early region of T7 DNA by *E. coli* RNA polymerase, relatively little is known of the molecular mechanisms of transcription of the late regions of either T3 or T7 DNA by their respective phage polymerases. Both T7 and T3 RNA polymerases initiate RNA synthesis *in vitro* on their respective phage genomes exclusively with GTP (Maitra and Huang, 1972; Chamberlin and Ring, 1973). The two phage polymerases differ in that the T7 polymerase only transcribes late T7 genes *in vitro* (Summers and Siegel, 1970), whereas the T3 polymerase transcribes both early and late regions of the T3 DNA (Dunn *et al.*, 1972). The *in vitro* RNA products of T3 RNA polymerase, although quite heterogenous in size, form a number (about 9) of discernible peaks on sucrose density gradient centrifugation which show many similarities to the *in vivo* late T3 mRNA species (Dunn *et al.*, 1972; Takeya and Fujisawa, 1973). This suggests no other protein factors, such as ρ factor or RNase III, are necessary for correct transcription by the T3 RNA polymerase.

Recently Golomb and Chamberlin have been able to demonstrate, by polyacrylamide gel electrophoresis, the presence of seven classes of RNA produced *in vitro* by T7 RNA polymerase using T7 DNA (Golomb and Chamberlin, 1974). These RNA species ranged in size from 5.5 to 0.2×10^6 molecular weight. Mapping of T7 late transcripts is difficult because there are no deletion mutants known in the region from 30 to 100% of the length of the T7 DNA (Studier, 1973). By using an exonuclease to partially shorten T7 DNA, however, it was demonstrated that the three largest T7 transcripts all had sequences complementary to the extreme right end of the T7 DNA (Golomb and Chamberlin, 1974). Thus, these three transcripts represent overlapping transcription units with a common terminator and promoters at approximately 56, 64, and 83% of the length of the T7 DNA. The remaining late transcripts, even if contiguous, could not account for the entire region from the start of the late T7 genes (about 20.2%) to 56%. Thus, the presence of promoters that only bind weakly with the T7 RNA polymerase is suggested. While this work is only preliminary, it is a start to understanding the transcription of the T7 late region.

Stability of T7 mRNA

The fact that discrete size classes of T7 mRNA can be observed in polyacrylamide gel electrophoresis indicated these mRNA species are unusually stable (Summers, 1969). Whereas *E. coli* mRNA is known to have a half-life of 1 1/2 to 2 1/2 minutes, the half-life of T7 mRNA was found to be 15 to 20 minutes (Summers, 1970). Measurement of the half-life of *tryp* mRNA in T7-infected cells indicated that it was approximately the same as in uninfected cells (Marrs and Yanofsky, 1971). Thus, the stability of T7 mRNA cannot be due merely to a disruption of the normal host mRNA breakdown system. The actual explanation for the stability of T7 mRNA remains a matter of conjecture.

The finding that T7 mRNA is relatively stable seems in contradiction with the observation that Class I protein synthesis ceases about eight minutes after infection. A possible explanation has recently been suggested (Yamada *et al.*, 1974). The half-life measured above used either polyacrylamide gel electrophoresis or RNA-DNA hybridization to measure T7 mRNA and, thus, measured chemically stable molecules. When the functional stability of T7 early mRNA to act in programming *in vitro* protein synthesis was measured, it was found that its half life was only six minutes, quite a bit shorter than that for the chemical stability (Yamada *et al.*, 1974). The mechanism by which T7 early mRNA species become functionally inactive, while still maintaining their basic chemical structure, is unknown. If these findings are verified, however, it does indicate that translational control mechanisms, as well as transcriptional controls, play a role in the regulation of gene expression in T7-infected cells.

The basic regulatory features of transcriptional control in T7-infected cells are fairly well understood. Furthermore, the actual molecular mechanism of the transcription of the early region of T7 DNA is known in some detail. Several questions on control in this system still exist. For instance, is the host RNA polymerase inactivated to shut off host RNA synthesis, and if so, how? How is the synthesis of one set of late T7 proteins (Class II) turned off midway through the infective cycle? What mechanism is involved in controlling the quantities of each individual RNA transcript synthesized? The answers to these and similar questions should provide much more interesting information on the mechanisms of gene transcription.

The Temperate Coliphage Lambda

The bacteriophage λ is undoubtedly the most extensively studied phage system, especially in terms of genetic analyses, known today. Being a temperate bacteriophage, it presents many more control problems than virulent phages for it can be present in *E. coli* in either of two entirely different life styles. In the lysogenic state, the λ DNA becomes integrated into the bacterial chromosome at a specific site and then is replicated in concert with the bacterial DNA. In this state, very few gene products are synthesized; the main one being the product of the cI gene, λ repressor, which is responsible for the maintenance of the lysogenic state. In the virulent state, either after phage infection or induction of the prophage, the λ DNA is sequentially transcribed into various classes of RNA, leading to an orderly production of phage proteins for development. Due to the large number of fine and coarse control mechanisms which have been found to regulate λ infection, it will be impossible to discuss this system in detail. Recently, an extensive combination of review articles and research papers on λ have been published, making such a discussion unnecessary (Hershey, 1971). The general features of control during lytic infection of λ will be outlined and the mechanism of action of λ repressor examined in some detail.

Genetic Control during Lytic Development of λ

The DNA of λ has a molecular weight of 31 X 10^6, contains the four usual bases, and has 5'-single stranded ends twelve nucleotides in length. After injection into the cell, the homologous 5'-ends (sticky ends) cause circularization of the DNA, which subsequently becomes covalently joined. The genes of λ may be broken down into four functional groups. The late genes determine phage head and tail structural components, as well as genes for cell lysis, and are transcribed off the r-strand of λ DNA. The early genes include the recombination genes, which determine enzymes responsible for integration and excision of λ DNA from the host chromosome, and replication genes O and P, which are required for phage DNA replication. The recombination genes are transcribed off the l-strand and the replication genes off the r-strand of λ DNA. Finally, there are several regulatory genes of λ, including the cI, cII, cIII, *tof* or *cro*, N and Q genes.

In the presence of gene N mutants, only the mRNA from N gene, directly to the left of the cI gene, and the *tof* gene, directly to the right of the cI gene, are transcribed (termed "immediate early" stage) (Kourilsky *et al.*, 1968). In the presence of normal gene N product, transcription of the recombination and replication genes, as well as regulatory gene Q, occurs (termed "delayed early" stage). After replication of λ DNA, the genes of the late region are transcribed. This does not occur to a significant extent, however, in mutants of gene Q. Thus, both gene N and Q products are positive regulatory elements for λ gene transcription. The sequential production of these positive regulatory elements leads to a sequential control of RNA transcription.

The sites of action of gene N product have been determined by the isolation of mutants which no longer require the action of N protein for the production of certain gene products. Mapping of these "bypass" mutants has indicated three distinct sites of action for N protein: one located to the right of gene cI allows transcription of replication genes; a second located to the left of gene N allows transcription of the recombination genes; and a third to the right of the replication genes permits the formation of Q protein (and, thus, indirectly late proteins). These studies indicate that the genes in these three regions are each controlled by a single point at one end of the region and this site is where gene N product exerts its effect.

There is good evidence indicating that the N protein may act as an "antiterminator" protein to allow transcription to proceed into the delayed early regions from immediate early promoters. The _in vitro_ transcription of λ DNA by _E. coli_ RNA polymerase in the presence of the termination factor, ρ, led to the production of two predominant species of 7S and 12S mRNA (Roberts, 1969). The 12S RNA was transcribed from the l-strand of λ DNA, immediately to the left of the cI gene, and is thought to be the N gene transcript. The 7S RNA was transcribed from the r-strand of λ DNA, immediately to the right of the cI gene in the region of the _tof_ gene. These two transcripts synthesized by _E. coli_ RNA polymerase are probably the same as the _in vivo_ immediate early RNA species. When the _in vitro_ transcription of λ DNA was carried out in the absence of ρ factor, a broad size distribution of RNA from 5 to 35S was found (Roberts, 1969). These results led Roberts to postulate that N protein might act by antagonizing the action of the termination factor, ρ, and thus, allowing

E. coli RNA polymerase to proceed past the normal termination sites for the 7S and 12S immediate early RNA.

Luzzati has shown that the N protein is not a sufficient prerequisite, by itself, for the in vivo transcription of delayed early genes from a λ prophage (Luzatti, 1970). It was also necessary that the action of λ repressor be lifted, and, thus, the negative control by λ repressor was epistatic to positive control by N protein. Since the repressor is known to act at only two sites adjacent to the cI gene, operators 0_l and 0_r (see below), this observation indicated that the production of delayed early products requires prior initiation at the immediate early promoters. This conclusion is consistent with the anti-terminator model for N protein action in which no specific promoters for the delayed early region are present.

Recently, more direct evidence of the readthrough model has been obtained by competition-hybridization experiments (Portier et al., 1972). These studies found that mRNA isolated in vivo from the recombination region (delayed early transcript) would essentially completely compete with in vivo mRNA for the N gene (immediate early transcript). Thus, the delayed early mRNA was present on the same molecule as is the immediate early RNA sequences, indicating new initiation did not occur.

The molecular mechanism by which gene N causes readthrough of the normal termination signals of the immediate early region is unknown. Mutants of E. coli have been isolated which fail to respond to N protein, and, thus, do not induce λ early gene transcription (Pironio and Ghysen, 1970; Georgopoulos, 1971). One of these mutants

was shown to contain an altered form of RNA polymerase (Georgopoulos, 1971). This suggested that N protein might interact with the *E. coli* RNA polymerase directly to cause its effect. A clear answer of the mechanism, however, will require an *in vitro* RNA transcription system in which the N protein can be isolated and its biochemical role defined. While such a system is not presently available, a first step has been made by the recent development of an *in vitro* assay for N protein activity (Dottin and Pearson, 1973; Greenblatt, 1973). Furthermore, initial studies on the isolation of a modified form of RNA polymerase after λ infection have been made (Brown and Cohen, 1974). Thus, the molecular mechanism of N protein may be understood in the near future.

The product of regulatory gene Q is necessary for the transcription of all late λ genes (Skalka *et al.*, 1967). This transcription occurs largely after λ DNA replication is complete, although replication is not an essential prerequisite for late transcription. One essential site for the production of all late gene transcription has been found to lie immediately to the right of gene Q on λ DNA (Herskowitz and Signer, 1970a). This strongly indicated that all late transcription is sequential, starting from a single promoter site adjacent to gene Q. These results implied, but gave no direct evidence, that the essential site is the site of action of gene Q product in turning on late transcription.

Compared to the action of gene N product, little is known about the mechanism of Q protein activation of late gene transcription. The production of late proteins was found to occur in prophages deleted in the region directly adjacent to the late genes in transactivation experiments (Herskowitz and Signer, 1970b). Thus, late transcription most likely involved initiation of new RNA chains, rather than readthrough from the early region (Herskowitz and Signer, 1970b). Q protein could either directly participate to cause new initiation or indirectly activate this initiation. As was the case with N protein, the elucidation of the mechanism of action of Q protein will require an *in vitro* system in which Q protein can be isolated and its biochemical role investigated. Naono and Tokuyama have reported the preparation of a rather crude RNA polymerase fraction from post-replicative induced λ lysogens, which was capable of enhancing late gene transcription *in vitro* (Naono and Tokuyama, 1970). This RNA polymerase activity was not found in an identically induced lysogen containing a mutation of gene Q. Attempts to purify this activity, however, resulted in the loss of the late RNA specificity. Little is known, therefore, of the structure of the RNA polymerase or the relation of gene Q product to it.

A discussion of the general controls of the λ lytic cycle would be incomplete without a mention of the negative regulatory mechanisms acting at the level of transcription. After induction of λ prophage, the synthesis of gene cI mRNA for λ repressor is rapidly terminated (Eisen and Ptashne, 1971). Mutants lacking this function (termed *cro* gene for control of repressor) mapped to a region immediately to the right of the cI gene. A second shutoff of λ DNA transcription was found to occur about eight to ten minutes after infection and affect the transcription of gene N and, consequently, the λ delayed early genes (Eisen and Ptashne, 1971). This gene (tof for turn-off function) was also found to map immediately to the right of gene cI (Herskowitz and Signer, 1970b). When it was found that mutants of the cro gene were also defective in turning-off early transcription, it became apparent that these two functions are controlled by the same gene (Eisen *et al.*, 1970). Little is known about the mechanism of action of this negative regulatory protein. It has been proposed that it may act as a classical repressor to turn off transcription from the two operons it controls (Eisen and Ptashne, 1971).

Phage λ does not exert complete turn off of the host macromolecular synthesis characteristic of typical virulent phage infections (Cohen and Chang, 1970). Such a complete turnoff might be detrimental to a temperate phage such as λ which depends on normal cell functions when in the lysogenic state. Partial inhibition of host DNA, RNA, and protein synthesis, which is more pronounced at higher multiplicities of infection, is seen after λ lytic infection or induction of a λ lysogen (Wu *et al.*, 1971; Cohen and Chang, 1970). This partial shutoff is probably necessary for a more efficient production of phage particles. The shutoff of host RNA synthesis is under the control of a phage protein, since it does not occur in gene N mutants. The gene responsible for this repression, as well as the mechanism of action, are completely unknown.

Control by λ Repressor

The regulatory genes cI, cII, and cIII are all required for the establishment of the lysogenic state in λ infection. The controls

on whether a λ-infected cell enters the lysogenic state, or undergoes normal lytic infection, are very complex and not well understood (for review see Echols, 1971). A discussion of this topic is, therefore, beyond the scope of this survey. The gene cI product alone, however, is required for the maintenance of the lysogenic state, and it is this process that will be more closely examined.

The sites of action of the gene cI product, λ repressor, have been identified by the isolation of multiple mutants which are no longer sensitive to the action of λ repressor. These mutants, called virulent mutants, contain three distinct mutations. One mutation maps immediately adjacent to the left of gene cI and is constitutive for the production of N protein. The other two mutations are tightly linked and map immediately to the right of gene cI. These mutations are thought to define the two sites where λ repressor acts by binding to the λ DNA, the operators O_l and O_r. The binding of λ repressor at these operators was postulated to result in the blockage of RNA transcription from the two promoters, P_l and P_r, which are responsible for immediate early RNA synthesis. Since N protein is a gene product of transcription from promoter P_l, this blockage would result in all further λ transcription being repressed. The gene cI and its adjacent operators O_l and O_r constitute the immunity region of phage λ DNA, the portion responsible for the immunity of λ lysogens to superinfections by λ and other closely-related phages. The direct verification of the above model for the action of λ repressor became possible when repressors of both λ phage, and the related heteroimmune phage 434,

were isolated and purified by Ptashne and coworkers (Ptashne, 1967a; Pirrotta and Ptashne, 1969).

The λ and 434 repressors were purified from lysogenic cells, which had been UV-treated to reduce host protein synthesis and superinfected to maximize repressor synthesis (Ptashne, 1967a; Pirrotta and Ptashne, 1969). The repressor was identified by means of a differential labeling technique between cells containing normal repressor and an amber mutant in gene cI. A protein was found which was specifically labeled only in wild type gene cI containing lysogens. This protein had a molecular weight of 26,000 for the 434 repressor and 27,000 for the λ repressor (Ptashne, 1967a; Pirrotta and Ptashne, 1969), but was thought to be active in the dimeric form (Chadwick et al., 1970). The following properties of the protein were consistent with its designation as the repressor: (1) It was not found in amber mutants of gene cI and was found in an altered form in temperature-sensitive mutants of gene cI (Ptashne, 1967a). (2) The repressor from λ would bind tightly to λ DNA, but not DNA from the heteroimmune 434 phage (Ptashne, 1967b). The opposite specificity was seen for the 434 repressor (Pirrotta and Ptashne, 1969). (3) DNA isolated from the putative operator mutant, λ vir, showed a much lower binding affinity than wild type λ DNA for repressor. Furthermore, mutants containing only one mutant operator and one normal operator displayed intermediate binding levels (Ptashne and Hopkins, 1968). (4) The *in vitro* transcription of 7S and 12S immediate early RNA by *E. coli* RNA polymerase in the presence of termination factor, ρ, could be blocked by the addition of λ repressor (Steinberg and Ptashne, 1971;

Wu et al., 1971). Thus, it was concluded that the protein isolated was a repressor which acted by binding to specific sites on the λ DNA to block RNA transcription.

The molecular mechanism by which the repressor blocks RNA transcription is unknown, but is thought to involve an early step in the RNA synthetic process. The sites of action of repressor, the operators O_l and O_r, are closely linked to the promoters, P_l and P_r, for RNA synthesis. In vitro RNA synthesis from rifampicin-resistant complexes of E. coli RNA polymerase and λ DNA is no longer sensitive to the subsequent addition of repressor (Wu et al., 1971). Finally, the binding of E. coli RNA polymerase to λ DNA blocked the binding of λ repressor (Chadwick et al., 1970). This observation led to the hypothesis that the reverse might be true; that is, repressor might block binding of RNA polymerase to the λ DNA. Further evidence on this hypothesis will be necessary for verification.

The general pattern of control of RNA synthesis after induction of λ prophage can now be outlined. (1) The λ repressor binds to two operators immediately adjacent to its structural gene and, thus, prevents transcription at the immediate early promoters. The blockage of immediate early RNA synthesis effectively blocks all further λ transcription because gene N protein is not produced. (2) After induction, transcription of immediate early region gives rise to the gene N protein and tof gene product. Propagation of RNA polymerase along the λ genome is stopped at two ρ-dependent termination signals at the end of the immediate early gene. The tof gene product shuts off further production of gene cI mRNA; so that repressor cannot interfere

with the lytic cycle. (3) Gene N protein acts as an "antitermination" factor to activate transcription at three sites giving rise to recombination, replication, and gene Q product. λ DNA is excised from the bacterial chromosome and replicated. (4) Gene Q product causes initiation of transcription of all late genes by acting at a single promoter giving rise to structural components of λ. This outline indicates how the interaction of different control mechanisms can intermesh to cause the sequential production of gene products necessary for efficient production of infectious λ particles.

Other Bacteriophages

While it is impossible to discuss every phage system which has been studied, two other specific phages which provide rather interesting examples of control mechanisms will be mentioned. The helper-dependent or satellite phage P4 of *E. coli* can only multiply in the presence of the temperate phage P2 (Calendar, 1970). The helper can be present as a prophage, in which case only P4 phage are produced, or as a coinfecting phage, which leads to the production of both phages. The phage P4 requires most, or all, of the P2 late gene products (Six and Lindquist, 1970). P4 encapsulates its DNA in P2 head proteins, and, yet, the morphological structure of the head is different.

Normal expression of late functions in lytic development of phage P2 requires two early gene functions, A and B, as well as P2 DNA replication. The phage P4, however, can activate P2 late transcription in mutants of genes A or B, or when P2 phage DNA

replication does not occur (Six and Lindquist, 1971). These results suggest that P4 bypasses the normal control of P2 development and activates helper late transcription by a unique mechanism.

One potential mechanism for bypassing helper control would be the synthesis of a RNA polymerase activity coded for by P4 DNA, but specific for P2 late genes. It was found that P4 induces the synthesis of a novel rifampicin-resistant RNA polymerase (Barrett *et al.*, 1972). This RNA polymerase would transcribe the synthetic template, poly (dC)•poly (dG), to give rise to poly (rG) exclusively. Unfortunately, the P4-induced RNA polymerase would not utilize either P2 or P4 DNA (or any other natural DNA tested) as an *in vitro* template. Thus, the possible role of this enzyme in late helper gene activation is questionable. It has also been postulated that this RNA polymerase may play a role in P4 DNA replication by providing the primer for the initiation of DNA synthesis (Barrett *et al.*, 1972).

The phage T5 of *E. coli* is unique in that it injects its genome in two steps. After adsorption, only 8% of the total DNA of T5 is injected into the cell. The synthesis of "pre-early" mRNA and protein is directed by this segment of DNA (Moyer and Buchanan, 1969; McCorquodale and Buchanan, 1968). Certain of the pre-early products are required before the injection of the remaining 92% of the T5 genome occurs (Lanni, 1969). Hence, an obligatory delay in the transcription of the majority of the T5 genome is caused by the physical sequestration of that portion of the DNA.

After the complete T5 genome has been injected, the synthesis of "delayed early" and late classes of RNA and proteins occurs (Moyer and Buchanan, 1969; McCorquodale and Buchanan, 1968). Recently it has been demonstrated that the synthesis of a phage-specific 5'-exonuclease is necessary for the proper turning-on of late gene transcription (Chinnadurai and McCorquodale, 1973). This requirement for 5'-exonuclease is not due to its role in DNA replication, as late transcription can occur from unreplicated DNA, if a functional 5'-exonuclease is present (Hendrickson and McCorquodale, 1971). Therefore, it has been postulated that a modified form of T5 DNA, possibly one containing single-stranded nicks or gaps, is necessary for T5 late transcription (Chinnadurai and McCorquodale, 1973). This hypothesis is similar to the requirement of a replicating form of DNA for T4 late gene transcription (Riva *et al.*, 1970a), although it is not known whether the exact structures of the DNA molecules necessary to allow transcription to proceed are the same.

Concluding Remarks

As can be seen by this review, a large number of distinct control mechanisms have evolved for the regulation of bacteriophage development. These controls act mainly at the level of transcription and include both positive and negative regulatory events.

The clearest example of negative control of transcription is the action of λ repressor in blocking transcription of the lytic genes of λ DNA. The λ repressor has been purified and shown to act *in vitro* in the same manner that it does *in vivo*. The λ repressor is a prototypical repressor as defined by Jacob and Monod; it binds to DNA at specific sites (operators) and by virtue of this binding blocks transcription from the adjacent promoters (Jacob and Monod, 1961). The mechanism of the induction of λ repressor is unclear. Other examples of negative control have been discussed: the turning-off of mRNA for λ repressor and λ early proteins by the tof gene product; the turning-off of SP01 early mRNA synthesis by the TF1 factor; and the turning-off of host RNA transcription by a number of phages. While any of these events may involve a repressor, the mechanisms await further experimentation for elucidation.

The clearest example of positive control of transcription is the synthesis of a new RNA polymerase after T3-or T7-infection of *E. coli*. Due to the unique initiation specificity of the T3 and T7 RNA polymerases, a dramatic shift occurs in RNA synthesis after synthesis of these enzymes. The shift in transcription from early to middle classes of RNA in SP01 infection is thought to be due to an altered initiation specificity of the host RNA polymerase after association with phage-specific polypeptides. This association of new polypeptide factors to host RNA polymerase affecting transcriptional activity may play an important role in regulation, as such polypeptides have been seen associated with RNA polymerase after infections by T4, SP82, and φ29 (Holland and Whiteley, 1973), as well as during sporulation in *B. subtilus* (Greenleaf and Losick, 1973). In the case of T4, one of these polypeptides has been shown to be the product of the T4 regulatory gene--gene 33. The actual molecular mechanism by which these polypeptides affect transcription is still under investigation.

Two general modes of positive regulation of gene transcription have been demonstrated. One mechanism involves initiation of RNA synthesis at previously unused promoters. This mechanism could involve the alteration of the host RNA polymerase or synthesis of a new RNA polymerase. The second mechanism involves the readthrough of RNA polymerase past normal termination sites. The gene N protein of λ is thought to act as an "antitermination" factor to allow RNA polymerase to proceed past normal immediate early gene termination sites into the delayed early gene region. The shift from immediate early to delayed early RNA synthesis in T4 infection may also involve this mechanism to a certain degree.

In the cases of late gene transcription in T4- and T5-infected cells, it has also been demonstrated that some structural modification of the phage DNA may be necessary to turn on the transcription of certain viral genes. Thus, until the phage DNA assumes a certain configuration, as a consequence of some phage protein expressed earlier in infection, the synthesis of a certain class of RNA does not occur.

Despite the large number of control mechanisms seen in various bacteriophage systems, several generalizations concerning phage development can be made. Immediately after infection, the normal host RNA polymerase transcribes a certain limited number of viral genes. These first phage functions are generally regulatory in nature and involve the modification of the transcriptional machinery to alter its specificity. Subsequently, phage genes are expressed in classes which are generally related in function and clustered in one or a few regions of the phage genome. The phage genes involved in viral DNA replication are generally expressed at early times after infection, but their synthesis is dependent on earlier phage protein synthesis. After DNA replication, those phage products concerned with synthesis and assembly of the phage particle, maturation of viral DNA, and cell lysis are produced. While each change in gene expression is probably the result of a certain individual control mechanism, it is the interaction of the various elements of regulation which leads to the orderly production of phage products necessary for efficient phage production.

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ABSTRACT

Infection of *P. putida* by the bacteriophage gh-1 induced the synthesis of a novel DNA-dependent RNA polymerase. This gh-1-induced RNA polymerase was purified to near homogeneity. It was shown to be distinct from the host RNA polymerase ($\alpha_2\beta\beta'\sigma$) physically and in respect to many of its catalytic properties. The gh-1 polymerase was composed of a single polypeptide of approximately 98,000 molecular weight. RNA synthesis by the gh-1 polymerase was highly resistant to inhibition by rifampicin or streptolydigin, but could be inhibited by relatively low concentrations of monovalent ions or the rifamycin derivative AF/013. The antibiotic derivative, 3'-deoxyadenosine 5'-triphosphate, inhibited the gh-1 polymerase by competing for a single binding site with ATP. The phage polymerase was extremely sensitive to this inhibitor, exhibiting an apparent K_I value (2×10^{-8} *M*) about 100 times lower than that for the host RNA polymerase. The gh-1 polymerase showed a highly specific template requirement for DNA from the homologous gh-1 phage.

INTRODUCTION

When a bacterial cell becomes infected with a virulent bacteriophage, a shift in RNA synthesis occurs from entirely host-specific (transcription from the host DNA) to largely phage-specific (transcription from the viral DNA). There are two general types of mechanisms by which this shift in transcription can occur. In one mechanism, the host DNA-dependent RNA polymerase is utilized throughout the infectious cycle for the transcription of all classes of viral genes. Modifications of the host RNA polymerase in the viral-infected cell, however, alter the specificity of the enzyme to program changes in transcription during the infectious cycle. This mechanism most likely occurs in T4 and λ bacteriophage infections of *Escherichia coli* (Haselkorn *et al.*, 1969; Takeda, *et al.*, 1969), SP01 and SP82 infections of *Bacillus subtilus* (Geiduschek and Sklar, 1969; Spiegelman and Whiteley, 1974), and ϕ29 bacteriophage infection of *Bacillus amyloliquefaciens* (Holland and Whiteley, 1973). The exact nature of the modification causing altered specificity of the host RNA polymerase is unknown. Several chemical alterations of the subunits of the host RNA polymerase have been demonstrated after T4 infection of *E. coli* (Seifert *et al.*, 1971; Goff and Weber, 1970; Travers, 1969). Furthermore, T4 specific polypeptides, some of which have been shown to be the products of T4 regulatory genes, have been found associated with the host RNA polymerase in T4-infected cells (Stevens, 1972; Horvitz, 1973). It has not been demonstrated, however, which if any, of these modifications confers altered transcriptional specificity to the host RNA polymerase.

The second mechanism to account for the shift in DNA transcription after bacteriophage infection involves the synthesis of a new, viral-coded DNA-dependent RNA polymerase. This mechanism has been shown to occur in both T3- and T7-infection of _E. coli_ (Chamberlin _et al._, 1970; Summers and Siegel, 1971; Maitra, 1971; Dunn _et al._, 1971). The new RNA polymerases synthesized after infection by these coliphages are quite different from the host RNA polymerase in both structure and catalytic properties. These phage-induced RNA polymerases are composed of single polypeptides of approximately 108,000 to 110,000 molecular weight (Chamberlin _et al._, 1970; Dunn _et al._, 1971). The _E. coli_ RNA polymerase is composed of five subunits, $\alpha_2\beta\beta'\sigma$, with a combined molecular weight of 470,000 (Burgess, 1969). The phage-induced RNA polymerases show highly stringent template specificities _in vitro_ for their homologous phage DNA, whereas the host RNA polymerase can utilize DNA from the variety of sources (Chamberlin _et al._, 1970; Maitra, 1971; Dunn _et al._, 1971). A comparison of other properties of these two types of RNA polymerases has been recently presented (Bautz, 1973).

We have examined the regulation of RNA synthesis after the infection of _Pseudomonas putida_ by the bacteriophage gh-1. gh-1 is a small, virulent bacteriophage, isolated in this laboratory, with a linear, double-stranded DNA having a molecular weight of 23 x 10^6 (Lee and Boezi, 1966). In this paper, we report that gh-1 infection of _P. putida_ induces the synthesis of a new DNA-dependent RNA polymerase. This gh-1-induced RNA polymerase has been purified and its structure and catalytic properties studied. A preliminary report on some of this work has been previously presented (Towle *et al.*, 1973).

MATERIALS AND METHODS

Materials

Whatman DEAE-cellulose (DE-52) and phosphocellulose (P-11) were purchased from Reeve Angel. Dithiothreitol, calf thymus DNA, yeast glucose-6-phosphate dehydrogenase, and unlabeled nucleoside triphosphates were obtained from P-L Biochemicals. ^{3}H-labeled ribonucleoside triphosphates were from Schwarz-Mann and [γ-^{32}P]-labeled ATP and GTP from New England Nuclear. Poly(dC)•poly(dG) and poly [d(A-T)] were purchased from Miles Laboratories. *E. coli* alkaline phosphatase, beef heart lactate dehydrogenase, bovine hemoglobin, rabbit muscle phosphorylase a, bovine serum albumin, and chloramphenicol were obtained from Sigma. Beef liver catalase was from Worthington Biochemicals. Blue dextran 2000 was purchased from Pharmacia Fine Chemicals and Bio-Gel P-200 from Bio-Rad Laboratories. T4 DNA and T7 DNA were the kind gifts of Dr. Loren Snyder, Department of Microbiology and Public Health, Michigan State University. Rifamycin derivatives were the gifts of Dr. Luigi Silvestri, Gruppo Lepetit, Inc., Milan, Italy. ^{3}H-labeled ribosomal RNA, 3'-deoxyadenosine, 3'-deoxyadenosine 5'-diphosphate, 3'-deoxyadenosine 5'-triphosphate, and 3'-*O*-methyladenosine 5'-triphosphate were the very generous gifts of Ron Desrosiers and Dr. Fritz Rottman of this department. All other materials were obtained from sources previously described (Johnson *et al.*, 1971; Gerard *et al.*, 1972).

Growth of gh-1-Infected P. putida

Pseudomonas putida (ATCC 12633) was grown at 33° C in a medium containing, in grams per liter: yeast extract, 5; tryptone, 5; glucose, 5; NaCl, 8; Na_2HPO_4, 6; KH_2PO_4, 3. After cell growth had reached mid-logrithmic phase, gh-1 phage were added to a multiplicity of 5 plaque-forming units per cell. After ten minutes of incubation, the culture was poured onto a half volume of crushed ice (-20° C) and collected immediately by centrifugation at 0° C. Infected cells were quick-frozen in an acetone-dry ice bath and stored at -20° C.

Purification of gh-1-Induced RNA Polymerase

All procedures were performed at 0° to 4° C. Frozen gh-1-infected P. putida (30 grams wet weight) were ground in a mortar and pestle in two volumes of acid-washed glass beads until cell breakage occurred. The cell homogenate was extracted in four to six volumes of buffer containing 10 mM Tris•HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol (Buffer A; Initial Extract Fraction). This fraction was centrifuged at 105,000 x g for 2 hr. The pellet, which contained 75 to 90% of the RNA polymerase activity, was extracted with 1 M NH_4Cl in Buffer A and centrifuged at 105,000 x g for 1 1/2 hr. The supernatant solution was dialyzed for 12 hr against Buffer A minus $MgCl_2$ (NH_4Cl Wash Fraction) and applied to a DEAE-cellulose column (4 x 16 cm) equilibrated with the same buffer. The RNA polymerase activity was eluted with a linear gradient of 1 liter from 0 to 0.4 M KCl in Buffer A minus $MgCl_2$. A single peak of RNA polymerase activity eluted at about 0.17 M KCl, as determined by conductivity measurements. The fractions containing the majority of the RNA polymerase activity were pooled and dialyzed against a buffer containing 20 mM potassium phosphate, pH 7.5, 5 mM 2-mercaptoethanol, 15% (v/v) glycerol (Buffer B) for 12 hours (DEAE Fraction). The dialyzed fraction was applied to a phosphocellulose column (2 x 12 cm) equilibrated with Buffer B. The column was eluted with a linear gradient of 400 ml from 0 to 0.6 M KCl in Buffer B. RNA polymerase activity appeared as a single peak at about 0.35 M KCl. The fractions with the majority of RNA polymerase activity were pooled and concentrated to a volume of approximately 2 ml using an Amicon Micro-Ultrafiltration System, Model 8MC, with a PM-30 Diaflo membrane. This fraction (Phosphocellulose Fraction) was dialyzed against a buffer containing 20 mM potassium phosphate, pH 7.5, 0.5 mM dithiothreitol, 0.2 M KCl, 7.5% (v/v) glycerol for 8 hr before being layered on the top of a 1.5 x 85 cm Bio-Gel P-200 (50-150 mesh) column. The Bio-Gel column was equilibrated and developed in a buffer containing 20 mM potassium phosphate, pH 7.5, 0.5 mM dithiothreitol, 0.2 M KCl, 5% (v/v) glycerol. The fractions with RNA polymerase activity were again pooled, concentrated by ultrafiltration, and dialyzed against a buffer containing 50 mM Tris•HCl, pH 8.0, 1 mM dithiothreitol, 50% (v/v) glycerol (Bio-Gel Fraction). This fraction was stored at -20° C.

For purposes of further purification, the Bio-Gel Fraction was dialyzed against a buffer containing 20 mM Tris•HCl, pH 8.0, 0.5 mM dithiothreitol, 0.2 M KCl, 5% (v/v) glycerol for 12 hours. Samples of 0.10 to 0.15 ml were layered onto the top of 4.8 ml 10 to 30% glycerol gradients made in 20 mM Tris•HCl, pH 8.0, 0.5 mM dithiothreitol, 0.2 M KCl. Centrifugation was performed at 44,000 RPM in a Spinco SW 50.1 rotor at 2° C for 13 hr. Fractions of 0.16 ml were collected and small aliquots analyzed for activity. Fractions with the majority of RNA polymerase activity were pooled and dialyzed against a buffer containing 50 mM Tris•HCl, pH 8.0, 1 mM dithiothreitol, 50% (v/v) glycerol (Glycerol Gradient Fraction).

Assay for RNA Polymerase Activity

The assay of RNA polymerase activity measured the incorporation of CMP into a form insoluble in trichloroacetic acid. The standard reaction mixture contained in a final volume of 0.125 ml: 40 mM Tris•HCl, pH 8.0, 1 mM dithiothreitol, 10 mM $MgCl_2$, 400 μg/ml bovine serum albumin, 0.4 mM each of ATP, ^{3}H-CTP, GTP and UTP, 50 μg/ml gh-1 DNA and RNA polymerase, as indicated. The specific activity of ^{3}H-CTP was 1×10^4 CPM/nmole. In experiments in which the apparent Km value of CTP was measured, ^{3}H-UTP was used as the labeled nucleoside triphosphate at the same specific activity. Reactions were initiated by the addition of enzyme and incubated for 10 min at 30° C. Termination of the reaction, filtration onto nitrocellulose membrane filters, and analysis of the filters for radioactivity were as previously described (Johnson et al., 1971). One unit of enzyme activity was equal to the incorporation of one nmole of CMP in 1 hr. The specific enzyme activity was the number of units per milligram of protein as determined by the method of Lowry using bovine serum albumin as the standard (Lowry et al., 1951).

Assay of Other Enzyme Activities

E. coli alkaline phosphatase was assayed by following the rate of release of p-nitrophenol from p-nitrophenyl phosphate, as determined spectrophotometrically at 410 nm. The sedimentation coefficient of alkaline phosphatase was taken as 6.1 S (Schlesinger and Barrett, 1965) and the molecular Stokes radius as 29.2 angstroms (Laurant and Killander, 1965). Glucose-6-phosphate dehydrogenase was assayed by following the reduction of $NADP^+$ in the presence of glucose-6-phosphate, as measured by the increase in absorbance at 340 nm. The diffusion coefficient of glucose-6-phosphate dehydrogenase was taken as 5.77×10^{-7} cm^2 sec^{-1} (Yue *et al.*, 1967). Lactate dehydrogenase was assayed by following the oxidation of NADH in the presence of pyruvate, as determined spectrophotometrically at 340 nm. The sedimentation coefficient of lactate dehydrogenase used was 7.4 S and the diffusion coefficient 5.05×10^{-7} cm^2 sec^{-1} (Jaenicke and Knof, 1968). The molecular Stokes radii of lactate dehydrogenase and glucose-6-phosphate dehydrogenase were determined from their respective diffusion coefficients as described by Siegel and Monty (1966).

RNase activity was assayed by determining if any change occurred in the sucrose density gradient sedimentation profile of ^3H-labeled ribosomal RNA after incubation at 30° C for 20 min with 6 μg/ml or 60 μg/ml of gh-1 polymerase (Bio-Gel Fraction). DNase was assayed similarly using native ^{32}P-labeled gh-1 DNA. RNase III activity was assayed by the procedure of Robertson *et al.* (1968) using ^3H-labeled poly[r(A,U)] as substrate.

Preparation of Bacteriophages and DNA

P. putida bacteriophage gh-1 was purified from cell lysates by two rounds of differential centrifugation, followed by DEAE-cellulose chromatography (Lee and Boezi, 1966). *E. coli* bacteriophage T3 was purified from cell lysates by differential centrifugation, followed by banding in a preformed CsCl density gradient. All bacteriophage DNA preparations were purified by the method of Thomas and Abelson (1966). *P. putida* DNA was prepared by the procedure of Thomas *et al.* (1966). Commercially obtained calf thymus DNA was further purified by two SDS-phenol extractions, followed by extensive dialysis.

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed using a modification of the procedure of Shapiro *et al.* (1971), as described by Johnson *et al.* (1971). Samples of 2 to 15 μg of protein in 50 μl or less were layered on 0.5 x 10 cm 5% (w/v) polyacrylamide gels. Electrophoresis was performed for six to eight hr at 4 volts/cm of gel length. Gels were stained for protein with 0.4% (w/v) Coomassie brilliant blue in 10% (w/v) trichloroacetic acid, 33% (v/v) methanol for 8 to 12 hr. Destaining was performed on a diffusion destainer in 10% trichloroacetic acid, 33% methanol for 6 hr. Gels were removed from the diffusion destainer and incubated in 10% trichloroacetic acid at 30° C until the background was clear (about 4 hr). Gels were stored at 4° C in 10% trichloroacetic acid.

Other Methods

Extracts of either gh-1-infected or uninfected *P. putida*, which were used to assay RNA polymerase activity directly, were made by suspending cells in two volumes of Buffer A. These suspensions were sonicated for 1 1/2 minutes (in 30 second bursts) at a setting of 70 on a Biosonik sonicator and then centrifuged at 16,000 x g for 20 minutes to remove cellular debris. RNA polymerase assays were performed with varying amounts of extract to ensure the enzyme activity was linearly proportional to the protein concentration.

The purification of *P. putida* RNA polymerase was performed by the method of Johnson *et al.* (1971). The preparation of RNA polymerase used in these studies was more than 95% pure, as determined by SDS-polyacrylamide gel electrophoresis.

RESULTS

A Novel RNA Polymerase Activity in Bacteriophage gh-1-Infected P. putida

The first evidence that a novel RNA polymerase is synthesized after gh-1-infection of P. putida was obtained from measurements of the RNA polymerase activity in extracts of uninfected and gh-1-infected cells. In extracts from uninfected cells, RNA polymerase activity was inhibited 97% by the addition to the reaction mixture of the antibiotics-rifampicin and streptolydigin (Table I). This activity is largely, if not entirely, due to the P. putida RNA polymerase, which is known to be sensitive to these antibiotics (Johnson et al., 1971). In extracts from gh-1-infected cells, the specific activity of RNA polymerase was eleven times greater than the specific activity in extracts from uninfected cells. Furthermore, this activity from infected cells was inhibited only 4% by the addition to the reaction mixture of the two bacterial RNA polymerase inhibitors. Addition to the reaction mixture of actinomycin D and nogalamycin, which inhibit RNA synthesis by binding to DNA, almost completely inhibited the activity from extracts of gh-1-infected cells (Bhuyan and Smith, 1965; Goldberg et al., 1962). This activity is, therefore, due to a DNA-directed process. In extracts from cells infected with gh-1 in the presence of chloramphenicol, the specific activity of RNA polymerase was essentially the same as that in uninfected cells. This

TABLE I.--Specific Activity of RNA Polymerase in Extracts of Uninfected and Bacteriophage gh-1-Infected *P. putida*.[a]

Components of the Reaction Mixture	Specific Activity of RNA Polymerase (units/mg)		
	Extract of Uninfected *P. putida*	Extract of *P. putida* Infected with gh-1	Extract of *P. putida* Infected with gh-1 in the Presence of Chloramphenicol[b]
Standard	17	193	15
Standard plus Rifampicin and Streptolydigin	0.6	186	0.5
Standard plus Actinomycin and Nogalamycin	0.3	1.7	0.4

[a] Components of the standard reaction mixture and preparation of cell extracts were as described in Materials and Methods. Rifampicin and streptolydigin, when added to the reaction mixture, were at concentrations of 5 µg/ml and 100 µg/ml, respectively. Actinomycin and nogalamycin, when present, were both at a concentration of 10 µg/ml. Reactions were initiated by the addition of extract to a final protein concentration between 50 and 400 µg/ml. Reactions were incubated and treated as described in Materials and Methods.

[b] Chloramphenicol was added to the growth medium to a final concentration of 100 µg/ml one minute before the addition of gh-1 phage.

activity also was sensitive to rifampicin and streptolydigin. Thus, protein synthesis is necessary for the appearance of the rifampicin and streptolydigin resistant RNA polymerase activity. While other interpretations are possible, these results can most readily be explained by the synthesis of a novel DNA-dependent RNA polymerase after gh-1 infection of *P. putida*. This explanation was verified by the purification of the gh-1-induced RNA polymerase and a study of its structure and catalytic properties.

Purification of the gh-1-Induced RNA Polymerase

The results of the purification of the gh-1-induced RNA polymerase, performed as described in Materials and Methods, are shown in Table II. The Bio-Gel Fraction which was used for many of the catalytic studies reported below, had a specific enzyme activity of 42,000 units/mg. This represents a 280-fold purification from the Initial Extract Fraction. An accurate determination of the specific enzyme activity of the Glycerol Gradient Fraction could not be made due to the difficulty of determining protein concentration by the method of Lowry at the relatively low level present in this fraction. An estimate of the protein concentration of the Glycerol Gradient Fraction, however,

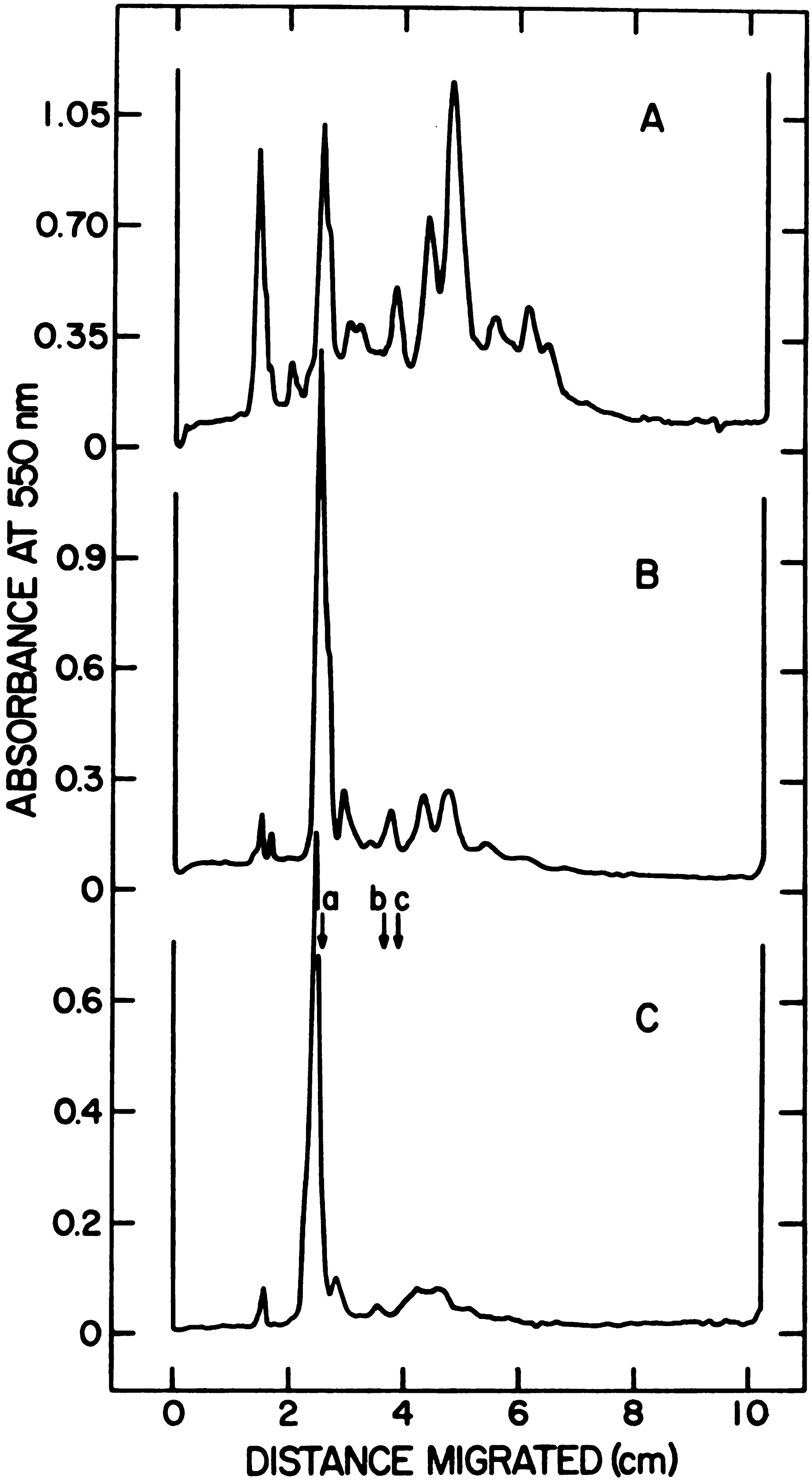
TABLE II.--Summary of Purification.[a]

Fraction	Total Protein (mg)	Total Enzyme Activity (units $X10^{-4}$)	Recovery of Enzyme Activity (%)	Specific Activity (units/mg)
Initial Extract Fraction	2900	44	100	150
NH_4Cl Wash Fraction	1300	39	89	300
DEAE Fraction	330	21	48	640
Phosphocellulose Fraction	7.2	6.3	14	8700
Bio-Gel Fraction	0.62	2.6	6	42000
Glycerol Gradient Fraction	(0.23)[b]	2.0	5	(86000)[b]

[a] Summary of purification of gh-1-induced RNA polymerase from 30 gr (wet weight) of gh-1-infected *P. putida* as described in Materials and Methods.

[b] Based on protein concentration determination made from SDS-polyacrylamide gel electrophoresis of sample as described in Results.

Figure 1.--SDS-polyacrylamide gel scans of fractions from the purification of gh-1-induced RNA polymerase. Samples of the Phosphocellulose Fraction (A, 12.7 μg), Bio-Gel Fraction (B, 9 μg), and Glycerol Gradient Fraction (C, approximately 5 μg) of gh-1 polymerase were subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. Electrophoresis was performed at 4 volts per cm of gel length for 6.25 hours at 25° C. After staining and destaining, the gels were scanned at 550 nm on a Gilford linear transport. The direction of migration was from left to right. The arrows indicate the peak positions of the reference proteins--phosphorylase a (a), bovine serum albumin (b), and catalase (c).


A
B
C
a
b
c
1.05
0.70
0.35
0
0.9
0.6
0.3
0
0.6
0.4
0.2
0
ABSORBANCE AT 550 nm
0
2
4
6
8
10
DISTANCE MIGRATED (cm)

could be made from the SDS-polyacrylamide gel electrophoresis of this fraction (Figure 1C). By measuring the area under the peaks of the scan at 550 nm of the SDS-polyacrylamide gel and comparing with the area under the peaks of known amounts of the reference proteins, the amount of protein present in the gel could be determined. This determination is dependent on the demonstration that the amount of stain absorbed by the SDS-polyacrylamide gel is linearly related to the amount of protein present (Johnson et al., 1971). From this estimate of protein concentration, a specific enzyme activity of 86,000 units/mg was calculated for the Glycerol Gradient Fraction.

Analysis of the Bio-Gel Fraction for RNase and DNase activities, contaminants of RNA polymerase preparations which can alter the observed RNA polymerase activity, were negative. The Bio-Gel Fraction also did not contain any RNase III activity, the enzyme involved in the "sizing" of T7 early mRNA in E. coli (Dunn and Studier, 1973a; Dunn and Studier, 1973b). The Bio-Gel Fraction is also free of any host RNA polymerase activity. The slowest migrating polypeptides on SDS-polyacrylamide gel electrophoresis of the Bio-Gel Fraction migrated significantly ahead of the β and β' subunits of purified P. putida RNA polymerase. The Phosphocellulose and Bio-Gel Fractions could be stored at -20° C in buffer containing 50% glycerol for several months with little loss of activity, if the protein concentration was equal to or greater than 0.5 mg/ml.

Analysis of the Phosphocellulose Fraction, the Bio-Gel Fraction, and the Glycerol Gradient Fraction was performed by SDS-polyacrylamide gel electrophoresis (Figure 1). The Glycerol Gradient

Fraction contained one major polypeptide which comprised approximately 80%, by weight, of the total protein present (Figure 1C). No other polypeptide present comprised more than 8% of the total protein. The molecular weight of the major polypeptide was determined by comparison of its mobility to the mobility of the reference proteins--phosphorylase a (subunit molecular weight of 94,000), catalase (68,000), and bovine serum albumin (60,000). Using a standard curve of the logarithms of the molecular weights of the reference proteins to the distances of migration, a molecular weight of 97,000 was estimated for the major polypeptide of the Glycerol Gradient Fraction. This major polypeptide is thought to be the only polypeptide comprising the gh-1 polymerase. It is the only polypeptide which increased in relative purity in the last two steps of the purification procedure. Its increase in purity parallels the increase in specific enzyme activity of gh-1 polymerase in these last two steps. Finally, the molecular weight of the gh-1 polymerase, as determined by SDS-polyacrylamide gel electrophoresis, is consistent with a determination of 98,000 made by glycerol gradient centrifugation and gel filtration (see below). The gh-1 polymerase polypeptide was 10 to 15%, by weight, of the total protein in the Phosphocellulose Fraction (Figure 1A) and 50 to 55% of the Bio-Gel Fraction (Figure 1B).

Molecular Weight and Structure of the gh-1-Induced RNA Polymerase

The molecular weight of the gh-1 polymerase was calculated using experimentally obtained values for its sedimentation coefficient and molecular Stokes radius. A molecular weight value calculated in

this manner is not dependent on assumptions concerning the shape of the macromolecule (Siegel and Monty, 1966).

The sedimentation coefficient of gh-1 polymerase was determined by sedimentation velocity centrifugation in a 10 to 30% glycerol gradient (Figure 2). The reference proteins, alkaline phosphatase, lactate dehydrogenase, and P. putida RNA polymerase, were centrifuged under identical conditions. Based on the sedimentation coefficients of the reference proteins, the gh-1 polymerase exhibited a sedimentation coefficient of 6.1 ± 0.2 S.

The molecular Stokes radius of the gh-1-induced RNA polymerase was obtained by gel filtration on a Bio-Gel P-200 column (Figure 3). The reference proteins, alkaline phosphatase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, and hemoglobin, were chromatographed under identical conditions to standardize the column. Using the relationship derived by Ackers (1964) between molecular radius and distribution coefficient, the molecular radius of gh-1 polymerase was calculated to be 38 angstroms. By combining the molecular radius determined by gel filtration and a sedimentation coefficient from sedimentation velocity centrifugation and assuming a $\bar{v}$ of 0.73 ml/gm, a molecular weight estimate of 98,000 can be calculated for the gh-1 polymerase. This value for the molecular weight is in good agreement with the value of 97,000 obtained using SDS-polyacrylamide gel electrophoresis. Together these results indicate that the gh-1-induced RNA polymerase is composed of a single polypeptide with a molecular weight of 95,000 to 100,000.

Figure 2.--Glyerol gradient centrifugation of gh-1-induced RNA polymerase. gh-1 polymerase (Phosphocellulose Fraction, 18 μg) was mixed with 150 μg of bovine serum albumin and diluted to 0.15 ml in a buffer containing 20 m*M* Tris·Cl, pH 8.0, 0.5 m*M* dithiothreitol, and 0.2 *M* KCl. After dialysis for 6 hours against the same buffer, a 0.1 ml sample was layered on a 4.8 ml 10 to 30% linear glycerol gradient prepared in the above buffer and containing 0.5 mg/ml of bovine serum albumin. On three parallel gradients, 0.1 ml samples of the reference proteins--*P. putida* RNA polymerase holoenzyme (80 μg), *E. coli* alkaline phosphatase (70 μg), and beef heart lactate dehydrogenase (7 μg)--in the same buffer were layered. All gradients were centrifuged for 13 hours at 4° C in a Spinco SW50L rotor at 45,000 RPM in the same run. After centrifugation, thirty-two fractions of 0.16 ml were collected from each gradient and enzyme assays performed on the fractions as described in Materials and Methods. The arrows indicate the peak positions of the reference proteins--*P. putida* RNA polymerase ($\alpha_2\beta\beta'\sigma$), alkaline phosphatase (AP), and lactate dehydrogenase (LDH). The recovery of gh-1 polymerase activity was approximately 90%.

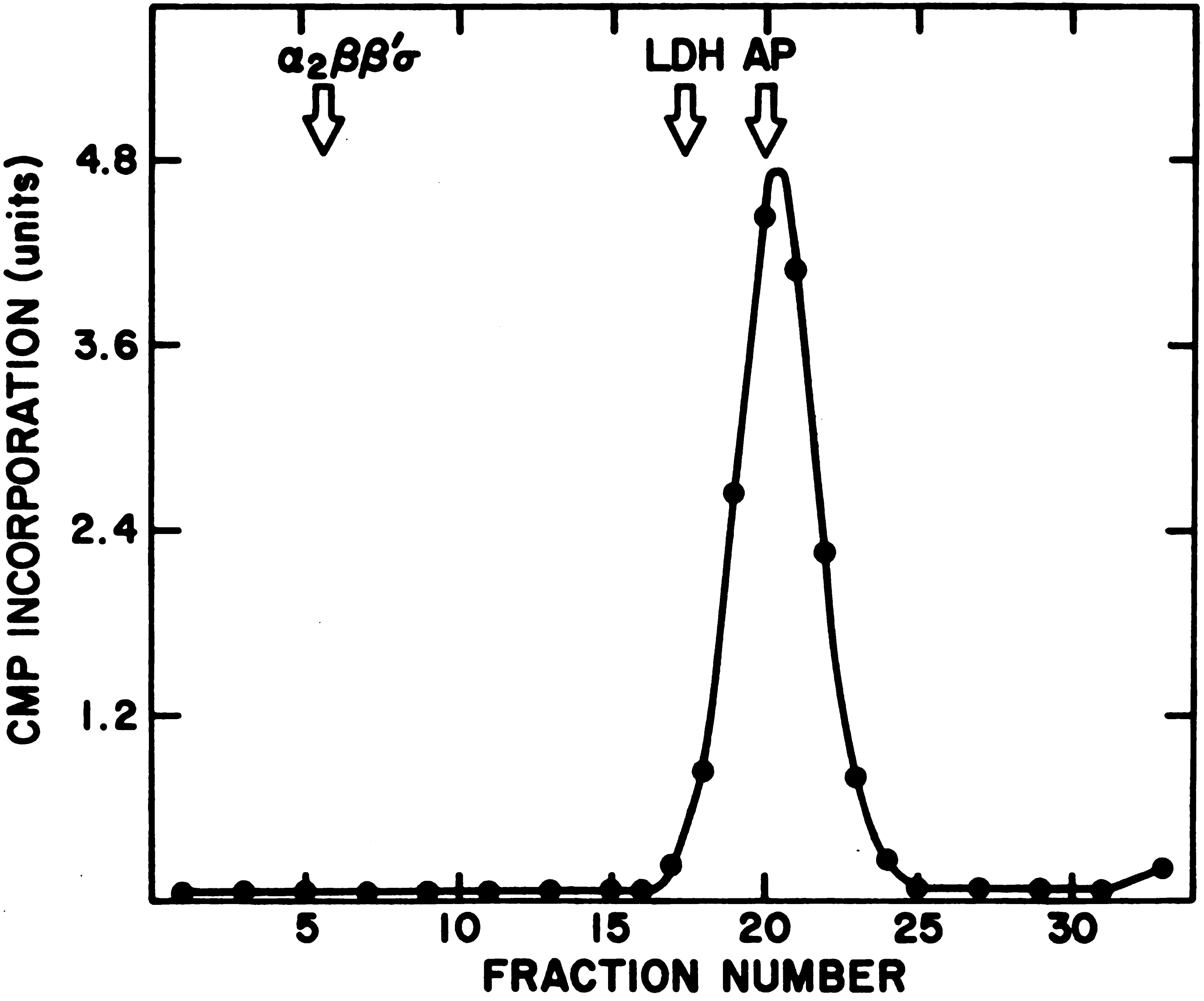
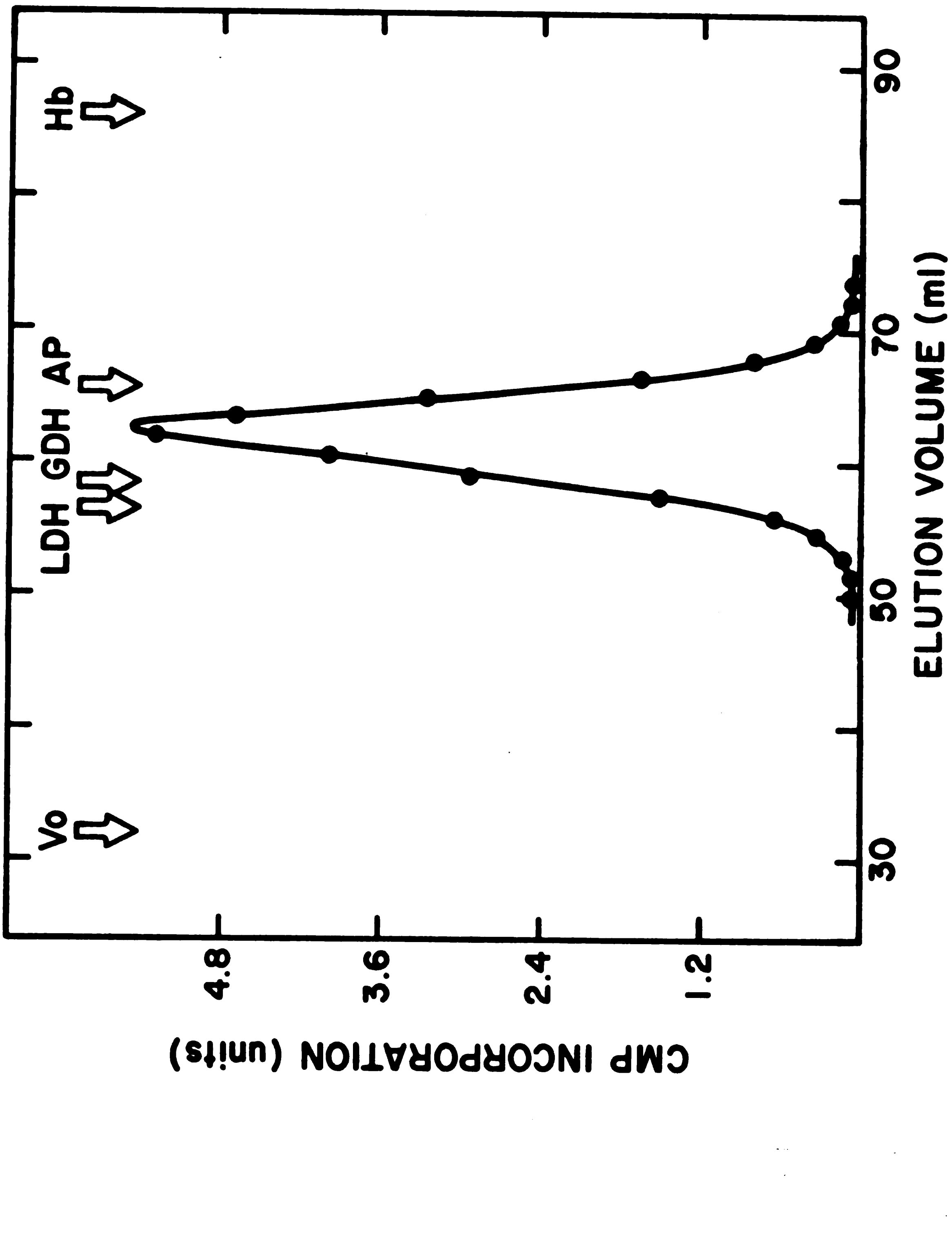

$\alpha_2\beta\beta'\sigma$
LDH
AP
CMP INCORPORATION (units)
4.8
3.6
2.4
1.2
5
10
15
20
25
30
FRACTION NUMBER

Figure 3.--*Gel filtration of gh-1-induced RNA polymerase on Bio-Gel P-200.* gh-1 polymerase (Phosphocellulose Fraction, 60 μg) was diluted to 1 ml in a buffer containing 20 m*M* potassium phosphate, pH 7.5, 0.5 m*M* dithiothreitol, 0.2 *M* KCl, and 7.5% (v/v) glycerol and dialyzed against the same buffer for 6 hours. The 1 ml sample was layered on the top of a 1.5 x 77 cm Bio-Gel P-200 (100-200 mesh) column which had been previously equilibrated with a buffer containing 20 m*M* potassium phosphate, pH 7.5, 0.5 m*M* dithiothreitol, 0.2 *M* KCl, 5% (v/v) glycerol, and 0.5 mg/ml bovine serum albumin at 4° C. The column was developed in the same buffer at a flow rate of 2.1 ml/hour and fractions of 1.5 ml were collected. To standardize the column, two samples containing markers were chromatographed under exactly the same conditions in subsequent runs. One sample contained the markers--0.2% (w/v) blue dextran 2000, *E. coli* alkaline phosphatase (1 mg), and beef heart lactate dehydrogenase (100 μg). The markers in the second sample were 0.2% (w/v) blue dextran 2000, yeast glucose-6-phosphate dehydrogenase (500 μg), and bovine hemoglobin (1.5 mg). Fractions from the column were analyzed for various enzyme activities as described in Materials and Methods. Blue dextran 2000 and hemoglobin were assayed spectrophotometrically at 650 nm and 410 nm, respectively. The peak position of the markers are shown by the arrows: blue dextran 2000--Vo, lactate dehydrogenase--LDH, glucose-6-phosphate dehydrogenase--GDH, alkaline phosphatase--AP, and hemoglobin--Hb.


Vo
LDH
GDH
AP
Hb
CMP INCORPORATION (units)
4.8
3.6
2.4
1.2
30
50
70
90
ELUTION VOLUME (ml)

Characterization of RNA Synthesis by the gh-1-induced RNA Polymerase Using gh-1 DNA as Template

The general requirements for *in vitro* RNA synthesis by the purified gh-1 polymerase were examined by varying the components of the standard reaction mixture (Table III). When the enzyme, the gh-1 DNA, one of the four ribonucleoside triphosphates, or the Mg^{2+} was removed from the reaction mixture, little or no RNA synthesis occurred. Near maximal enzyme activity was maintained over a broad concentration

TABLE III.--Characteristics of RNA Synthesis by gh-1-Induced RNA Polymerase.[a]

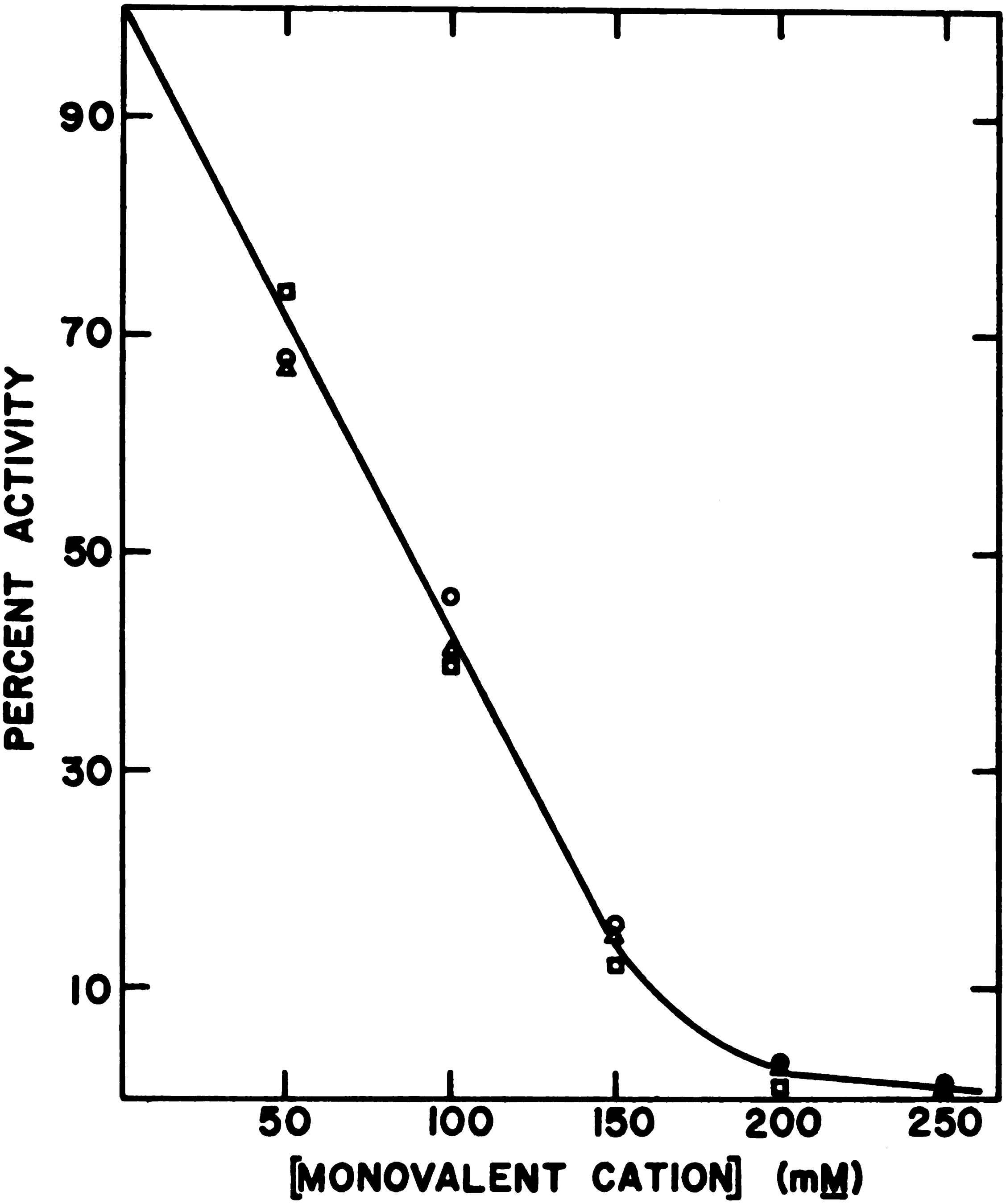
Components of the Reaction Mixture	nmol of CMP Incorporated per Hour
Standard	9.30
Minus Enzyme	0
Minus gh-1 DNA	0
Minus ATP or GTP or UTP	0 - 0.04
Minus $MgCl_2$	0
Minus $MgCl_2$; Plus $MnCl_2$ or $CaCl_2$ or $ZnCl_2$ (0.5-8 m*M*)	0
Plus 2 m*M* $MnCl_2$ or $CaCl_2$	0.84
Plus 2 m*M* $ZnCl_2$	0
Plus 85 m*M* KCl	4.45
Plus 200 m*M* KCl	0.18

[a] The components of the standard reaction mixture were as described in Materials and Methods. Where indicated, the appropriate component was removed from or added to the standard reaction mixture. Reactions were initiated by the addition of 2.4 µg/ml gh-1 polymerase (Bio-Gel Fraction). Incubation, termination, and analysis of reaction mixtures for CMP incorporation were as described in Materials and Methods.

range of 5 to 20 m*M* Mg^{2+} with the optimum activity occurring at about 10 m*M* (data not shown). No detectable RNA synthesis occurred when the Mg^{2+} was replaced in the standard reaction mixture by the divalent metal ions--Mn^{2+}, Zn^{2+}, or Ca^{2+}--at concentrations between 0.5 and 8 m*M* (Table III). In fact, the addition of any of these divalent metal ions at 2 m*M* to the reaction mixture containing Mg^{2+} inhibited the enzyme activity 93 to 100%. The activity of the gh-1 polymerase was also inhibited quite markedly by relatively low concentrations of monovalent ions. At a concentration of 85 m*M* KCl, the gh-1 polymerase activity was inhibited 50%; while at 200 m*M*, the reaction was essentially completely inhibited. An almost identical inhibition of enzyme activity was observed with either NaCl or NH_4Cl (Figure 4). This inhibition, therefore, is probably a general effect of ionic strength.

Apparent Km values for each of the four ribonucleoside triphosphates which are substrates for RNA synthesis were determined. For these studies, the concentration of three of the ribonucleoside triphosphates was fixed at a high level, greater than five times the Km for any ribonucleoside triphosphate. The concentration of the fourth ribonucleoside triphosphate was varied and the initial reaction rates measured at each concentration. To analyze the results, Michaelis-Menton kinetics were assumed applicable to this complex reaction, and the results were plotted in Lineweaver-Burk double reciprocal plots (1/V versus 1/[S]). All data were analyzed by a computer program to determine the highest correlation to a least squares straight line for the equation:

Figure 4.--The effect of varying concentrations of KCl, NaCl, and NH_4Cl on RNA synthesis by the gh-1-induced RNA polymerase. Standard reaction mixtures were prepared as described in Materials and Methods except KCl (○), NaCl (△), or NH_4Cl (□) was added to the final concentrations indicated. Reactions were initiated by the addition of gh-1 polymerase (Bio-Gel Fraction) to a final concentration of 2.4 µg/ml. Incubation, termination, and analysis of reaction mixtures for CMP incorporation were as described in Materials and Methods. The incorporation in reactions containing monovalent cations was compared to reactions containing no additions, in which 1.75 nmol CMP were incorporated by the gh-1 polymerase in 10 minutes.


PERCENT ACTIVITY
90
70
50
30
10
50
100
150
200
250
[MONOVALENT CATION] (m$\underline{M}$)

$$v = v_{max} - Km^n \; (v/[NTP]^n)$$

as n was varied in increments of 0.05 units (Dunne et al., 1973). An n value so determined is equivalent to the Hill coefficient, n, and should equal 1.0 if the double reciprocal plot is linear.

For the purine ribonucleoside triphosphate, ATP, the double reciprocal plot was linear (Figure 5A). The apparent Km value for ATP was 3.5 x 10^{-5} M. Likewise, the pyrimidine ribonucleoside triphosphates, CTP and UTP, yielded linear double reciprocal plots (data not shown). The apparent Km value for both of these substrates in the RNA polymerase reaction was 4.0 x 10^{-5} M. For the purine ribonucleoside triphosphate, GTP, however, the double reciprocal plot was curvilinear (Figure 5A). An n value of 1.2 for GTP was determined by the computer analysis. Thus, the best fit to a straight line was obtained when 1/V was plotted versus $1/[GTP]^{1.2}$ (Figure 5B). The kinetics of RNA synthesis at the lowest GTP concentration used in the Km study was linear for at least five minutes and showed no appreciable lag in initiation (data not shown). Thus, the higher order n value is not due to non-linear reaction rates at the lower substrate concentrations. The apparent Km value for GTP, using the higher order value of substrate concentration in the Michaelis-Menton equation, was 8.0 x 10^{-5} M or twice that seen for the other three ribonucleoside triphosphates.

The initiation of RNA synthesis by gh-1-induced RNA polymerase with gh-1 DNA as template was measured using [γ-^{32}P]-labeled purine ribonucleoside triphosphates. As shown in Figure 6, gh-1

Figure 5.--The effect of varying the concentration of a single nucleoside triphosphate on the activity of gh-1-induced RNA polymerase. Reaction mixtures for gh-1 polymerase were prepared as described in Materials and Methods except that the concentration of one nucleoside triphosphate was varied while the concentrations of the other three nucleoside triphosphates were kept constant at 0.4 mM. The reaction mixtures were prewarmed to 30° C and RNA synthesis initiated by the addition of gh-1 polymerase (Bio-Gel Fraction) to a final concentration of 2.4 μg/ml. After five minutes of incubation, the reactions were terminated and the incorporation of ^{3}H-CTP into acid-insoluble material determined as described in Materials and Methods. A, double reciprocal plot of 1/V versus 1/concentration of nucleoside triphosphate for ATP (○) and GTP (□). B, the data of A for GTP replotted as 1/V versus 1/concentration of GTP raised to the 1.2 power.

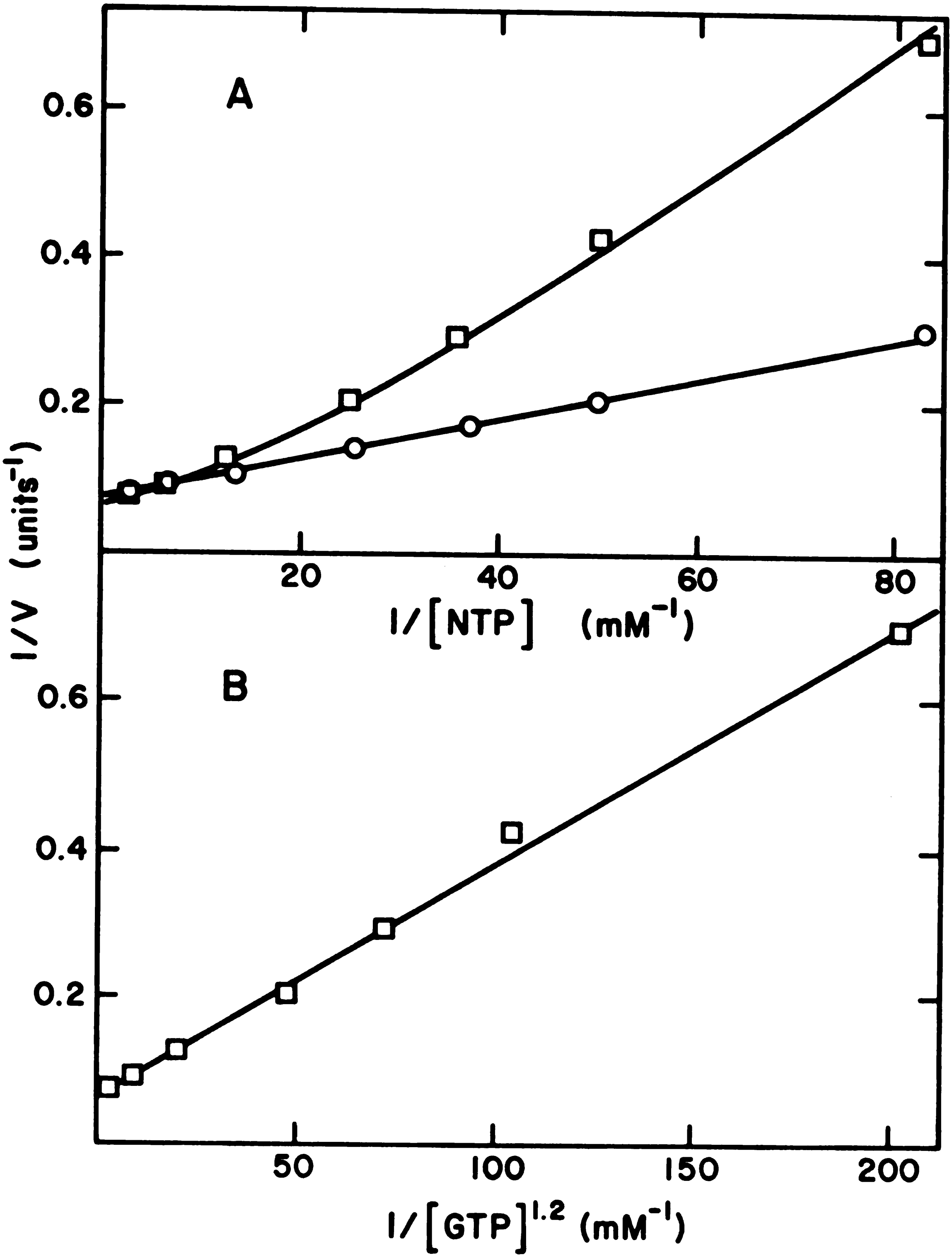
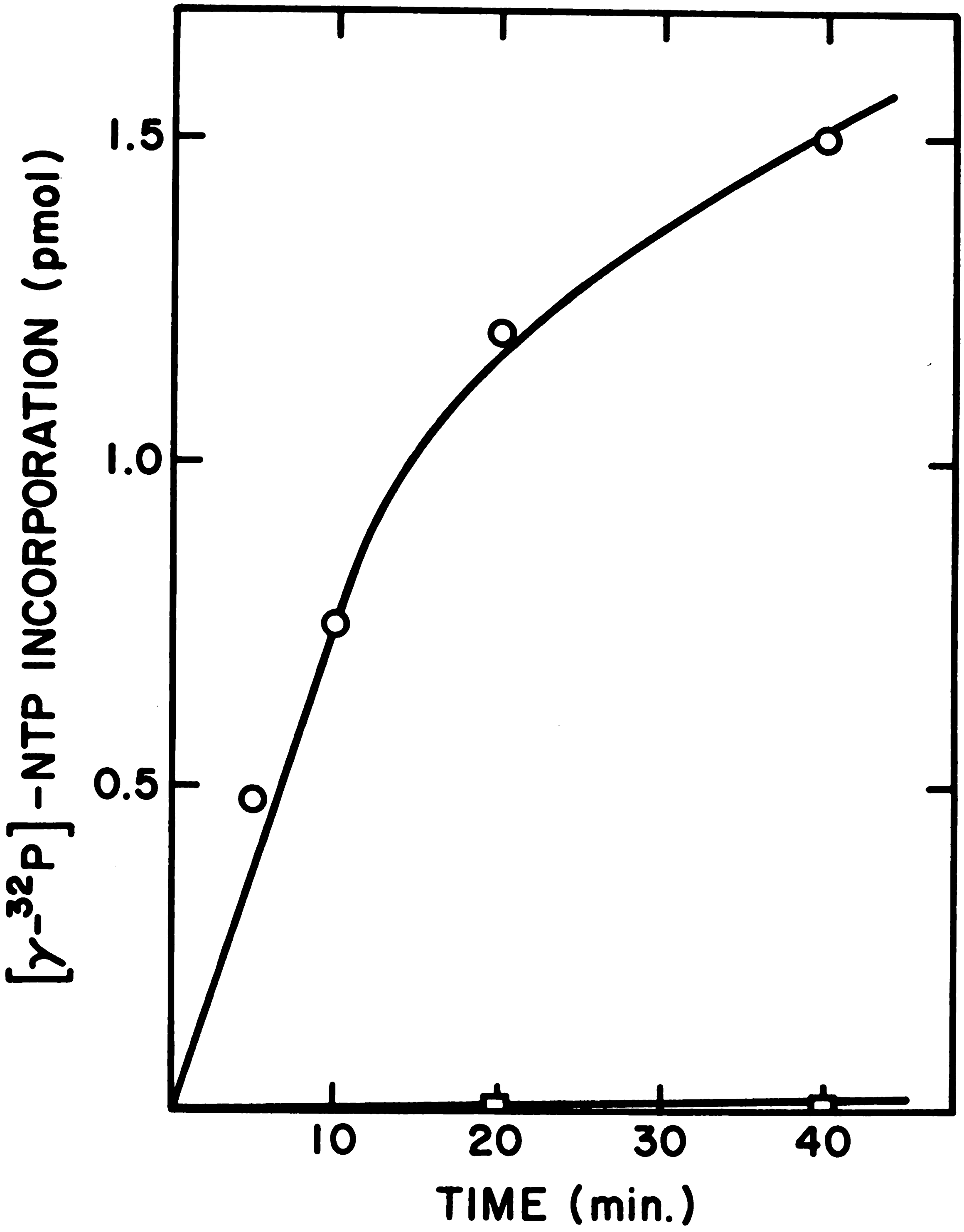

A
B
1/V (units^{-1})
0.6
0.4
0.2
20
40
60
80
1/[NTP] (mM^{-1})
0.6
0.4
0.2
50
100
150
200
1/[GTP]$^{1.2}$ (mM^{-1})

Figure 6.--The kinetics of incorporation of [γ-^{32}P]-GTP and [γ-^{32}P]-ATP into RNA by the gh-1-induced RNA polymerase. Reaction mixtures for gh-1 polymerase were prepared as described in Materials and Methods except that the final concentrations of ATP and GTP were lowered to 0.2 m*M*. Either the ATP (□) or GTP (○) was labeled with γ-^{32}P to a final specific activity of 2100 to 2500 CPM per pmol. In all assays in which [γ-^{32}P]ATP was the labeled substrate, 0.1 m*M* ADP was included in the reaction mixture to inhibit any trace amounts of polyphosphate kinase which might be present (Kornberg *et al.*, 1956). Reactions were initiated by the addition of 0.3 μg of gh-1 polymerase (Bio-Gel Fraction) and incubated at 30° C for the times indicated. The reactions were terminated and processed as described by Maitra *et al.* (1967).


1.5
1.0
0.5
10
20
30
40
TIME (min.)
$[\gamma\text{-}^{32}P]$-NTP INCORPORATION (pmol)

polymerase incorporated [γ-^{32}P] GTP into acid-insoluble material and this incorporation continued during the entire period of incubation. On the other hand, [γ-^{32}P] ATP was not incorporated significantly under the same conditions. The incorporation of [γ-^{32}P] GTP into acid-insoluble material did not occur in the absence of either enzyme or gh-1 DNA. The ^{32}P-labeled product, isolated from the reaction mixture after twenty minutes of incorporation, was rendered completely acid-soluble by treatment with either pancreatic RNase (1 μg/ml at 37° C for 0.5 hr) or alkali (1 N NaOH at 37° C for 6 hr). Thus, the [γ-^{32}P] GTP is being incorporated into RNA by the gh-1 polymerase in a DNA-dependent process. For the purine ribonucleoside triphosphates, gh-1 polymerase initiates RNA synthesis on gh-1 DNA exclusively with GTP.

Several antibiotics and antibiotic derivatives, which are inhibitors of host RNA polymerase activity, were added to the standard reaction mixture for gh-1 polymerase to test their effect on *in vitro* RNA synthesis catalyzed by the phage enzyme (Table IV). The antibiotics, actinomycin D and nogalamycin, inhibit RNA synthesis by intercalating into the DNA structure at G-C rich and A-T rich regions, respectively (Bhuyan and Smith, 1965; Goldberg *et al.*, 1962). These two antibiotics are effective inhibitors of the host and phage polymerases, as expected, since both catalyze DNA-dependent processes. The activity of the gh-1 polymerase is highly resistant to the antibiotics, rifampicin and streptolydigin, present at concentrations which markedly inhibit the host RNA polymerase. The effect of thirteen other derivatives of rifamycin on gh-1 polymerase activity was tested. The derivatives

TABLE IV.--Effect of Various RNA Synthesis Inhibitors on *P. putida* and gh-1-Induced RNA Polymerase Activities.[a]

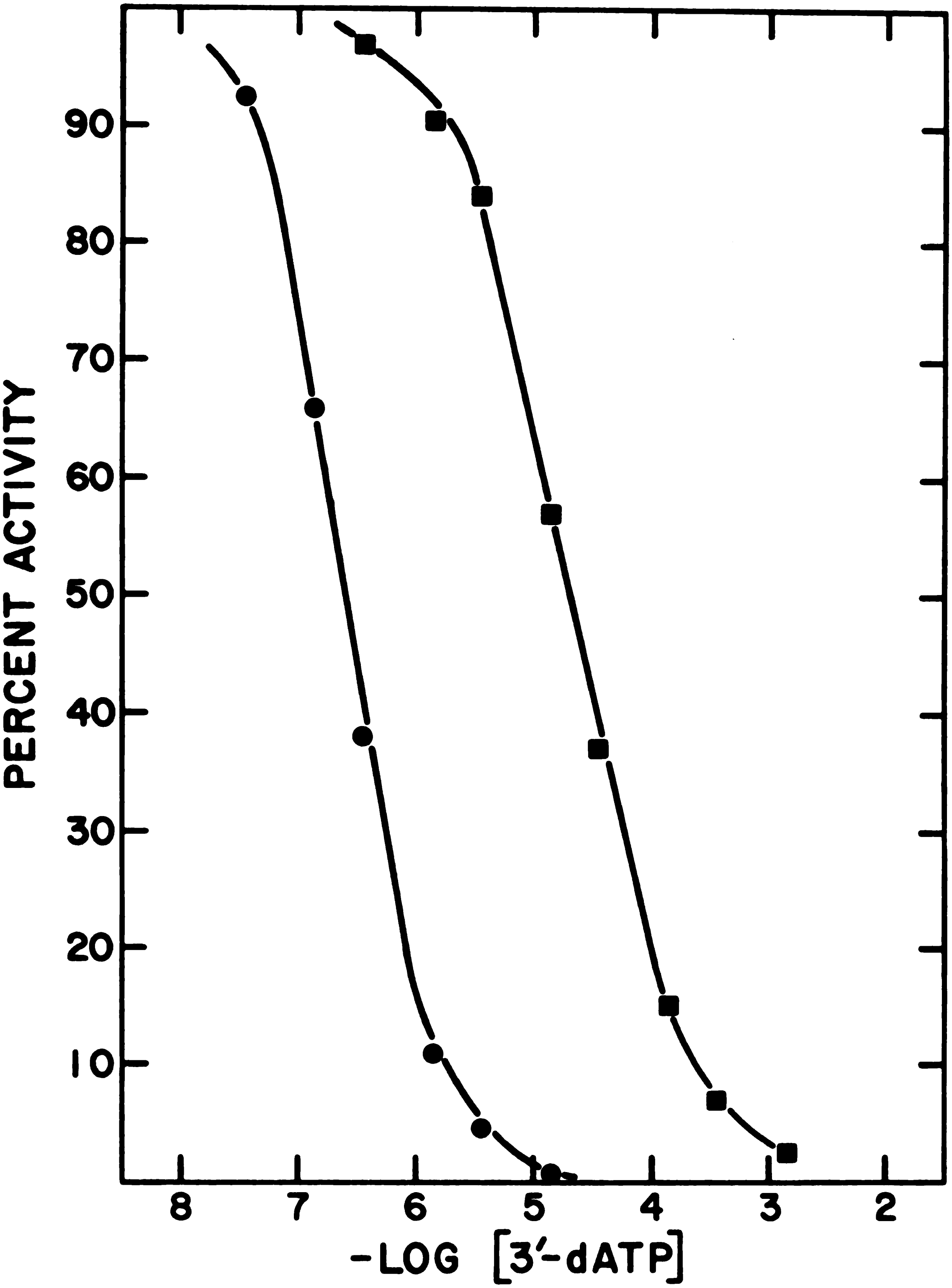
Addition to the Standard Reaction Mixture	Concentration (μg/ml)	Relative Activity (%)	
		gh-1 Polymerase	*P. putida* Polymerase
None	-	100	100
Actinomycin D	4	10	7
Nogalamycin	4	5	13
Streptolydigin	100	96	5
Rifampicin	10	95	0.5
Rifamycin AF/013 or AF/DNFI	35	50	0.5

[a] The components of the standard reaction mixture were as described in Materials and Methods. All inhibitors except streptolydigin were added from stock solutions in 10% (v/v) dimethyl sulfoxide. The final concentrations of dimethyl sulfoxide in the reaction mixtures, 0.4 to 0.8%, did not alter the overall incorporation of ^{3}H-CTP by either enzyme. Reactions were initiated by the addition of 2.4 μg/ml gh-1 polymerase (Bio-Gel Fraction) or 12.8 μg/ml *P. putida* RNA polymerase. Incubation, termination, and analysis of reaction mixtures for CMP incorporation were as described in Materials and Methods. In the standard reaction mixture with no additions, 1.85 nmol of CMP were incorporated by the gh-1 polymerase in 10 minutes and 0.95 nmole by the *P. putida* RNA polymerase.

examined, using the nomenclature of Gruppo Lepetit, were: AF/AOP, Rifamycin AG, AF/APR, AF/DEI, AF/DA-AMP, Rifamide, 4-Dessosi SV, PR/14, AF/013, AF/ABDP-cis, AF/AP, AF/BO, AF/DNFI, and PR/19 (for review of structures see Riva and Silvestri, 1972). All thirteen of these derivatives were effective inhibitors (> 95%) of RNA synthesis by the host RNA polymerase when present at a concentration of 10 μg/ml. When added to the gh-1 polymerase reaction mixture at 100 μg/ml, seven of the derivatives--AF/013, AF/DNFI, AF/BO, AF/AOP, AF/ABDP, PR/19, and AF/DEI--were found to inhibit polymerase activity to a significant degree (> 20%) (data not shown). The most effective inhibitors were AF/013 and AF/DNFI, which inhibited RNA synthesis by 50% at concentrations of 35 μg/ml and almost completely at concentrations of 80 μg/ml or more. The relative order of effectiveness of the rifamycin derivatives in inhibiting gh-1 polymerase activity was virtually the same as that observed for T7-induced RNA polymerase (Chamberlin and Ring, 1972). Even those rifamycin derivatives which were most effective inhibitors of the phage polymerase activities, however, were far more effective against the activity of the host RNA polymerase.

3'-Deoxyadenosine 5'-triphosphate, the triphosphate derivative of the antibiotic cordycepin, has been shown to be an _in vitro_ inhibitor of RNA synthesis by certain bacterial RNA polymerases (Shigeura and Boxer, 1964; Sentenac, _et al._, 1968). This ATP analog presumably inhibits RNA synthesis by being enzymatically incorporated into an RNA chain at a position normally occupied by an AMP residue. If incorporated, the 3'-dAMP would act as a chain terminator in RNA synthesis, since it does not contain a 3'-hydroxyl group necessary for the formation of the next phosphodiester bond. As shown in Figure 7, 3'-dATP, when added to the standard reaction mixture, inhibited RNA synthesis by both the gh-1-induced and _P. putida_ RNA polymerases. It was a much more effective inhibitor, however, of the gh-1 polymerase. The 3'-dATP concentration required to produce a given level of inhibition with the host RNA polymerase was about 80-times greater than that required to inhibit the gh-1 polymerase

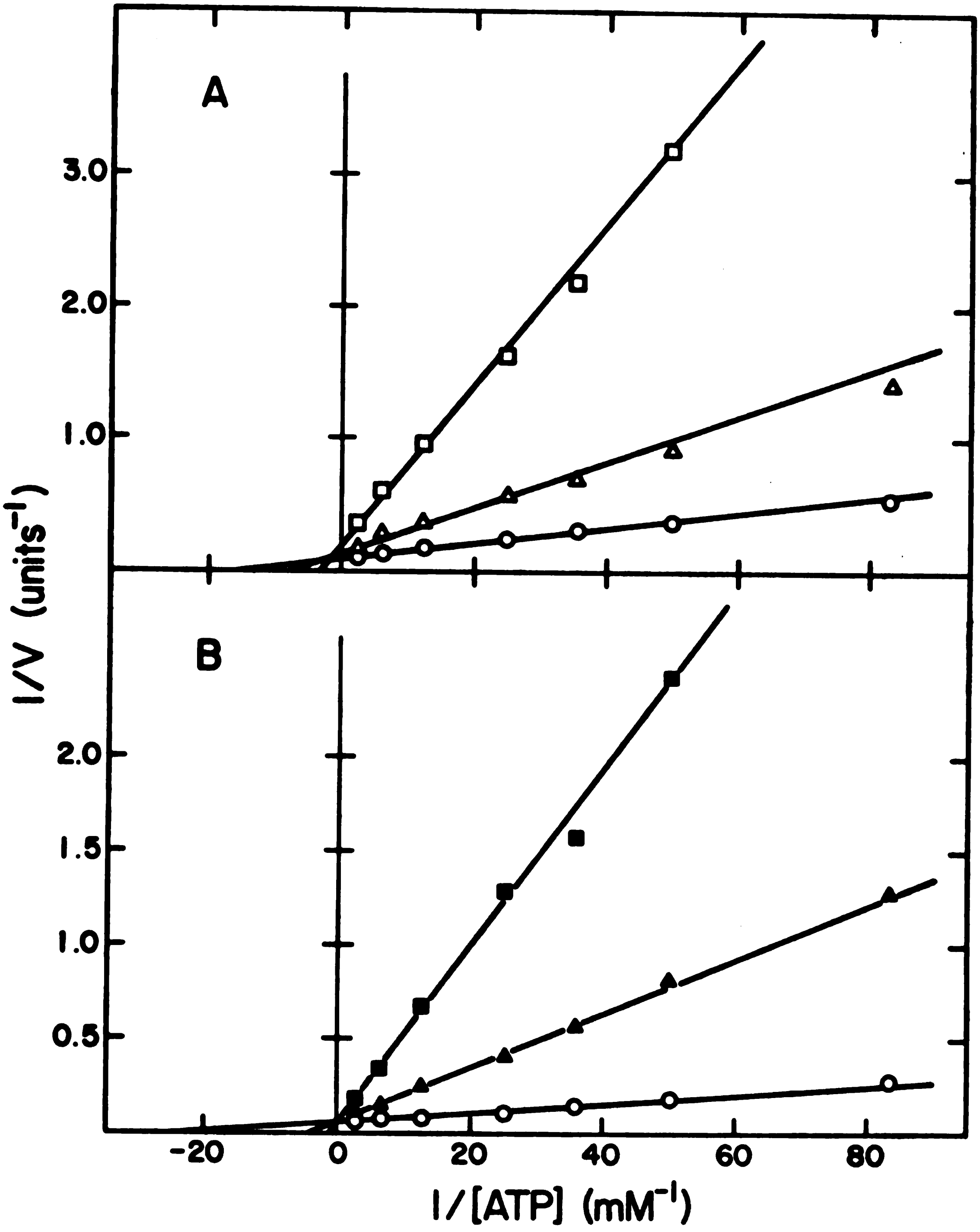
Figure 7.--The effect of 3'-deoxyadenosine 5'-triphosphate on in vitro RNA synthesis by P. putida and gh-1-induced RNA polymerases. Standard reaction mixtures were prepared as described in Materials and Methods except that 3'-dATP was added to some reactions as indicated. Reactions were initiated by the addition of either 0.3 µg of gh-1 polymerase (Bio-Gel Fraction) (○) or 1.6 µg of *P. putida* RNA polymerase (□). After ten minutes of incubation, the reactions were terminated and the incorporation of ^{3}H-CTP into acid-insoluble material determined as described in Materials and Methods. Incorporation in reactions containing various concentrations of 3'-dATP were compared to control reactions containing no 3'-dATP. For gh-1 polymerase, 100% activity (no 3'-dATP) was equal to 1.95 nmol of CMP incorporated in ten minutes and for *P. putida* RNA polymerase, 0.95 nmol.


PERCENT ACTIVITY
90
80
70
60
50
40
30
20
10
8
7
6
5
4
3
2
-LOG [3'-dATP]

to the same extent. Thus, at the concentration of ATP present in the standard reaction mixture, 0.4 m*M*, 50% inhibition of the host polymerase occurred at an ATP/3'-dATP molar ratio of 20, while the same degree of inhibition of the phage enzyme occurred at an ATP/3'-dATP molar ratio of 1600. By selecting the appropriate concentration of 3'-dATP, the gh-1 polymerase activity can be essentially completely inhibited, while the host polymerase activity is almost completely unaffected. Neither the nucleoside, 3'-deoxyadenosine (cordycepin), nor the diphosphate derivative, 3'-deoxyadenosine 5'-diphosphate, had any effect on either enzyme activity at concentrations up to 1 m*M* (data not shown).

Double reciprocal plots of 1/V versus 1/[ATP] in the absence and presence of 3'-dATP were experimentally determined to further study this interesting inhibitory effect (Figure 8). Within experimental error, 3'-dATP acted as a competitive inhibitor of ATP for both enzymes. The apparent Km values for ATP for both enzymes were similar: 6×10^{-5} *M* for the host enzyme and 3.5×10^{-5} *M* for the gh-1 polymerase. The apparent Ki values for 3'-dATP were, however, quite different: 2×10^{-6} *M* for the host enzyme and 2×10^{-8} *M* for the phage enzyme. Thus, the difference in sensitivity of the two enzymes towards 3'-dATP, as seen in Figure 7, was reflected in the relative difference of the apparent Ki values. These results indicate that 3'-dATP inhibited the polymerase by competing for a common binding site with ATP. This conclusion was substantiated by the finding that the poly(dC)•poly(dG)-primed polymerization of GTP by the gh-1 polymerase (see below) was not affected by the presence of 3'-dATP at levels which completely inhibit the gh-1 DNA-primed reaction (data not shown).

Figure 8.--The effect of varying the concentration of ATP in the absence and presence of 3'-deoxyadenosine 5'-triphosphate on in vitro RNA synthesis by gh-1-induced and P. putida RNA polymerases. Reaction mixtures were prepared as described in the legend to Figure 5 except that some reaction mixtures included 3'-dATP at the concentrations listed below. Reactions were initiated by the addition of either 1.6 μg *P. putida* RNA polymerase (A) or 0.3 μg gh-1 polymerase (B). After ten minutes of incubation, the reactions were terminated and the incorporation of ^{3}H-CTP into acid-insoluble material determined as described in Materials and Methods. Final concentrations of 3'-dATP in the reaction mixtures were: 0(○), 0.12 μ*M*(▲), 0.4 μ*M*(■), 8 μ*M*(△), or 40 μ*M*(□).


A
B
3.0
2.0
1.0
2.0
1.5
1.0
0.5
-20
0
20
40
60
80
1/V (units⁻¹)
1/[ATP] (mM⁻¹)

The inhibition of _in vitro_ RNA synthesis by 3'-dATP could either be due to the simple competition with ATP for a single binding site on the enzyme molecule or, in addition, the enzymatic incorporation of 3'-dATP into the growing RNA chain. If 3'-dATP becomes incorporated into the RNA chain, chain termination should occur and the RNA produced should by decreased in length. The size of the RNA synthesized by gh-1 polymerase utilizing gh-1 DNA in the absence or presence of 3'-dATP was examined (Figure 9). Incubation of _in vitro_ RNA synthesis reactions was for twenty minutes in this experiment to ensure that several rounds of transcription will have occurred. The size of the RNA produced in the presence of 3'-dATP was clearly much shorter than the RNA produced under standard reaction conditions. The mean size of the RNA synthesized in the absence of 3'-dATP was 17.4S, which roughly corresponded to a length of 1750 nucleotides. On the other hand, the RNA produced in the presence of 3'-dATP had a mean size of 10.8S or approximately 700 nucleotides in length. These results indicated that the presence of 3'-dATP in the standard reaction mixture caused premature termination of RNA synthesis by the gh-1 polymerase. While these results do not directly show that 3'-dATP becomes incorporated into RNA, they are certainly consistent with that presumptive conclusion.

Another structural analog of ATP is 3'-_O_-methyladenosine 5'-triphosphate (3'-AmTP). This ATP analog is similar to 3'-dATP in that it differs from ATP only at the 3'-position of the ribose moiety. 3'-AmTP was an inhibitor of _in vitro_ RNA synthesis by both the _P. putida_ and gh-1-induced RNA polymerases (Figure 10). The large differential inhibitory effect seen for these two RNA polymerases with 3'-dATP was

Figure 9.--Size of the RNA product synthesized by gh-1-induced RNA polymerase in the absence and presence of 3'-deoxyadenosine 5'-triphosphate. Standard reaction mixtures were prepared as described in Materials and Methods containing either no additions (●) or the addition of 3'-dATP to a final concentration of 4 μM (■). The reactions were initiated by the addition of gh-1 polymerase (Bio-Gel Fraction) to a final concentration of 6.2 μg/ml. After incubation for 20 minutes, reactions were terminated by the addition of 5 μl of 10% (w/v) SDS and then incubated for 3 minutes at 30° C. A solution of 2 M KCl was added to a final concentration of 0.1 M and the reactions were placed on ice for 5 minutes. The precipitated potassium salt of SDS was removed by centrifugation at 10,000 x g for 5 minutes. Samples of the supernatant solution of each reaction mixture (0.1 ml) were layered onto 4.8 ml linear 5 to 20% sucrose gradients made in 10 mM Tris•HCl, pH 8.0, 0.1 M KCl. A 0.1 ml sample of ^{3}H-labeled ribosomal and soluble RNA from Novikoff hepatoma cells was layered on a parallel gradient. Centrifugation was carried out at 45,000 RPM for 3 hours in a Spinco SW50.1 head at 4° C. After centrifugation, 30 fractions of about 0.16 ml were collected. Fractions were analyzed for ^{3}H-labeled trichloroacetic acid-insoluble material as described elsewhere (Gerard et al., 1972). The positions of 18S and 28S ribosomal RNA and soluble RNA are shown by the arrows.

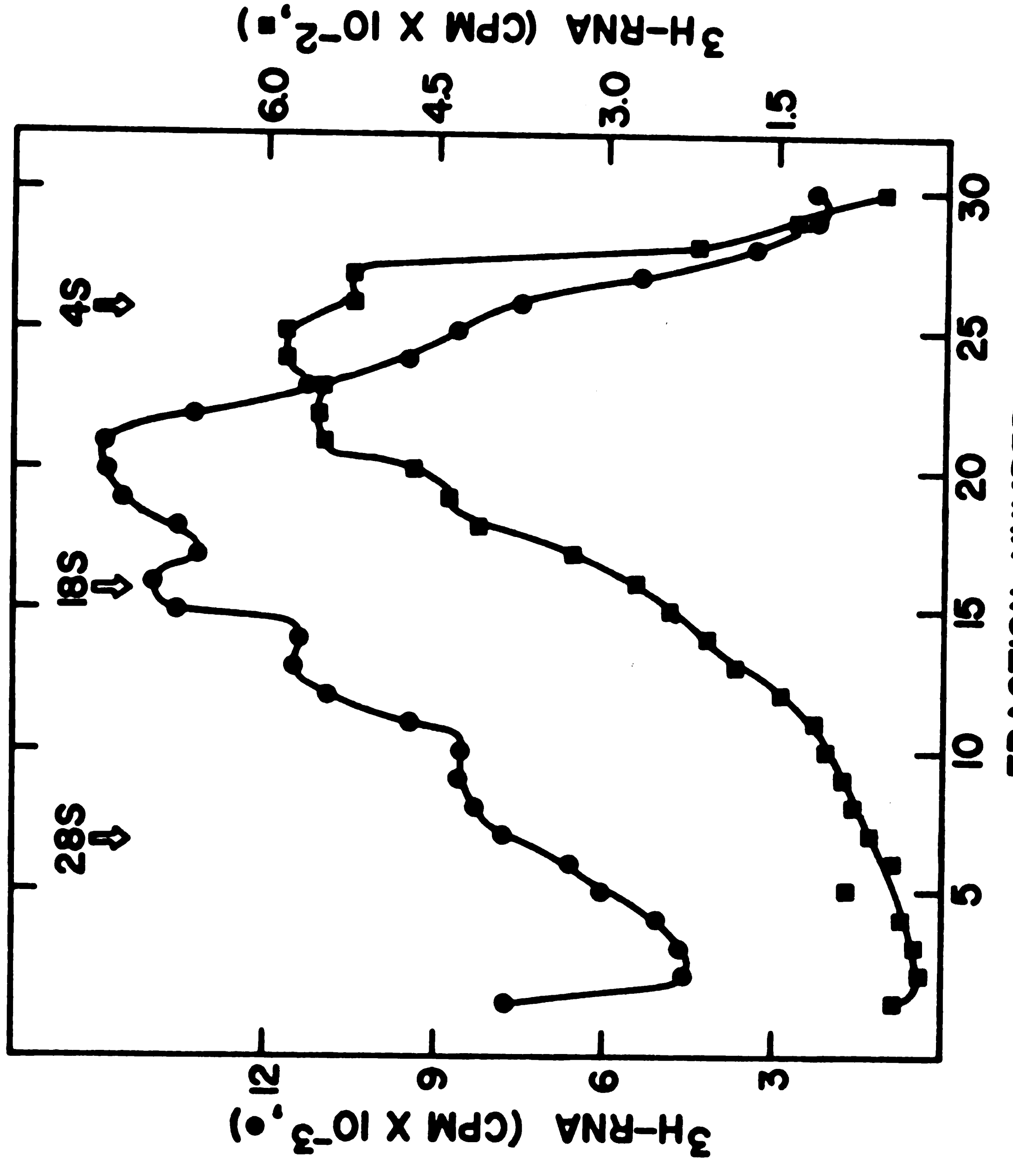
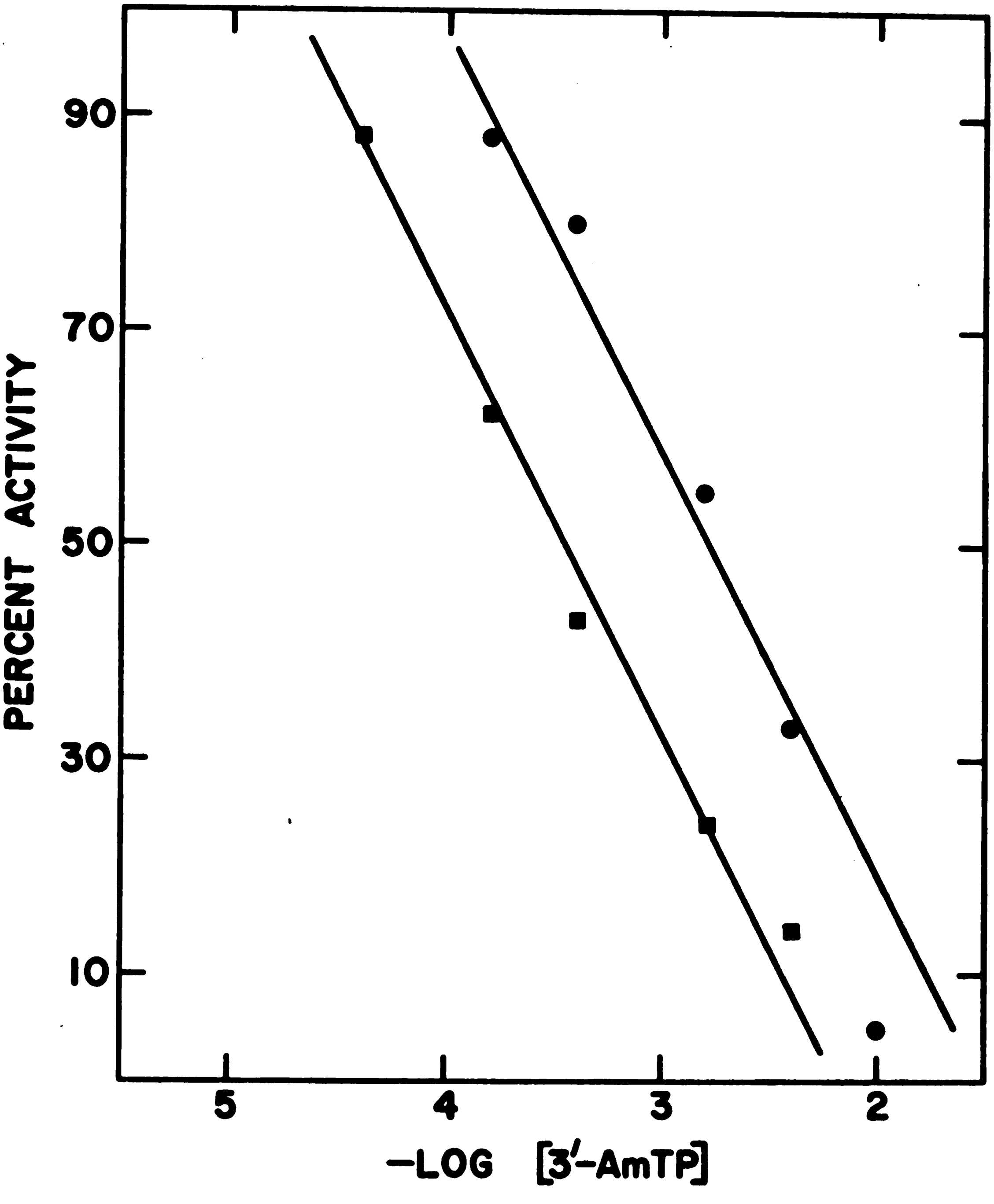

3H-RNA (CPM X 10-2, ■)
6.0
4.5
3.0
1.5
28S
18S
4S
5
10
15
20
25
30
FRACTION NUMBER
12
9
6
3
3H-RNA (CPM X 10-3, ●)

Figure 10.--The effect of 3'-O-methyladenosine 5'-triphosphate on in vitro RNA synthesis by P. putida and gh-1-induced RNA polymerases. Standard reaction mixtures were prepared as described in Materials and Methods except that 3'-AmTP was added to some reactions as indicated. Reactions were initiated by either 0.3 μg of gh-1 polymerase (Bio-Gel Fraction, ●) or 1.6 μg of *P. putida* RNA polymerase (■). After ten minutes of incubation, the reactions were terminated and analyzed for RNA synthesis as described in Materials and Methods. Incorporation in reactions containing various concentrations of 3'-AmTP were compared to control reactions containing no 3'-AmTP. For gh-1 polymerase, 100% activity (no 3'-AmTP) corresponded to 1.95 nmol of CMP incorporated in 10 minutes and for *P. putida* RNA polymerase, 0.95 nmol.


PERCENT ACTIVITY
90
70
50
30
10
5
4
3
2
–LOG [3′–AmTP]

not observed for 3'-AmTP. For both enzymes, inhibition by 3'-AmTP was competitive for ATP (data not shown). The apparent Ki value for 3'-AmTP calculated for the *P. putida* RNA polymerase was 4.1 X 10^{-5} *M*, or approximately 20-times higher than the apparent Ki value for 3'-dATP. For the gh-1 polymerase, the apparent Ki value of 3'-AmTP was 1.3 X 10^{-4} *M*; over three orders of magnitude greater than that of 3'-dATP. Thus, the 3'-*O*-methylated analog of ATP is not as efficient as an inhibitor of *in vitro* RNA synthesis as the 3'-H analog for these RNA polymerases.

Template Specificity of the gh-1-Induced RNA Polymerase

One of the most striking characteristics of the gh-1-induced RNA polymerase catalyzed reaction is the stringent template specificity. When DNA from many sources was tested, only the homologous phage gh-1 DNA was found to be an efficient template for *in vitro* RNA synthesis (Table V). The gh-1 polymerase would not utilize DNA from coliphages T3, T4, or T7; nor would it utilize calf thymus or *P. putida* DNA. When the gh-1 DNA was denatured, it became an inefficient template for the gh-1 polymerase. Thus, some feature inherent in the double-stranded structure of the gh-1 phage DNA is necessary for its function as an efficient template. Likewise, denatured DNA from either coliphage T3 or calf thymus supported little or no RNA synthesis. By contrast, the host RNA polymerase can utilize all of the above templates, although at varying efficiencies.

Several synthetic polydeoxyribonucleotides were tested as templates for RNA synthesis by the gh-1 polymerase (Table VI). The

TABLE V.--Template Specificity of gh-1-Induced and *P. putida* RNA Polymerase Towards DNA from Various Sources.[a]

DNA Template	Relative Activity (%)	
	gh-1 Polymerase	*P. putida* Polymerase
gh-1	100	100
T3	0.8	99
T7	< 0.5	137
T4	< 0.5	28
Calf Thymus	0.6	61
P. putida	< 0.5	29
Denatured gh-1	1.8	33
Denatured T3	1.2	22
Denatured Calf Thymus	< 0.5	25

[a] RNA polymerase reactions were prepared and run as described in Materials and Methods except that the gh-1 DNA was replaced by DNA from various sources as indicated. The final concentration of DNA in all cases was 50 μg/ml. The assays contained 4.3 μg/ml gh-1 polymerase (Bio-Gel Fraction) or 12.8 μg/ml *P. putida* RNA polymerase. With gh-1 DNA as template, the gh-1 polymerase incorporated 3.3 nmol of CMP in 10 minutes, and the *P. putida* RNA polymerase incorporated 1.4 nmol. Where indicated, DNA solutions were denatured immediately before use by heating for 10 minutes at 100° C, followed by rapid chilling at 0° C.

TABLE VI.--Template Specificity of gh-1-Induced and *P. putida* RNA Polymerases Towards Synthetic Polydeoxyribonucleotides.[a]

Template	Nucleoside Triphosphate Substrates	nmol of ^{3}H-NMP Incorporated per Hour	
		gh-1 Polymerase	*P. putida* Polymerase
gh-1	^{3}H-CTP,ATP,GTP,UTP	19.8	8.72
poly[d(A-T)]	^{3}H-ATP,UTP	0.22	21.3
poly(dC)•poly(dG)	^{3}H-CTP,GTP	0.23	0.83
poly(dC)•poly(dG)	^{3}H-GTP,CTP	1.66	7.57
poly(dA)	^{3}H-UTP	< 0.01	2.30
poly(dC)	^{3}H-GTP	1.87	1.58
poly(dI)	^{3}H-CTP	0.54	1.65
poly(dT)	^{3}H-ATP	3.39	11.5

[a] RNA polymerase reactions were prepared and run as described in Materials and Methods except that the template and nucleoside triphosphates were changed as indicated. Each nucleoside triphosphate was present as a final concentration of 0.4 m*M*. The following concentrations of template were employed: poly[d(A-T)], 3 OD_{260} units/ml; poly(dC)•poly (dG), 2.5 OD_{260} units/ml; poly(dA), poly(dC), poly(dI), and poly(dT), 50 μ*M* (expressed in terms of nucleotide phosphate). The assays contained either 4.3 μg/ml gh-1 polymerase (Bio-Gel Fraction) or 12.8 μg/ml *P. putida* RNA polymerase.

alternating copolymer, poly[d(A-T)], which was an efficient template for the host RNA polymerase, was not utilized by the gh-1 polymerase. The gh-1 polymerase utilized the homopolymer duplex, poly(dC)•poly(dG), to direct the polymerization of GTP at a rate seven times higher than the polymerization of CTP from this template. Several single-stranded polydeoxyribonucleotide homopolymers were also tested as templates for gh-1 polymerase. Either of the pyrimidine-containing polymers--poly(dT) or poly(dC)--would support the synthesis of the corresponding ribohomopolymers. Little or no template activity, however, could be detected with the purine-containing homopolymers--poly(dA) or poly(dI). Thus, with either single-stranded or double-stranded deoxyribonucleotide homopolymers, the gh-1 polymerase markedly prefers to utilize the pyrimidine-containing templates as compared to the purine-containing ones. It should be noted that the highest enzyme activity on any template other than native gh-1 DNA, namely that for poly(dT), was less than 5% of the enzyme activity on native gh-1 DNA, in terms of nmoles of nucleotide incorporated per hour per milligram of protein.

The Binding of gh-1-Induced RNA Polymerase to gh-1 DNA

The binding of gh-1 polymerase to gh-1 DNA was directly examined by determining whether a stable binary complex of enzyme and DNA could be formed. After mixing purified gh-1 polymerase and gh-1 DNA together, free enzyme was separated from DNA-bound enzyme by sedimentation velocity centrifugation in a glycerol gradient. When such experiments were performed with the host RNA polymerase, it was found that virtually all the enzyme in a mixture would bind to the gh-1 DNA and sediment as a binary complex at low salt (50 mM) conditions (Gerard *et al.*, 1972). In similar experiments using T7 RNA polymerase and T7 DNA, no stable binary complex was formed (Chamberlin and Ring, 1973). As seen in Figure 11, a large portion (about 60%) of the gh-1 polymerase in a mixture with gh-1 DNA cosedimented as a binary complex with the DNA. The remaining enzyme was present either in the position of unbound gh-1 polymerase or trailing behind the enzyme-DNA complex. The enzyme found to trail behind the complex probably represents enzyme molecules which were originally bound to the DNA, but were dissociated during centrifugation. Quantitatively similar results were obtained when the concentration of enzyme was either increased or decreased several-fold. Thus, the amount of binary complex formed was independent of enzyme concentration in the range studied. These results indicate that the gh-1 polymerase is not as efficient as the host RNA polymerase at forming a stable binary complex with gh-1 DNA, but is more efficient than the T7 RNA polymerase, which does not form any binary complex with its homologous T7 DNA.

The interaction between gh-1 polymerase and gh-1 DNA was further studied by determining the rates of *in vitro* RNA synthesis at varying concentrations of gh-1 DNA. As can be seen in Figure 12, the gh-1 polymerase reaction was essentially completely saturated at a concentration of 20 μg/ml gh-1 DNA. When the data of Figure 12 was replotted in a standard double reciprocal plot of 1/ the initial reaction rate versus 1/ the concentration of gh-1 DNA, a straight line was obtained. Based on a molecular weight of 23×10^6 for gh-1 DNA, the apparent Km value of gh-1 DNA calculated from the double reciprocal plot was about

Figure 11.--The binding of gh-1-induced RNA polymerase to gh-1 DNA. Reaction mixtures (0.125 ml) were prepared containing 40 m*M* Tris·HCl, pH 8.0, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 400 μg/ml bovine serum albumin, and either 2.4 μg/ml gh-1 polymerase (Bio-Gel Fraction, A); or 50 μg/ml ^{32}P-labeled gh-1 DNA (3900 CPM/μg, B); or both gh-1 polymerase and gh-1 DNA as above (C). The reactions were incubated for 1.5 minutes at 30° C and then cooled to 0° C in an ice bath. Samples of 0.1 ml were layered onto 4.8 ml linear 10 to 30% glycerol gradients prepared in the above buffer. All gradients were centrifuged for 3 hours at 4° C in a Spinco SW50.1 head at 50,000 RPM. After centrifugation, fractions of 0.16 ml were collected from each gradient. Fractions from the gradient with only gh-1 DNA (B) were analyzed directly for ^{32}P as described elsewhere (Gerard *et al.*, 1972). Samples of 25 μl from the other two gradients (A and C) were assayed for gh-1 polymerase activity as described in Materials and Methods. The final samples, precipitated with trichloroacetic acid, were analyzed for both ^3H (CMP incorporation) and ^{32}P (gh-1 DNA).

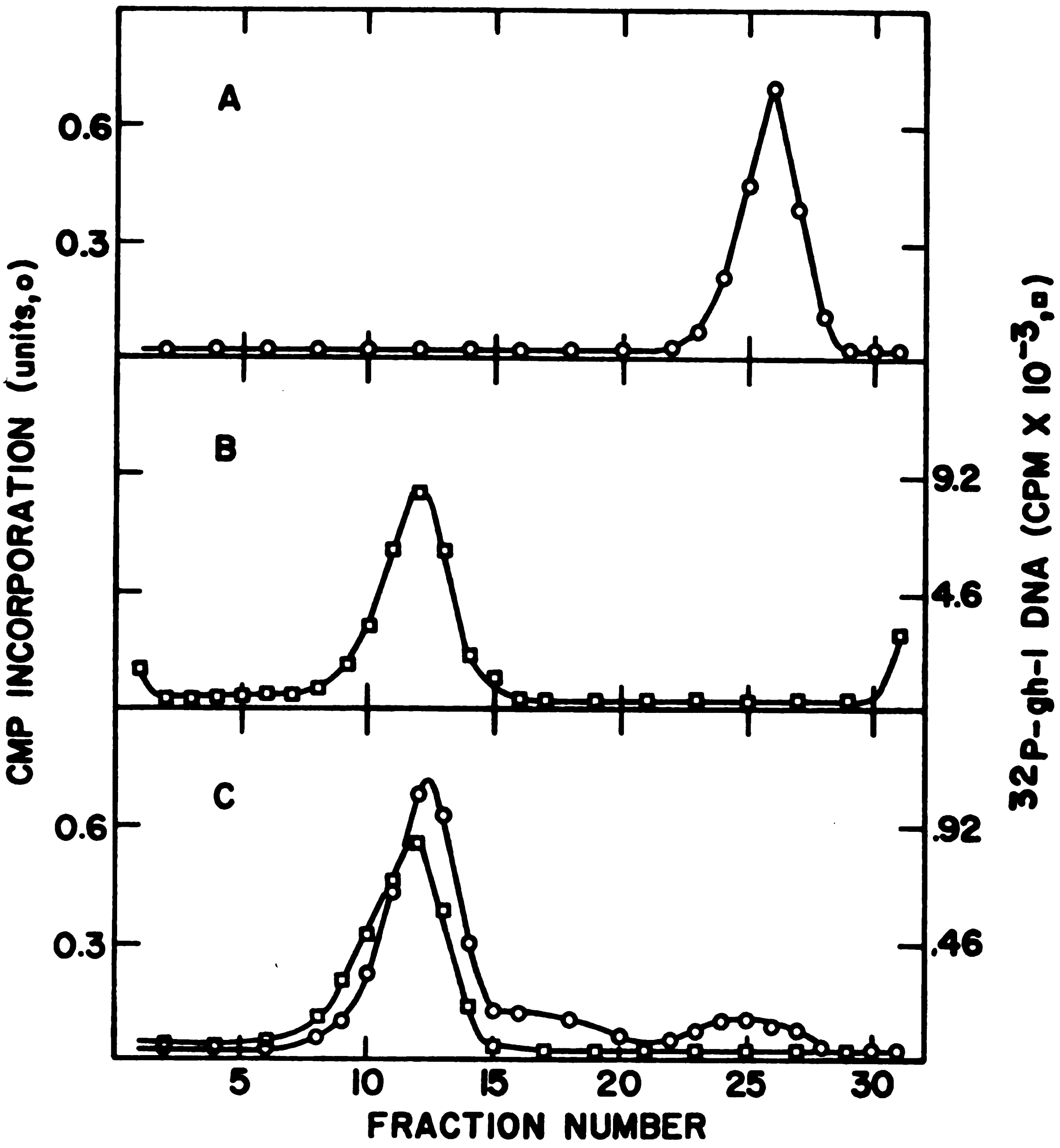
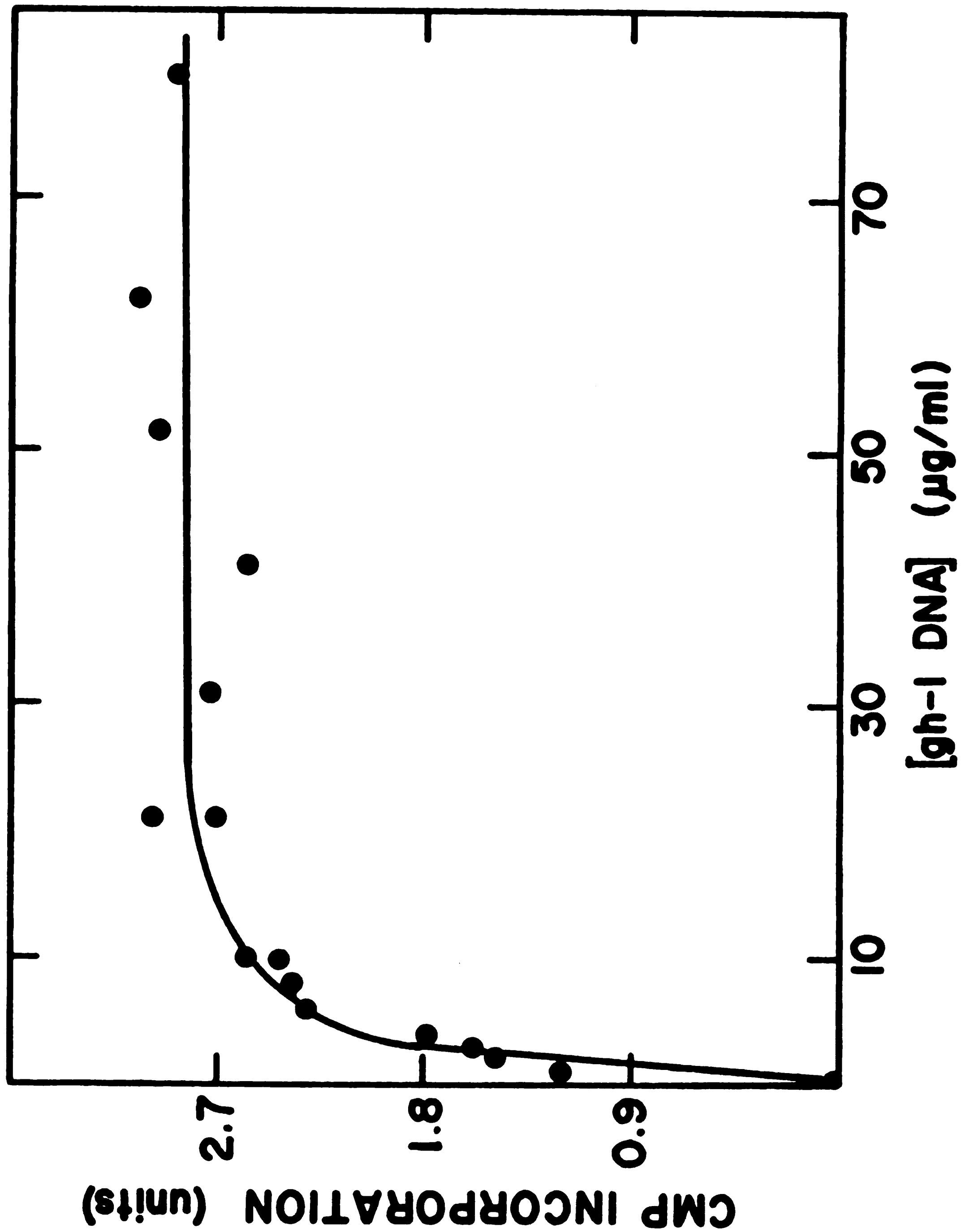

A
B
C
0.6
0.3
9.2
4.6
.92
.46
5
10
15
20
25
30
FRACTION NUMBER
CMP INCORPORATION (units,○)
32P-gh-1 DNA (CPM X 10^-3,□)

Figure 12.--*The effect of varying the concentration of gh-1 DNA on RNA synthesis by the gh-1-induced RNA polymerase*. Standard reaction mixtures were prepared for gh-1 polymerase as described in Materials and Methods except that the concentration of gh-1 DNA was varied as indicated. RNA synthesis was initiated by the addition of gh-1 polymerase (Phosphocellulose Fraction) to a final concentration of 2.2 μg/ml. After ten minutes of incubation, reactions were terminated and analyzed for CMP incorporation as described in Materials and Methods.

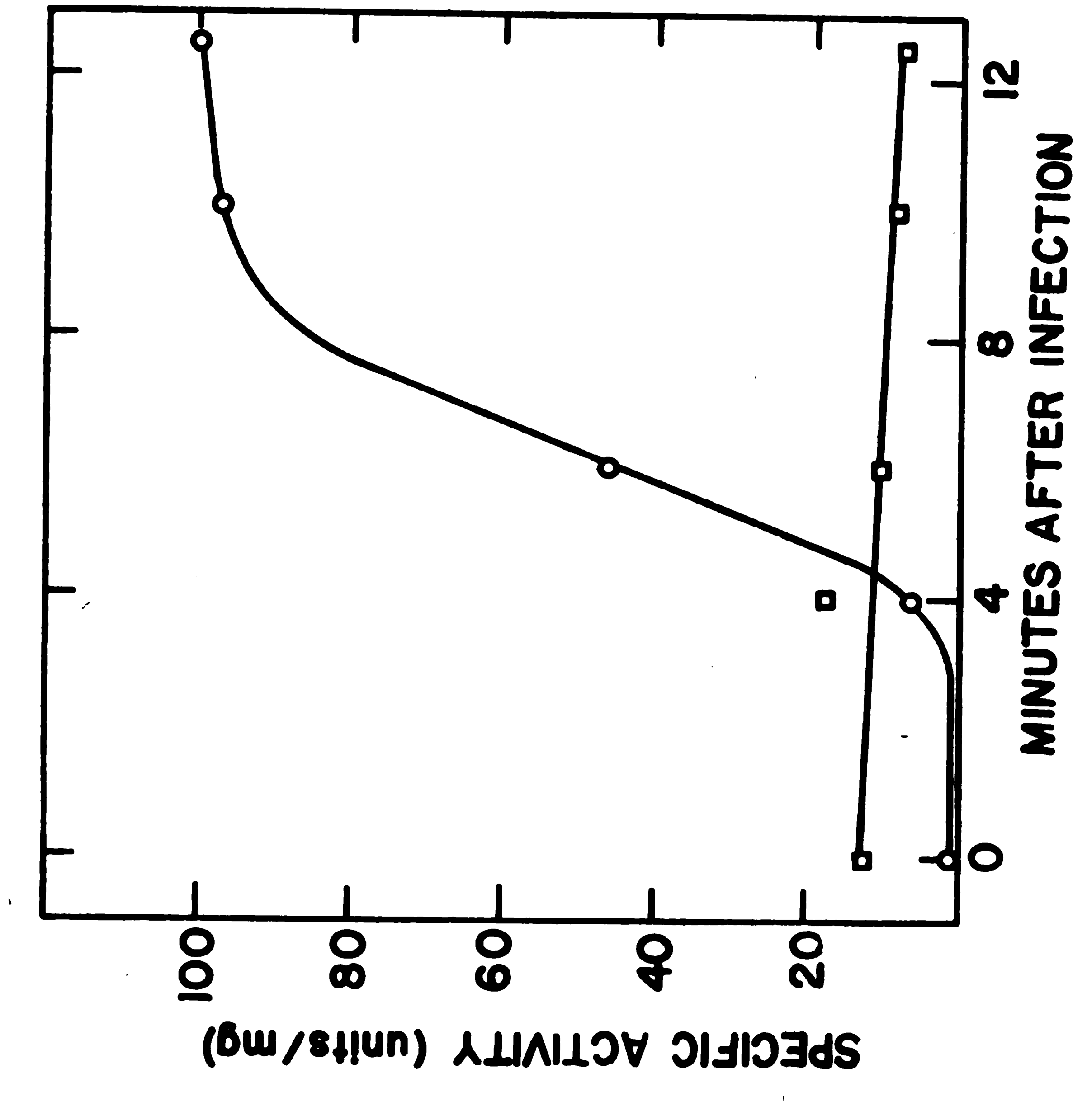

CMP INCORPORATION (units)
2.7
1.8
0.9
10
30
50
70
[gh-1 DNA] (µg/ml)

9×10^{-11} M. A similar determination of the apparent Km value for gh-1 DNA with the host RNA polymerase gave a value of 8×10^{-10} M, or about an order of magnitude higher than that for the gh-1 enzyme (data not shown). Assuming the saturation curves reflect the affinity of the enzyme for template, the gh-1 polymerase binds more tightly than the host RNA polymerase to gh-1 DNA. While this conclusion may seem to be inconsistent with the data on the formation of binary complexes, the conditions used for the two studies were different. For the formation of binary complexes, no RNA synthesis could occur because the four ribonucleoside triphosphates were not present. In the determination of the saturation curves for gh-1 DNA, however, RNA synthesis was measured. Thus, it is likely that the presence of the four ribonucleoside triphosphates resulted in the formation of a tighter complex between the gh-1 DNA and the gh-1 polymerase. While host RNA polymerase is more efficient at forming binary complexes in the absence of ribonucleoside triphosphates, under conditions of RNA synthesis the gh-1 polymerase shows a higher affinity for the gh-1 DNA.

The Time Course of Appearance of gh-1-Induced RNA Polymerase in gh-1-Infected *P. putida*

The induction of gh-1 polymerase in gh-1-infected cells was followed by measuring the levels of rifampicin and streptolydigin-resistant RNA polymerase activity in extracts of cells infected for varying lengths of time (Figure 13). The gh-1 polymerase activity first appeared in measureable amounts from four to six minutes after the onset of gh-1 infection. By ten minutes after infection the amount

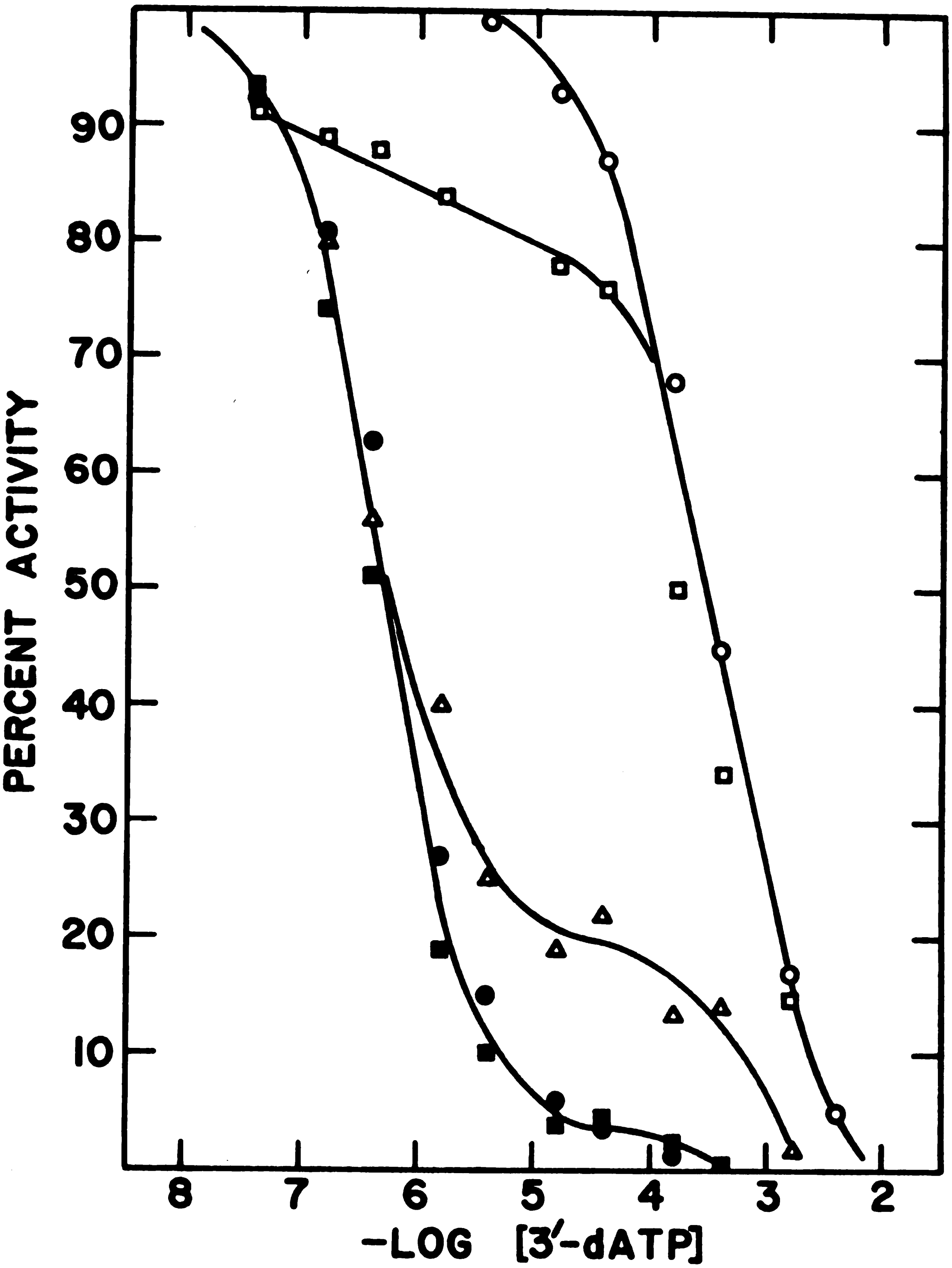
Figure 13.--Induction of gh-1-induced RNA polymerase after gh-1 infection of *P. putida*. *P. putida* was grown to a cell density of 1.4 X 10^9 cells/ml as described in Materials and Methods. After removing a sample for an uninfected control, gh-1 phage was added to a multiplicity of 5 plaque forming units per cell. At various times after the addition of phage, 400 ml samples were harvested directly onto an equal volume of ice at -20° C. Cells were immediately collected by centrifugation at 0° C and frozen at -20° C until further analysis. Extracts were prepared and assayed for enzyme activity and protein concentration as described in Materials and Methods. Samples from each time point were assayed in the standard reaction mixture and in the standard reaction mixture plus 5 µg/ml rifampicin and 100 µg/ml streptolydigin. The specific activity of the rifampicin and streptolydigin-resistant enzyme activity (○) is presumed to be the gh-1 polymerase, while that of the rifampicin and streptolydigin-sensitive enzyme activity (□) is presumed to be the host *P. putida* RNA polymerase.


SPECIFIC ACTIVITY (units/mg)
100
80
60
40
20
MINUTES AFTER INFECTION
0
4
8
12

of gh-1 polymerase had essentially reached its maximal level. The amount of P. putida host RNA polymerase, as measured by rifampicin and streptolydigin-sensitive RNA polymerase activity, declined slowly during the first 12.5 minutes of the infectious cycle.

The appearance of gh-1 polymerase was also followed by determining the sensitivity of the RNA polymerase activity in extracts of gh-1-infected cells to 3'-dATP. The gh-1-induced and host RNA polymerases can be differentiated by the unique sensitivity of each enzyme to 3'-dATP. The relative amounts of each RNA polymerase in a mixture can be estimated from the percentage of RNA polymerase activity in the mixture which displayed the sensitivity of either phage or host enzyme to 3'-dATP. The RNA polymerase activity in extracts of uninfected P. putida, which can only contain the host RNA polymerase, was slightly less sensitive to 3'-dATP than the purified host RNA polymerase (Figure 14). In extracts from cells infected for 4 minutes, 20% of the RNA polymerase activity no longer resembled the activity of uninfected cells, but was much more sensitive to 3'-dATP. This more sensitive RNA polymerase activity was presumably due to gh-1 polymerase activity. By 6 minutes after infection, the amount of presumptive gh-1 polymerase increased to 80% of the total RNA polymerase activity and by 10 minutes after infection, greater than 95%. The sensitivity to 3'-dATP of the RNA polymerase activity in extracts from 10 and 12.5 minute infected cells closely resembled the sensitivity of purified gh-1 polymerase. The relative percentages of gh-1 and host RNA polymerases estimated from the sensitivity to 3'-dATP of the RNA polymerase activity in

Figure 14.--The effect of 3'-dATP on RNA polymerase activity in extracts of uninfected and gh-1-infected *P. putida*. Uninfected and gh-1-infected *P. putida* were grown as described in the legend to Figure 13. Extracts of the various samples were prepared and assayed as described in Materials and Methods except that 3'-dATP was added to some reaction mixtures to the final concentrations indicated. Incorporation in reactions containing various concentrations of 3'-dATP was compared to respective controls with no 3'-dATP. ○ , uninfected; □ , 4' post infection; △ , 6' post infection; ●, 10' post infection; ■ , 12.5' post infection.


PERCENT ACTIVITY
90
80
70
60
50
40
30
20
10
8
7
6
5
4
3
2
-LOG [3'-dATP]

gh-1-infected cells were very similar to those obtained by the use of the bacterial RNA polymerase inhibitors, rifampicin and streptolydigin (cf., Figure 13). Thus, the 3'-dATP is a useful probe for differentiating between the two RNA polymerase activities.

DISCUSSION

The infection of P. putida by the bacteriophage gh-1 induces the synthesis of a novel DNA-dependent RNA polymerase. This gh-1-induced RNA polymerase has been purified to near homogeneity. It is composed of a single polypeptide chain with a molecular weight of about 98,000. The structure of the gh-1 polymerase is, thus, relatively simple compared to the structure of the host P. putida RNA polymerase, which is composed of five subunits, $\alpha_2\beta\beta'\sigma$, with a combined molecular weight of about 506,000 (Johnson *et al.*, 1971).

While the gh-1-induced and host P. putida RNA polymerases both catalyze the template-directed incorporation of ribonucleoside triphosphates into RNA, they differ in their response to several factors affecting RNA synthesis. Whereas the host polymerase can utilize either Mg^{2+} or Mn^{2+} to satisfy the divalent metal ion requirement, the phage polymerase can only utilize Mg^{2+}. Low concentrations of monovalent ions, which do not appreciably affect the activity of the host polymerase, inhibit the gh-1 polymerase markedly. The antibiotics, rifampicin and streptolydigin, inhibit the activity of the host enzyme at concentrations which do not affect the activity of the phage enzyme. The host RNA polymerase will utilize as an *in vitro* template every DNA with which it has been tested. The ability of the host polymerase to utilize a wide range of templates may be due to the diversity of sites it must recognize to perform its role in the transcription of the bacterial chromosome. On the other hand, the gh-1 polymerase is highly specific in its template requirement for DNA from the homologous gh-1 phage.

The infection of *E. coli* by the coliphages T3 or T7 has been shown to induce the synthesis of viral-specific RNA polymerases (Chamberlin *et al.*, 1970; Maitra, 1971; Dunn *et al.*, 1971). These coliphage-induced RNA polymerases are similar in structure to the gh-1 polymerase; both are single polypeptides of 108,000 to 110,000 molecular weight (Chamberlin *et al.*, 1970; Dunn *et al.*, 1971). The induction of a novel RNA polymerase activity has also been demonstrated after infection of *E. coli* by the helper-dependent bacteriophage P4 (Barrett *et al.*, 1972). The P4-induced RNA polymerase would synthesize polyriboguanylic acid from the duplex homopolymer, poly(dC)•poly(dG); however, no naturally-occurring DNA has yet been found to serve as an *in vitro* template for this enzyme. Its actual function, therefore, is still a matter of conjecture. These three phage-induced RNA polymerases of *E. coli* are the only bacteriophage-specific RNA polymerases which have been previously described.

A comparison of the catalytic properties of the gh-1-induced RNA polymerase with those of the T3 and T7 RNA polymerases shows that these three phage polymerases are quite similar (Chamberlin *et al.*, 1970; Maitra, 1971; Dunn *et al.*, 1971). All three phage polymerases cannot utilize Mn^{2+} as divalent metal ion in place of Mg^{2+}. The activities of the phage polymerases were highly resistant to inhibition by rifampicin and streptolydigin, but could be inhibited by the rifamycin derivative AF/013 at concentrations higher than 10 μg/ml (Chamberlin and Ring, 1972; Küpper *et al.*, 1973). Low concentrations of monovalent cations inhibited the activities of the three phage enzymes. Finally, all three phage-induced RNA polymerases showed highly stringent specificities for DNA from the homologous bacteriophage as *in vitro* templates.

The stringent template specificities of the gh-1, T3, and T7 RNA polymerases are quite interesting. All three polymerases can utilize pyrimidine-containing homopolymers, either single-stranded or as part of duplex pairs, as templates, but are far less efficient with the purine-containing homopolymers (Chamberlin and Ring, 1973; Maitra, 1971). The ability of the pyrimidine-containing polymers to serve as efficient templates may result from the preferential initiation by these enzymes with purine ribonucleoside triphosphates (Maitra and Huang, 1972). T7 polymerase can utilize T3 DNA approximately 50% as efficiently as T7 DNA; while T3 polymerase is about 10% as active on T7 DNA as its homologous T3 DNA (Chamberlin and Ring, 1973; Maitra, 1971; Dunn *et al.*, 1971). The gh-1 polymerase, however, will not utilize either T3 or T7 DNA as templates to any detectable degree. Thus, the exact nucleotide sequences of DNA necessary for either binding or initiation of RNA synthesis must be different between the coliphage-induced and the gh-1-induced RNA polymerases. The coliphage-induced RNA polymerases can utilize denatured or single-stranded DNA from many sources as templates for RNA synthesis at rates from 4 to 35% of the rates on native homologous phage DNA (Chamberlin and Ring, 1973; Salvo *et al.*, 1973). With the gh-1 polymerase, very little RNA synthesis is detected when any denatured templates are used.

The gh-1-induced RNA polymerase can initiate RNA synthesis on gh-1 DNA with the ribonucleoside triphosphate GTP. This nucleotide has an apparent Km value approximately twice as high as the other three ribonucleoside triphosphates. The Hill coefficient of GTP is 1.2, as opposed to 1.0 for ATP, CTP, and UTP. The higher apparent Km for GTP and its curvilinear double reciprocal plot may result from the role of GTP in the initiation process.

The process of RNA synthesis by bacterial and phage-induced RNA polymerases has been postulated to involve two binding sites for ribonucleoside triphosphates: an initiation site, which binds the 5'terminal ribonucleoside triphosphate during the initiation process and the 3'-terminal nucleotide of the growing RNA chain during elongation, and an elongation site, which binds the ribonucleoside triphosphate which is to be incorporated into the 3'-terminus of the growing RNA chain (Anthony *et al.*, 1969; McAllister *et al.*, 1973). These two sites may have very different Km values. The apparent Km value of any ribonucleoside triphosphate involved only in the elongation process will be the Km of the elongation binding site. For gh-1 polymerase, this value is apparently 35 to 40 µ*M* for the ribonucleoside triphosphates. The apparent Km value for any ribonucleoside triphosphate involved in both initiation and elongation processes should contain contributions from both binding sites. If the relative Km value of one of the two binding sites, however, is substantially higher than that of the other site, the apparent Km value for that ribonucleoside triphosphate will reflect mostly the higher Km binding site. This is apparently the case for *E. coli* and T3 polymerases, for which the apparent Km values of the ribonucleoside triphosphates involved in initiation are 10-times and 5-times higher, respectively, than the apparent Km values of ribonucleoside triphosphates involved only in elongation (Anthony et al., 1969; McAllister et al., 1973). For gh-1 polymerase, however, the apparent Km of the initiating ribonucleoside triphosphate GTP is only twice that of the ribonucleoside triphosphates involved solely in elongation. The small relative difference of the Km values probably indicates that the apparent Km value of GTP reflects contributions from both the initiation and elongation binding sites. The influence of the two Km values results in a double reciprocal plot for gh-1 polymerase being curvilinear with respect to GTP.

The inhibitor 3'-dATP was shown to compete with ATP for a common binding site on the gh-1 polymerase and host RNA polymerase molecules. The exact mechanism by which 3'-dATP inhibited in vitro RNA synthesis of the host and gh-1 RNA polymerases is unknown. The inhibition of in vitro RNA synthesis by 3'-dATP could be due to a simple competition with ATP for a single binding site. On the other hand, the inhibition could be due to the enzymatic incorporation of 3'-dATP into the growing RNA chain, thus causing chain termination. Once RNA synthesis has terminated, the RNA polymerase molecule would have to be released from the enzyme-DNA-nascent RNA complex and then bind to a proper initiation sequence in the DNA before it could once again participate in normal RNA synthesis. It is also possible that RNA polymerase molecules terminated by incorporation of 3'-dATP could be released less rapidly than RNA polymerase molecules terminated at natural termination sites. The determination of whether 3'-dATP is incorporated into RNA would be greatly facilitated by the use of radioactively labeled 3'-dATP. Experiments on the size of RNA transcribed *in vitro* by the gh-1 polymerase after incubation periods long enough to ensure several rounds of transcription revealed that the RNA synthesized in the presence of 3'-dATP was significantly shorter than in its absence. These experiments indicated that the 3'-dATP can cause premature termination of RNA synthesis by the phage enzyme. While these experiments do not directly demonstrate that 3'-dATP is incorporated into RNA by the gh-1 polymerase, they are presumptive evidence of this point.

The apparent Ki value for 3'-dATP for the gh-1 polymerase (2×10^{-8} *M*) is strikingly low compared to that for the host *P. putida* RNA polymerase (2×10^{-6} *M*) or for the eukaryotic RNA polymerase I and II isolated from Novikoff hepatoma tissue culture cells (1.4×10^{-5} *M* and 7×10^{-6} *M*, respectively, H. Towle and R. Desrosiers, unpublished data). This higher sensitivity of the gh-1 polymerase to 3'-dATP could indicate that it is not as competent at discriminating between the substrate analog, 3'-dATP, and the natural substrate, ATP, for binding to the active site as the other RNA polymerases. 3'-dATP provides a tool for selectively inhibiting gh-1 polymerase activity in the presence of host RNA polymerase activity in *in vitro* RNA synthesis.

In contrast to the large difference in the apparent Ki values for 3'-dATP for the gh-1 polymerase and host RNA polymerase, the ATP analog, 3'-AmTP, exhibited similar apparent Ki values for both enzymes.

The inhibition by 3'-AmTP was much less effective than with 3'-dATP for both RNA polymerases. Thus, the presence of the stereochemically bulkier 3'-*O*-methyl group probably results in a lower binding affinity for 3'-AmTP than 3'-dATP for the enzyme.

A comparison of the relative effectiveness of 3'-dATP and 3'-AmTP as inhibitors of RNA polymerases and poly A polymerases (ATP: RNA adenylyltransferases) from both a prokaryotic source, *P. putida*, and a eukaryotic source, Novikoff hepatoma cells, is shown in Table VII. It is immediately apparent that the gh-1 polymerase is by far the most sensitive of the seven enzymes tested with respect to 3'-dATP, while the poly A polymerases tested were the least sensitive to this inhibitor. A broad range of apparent Ki values for 3'-dATP can be seen for the seven polymerases. The apparent Ki values for 3'-AmTP are all greater than or equal to those for 3'-dATP. The apparent Ki values for 3'-AmTP are very similar to the apparent Km values for ATP for the enzymes tested. This similarity could indicate that these enzymes bind the natural substrate, ATP, and 3'-AmTP about equally as well. This physical interpretation of the Ki value must be taken with caution, however, due to the complexity of the reaction being catalyzed.

For T7-infection of *E. coli*, development of the bacteriophage requires both the host and phage-induced RNA polymerases (Chamberlin *et al.*, 1970; Summers and Siegel, 1971). The host polymerase transcribes approximately 20% of the length of the T7 DNA, giving rise to the early RNA species (Hyman, 1971). One of the products of this transcription is the mRNA for the T7 polymerase, which is then responsible for the

TABLE VII.--Comparison of the Apparent Ki Values of 3'-dATP and 3'-AmTP for Bacterial and Eukaryotic RNA Polymerases and Poly A Polymerases.

Enzyme (Source)	Km(ATP)	Ki(3'-dATP)	Km/Ki(3'-dATP)	Ki(3'-AmTP)	Km/Ki(3'-AmTP)
gh-1-induced RNA polymerase	3.5×10^{-5}	2×10^{-8}	2000	1.3×10^{-4}	0.3
P. putida RNA polymerase	6×10^{-5}	2×10^{-6}	30	4.1×10^{-5}	1.5
P. putida Poly A polymerase[a]	2×10^{-4}	6×10^{-4}	0.33	---	---
Novikoff hepatoma RNA polymerase I	3.5×10^{-5}	1.4×10^{-5}	2.5	8.8×10^{-5}	0.4
Novikoff hepatoma RNA polymerase II	4×10^{-5}	7×10^{-6}	6	3.6×10^{-5}	1
Novikoff hepatoma Poly A polymerase[a]	2×10^{-5}	2×10^{-5}	1	2×10^{-5}	1

[a] R. Blakesley, private communication.

transcription of the late region of coliphage T7 DNA (Summers and Siegel, 1971). In gh-1 infection of _P_. _putida_, two temporally appearing classes of RNA have been identified (J. Jolly, personal communication). The gh-1 polymerase transcribes from only one strand of gh-1 DNA, the biologically correct strand, and synthesizes both early and late gh-1 RNA (J. Jolly, personal communication). It is likely, therefore, that the gh-1 polymerase acts similarly to the T7 and T3 RNA polymerases to provide a positive control in turning-on transcription of viral genes.

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SECTION II

THE SENSITIVITY OF RNA POLYMERASES I AND II FROM NOVIKOFF HEPATOMA CELLS TO INHIBITION BY 3'-DEOXYADENOSINE 5'-TRIPHOSPHATE

ABSTRACT

The effects of 3'-deoxyadenosine 5'-triphosphate (3'-dATP) and 3'-*O*-methyladenosine 5'-triphosphate (3'-AmTP) on *in vitro* RNA synthesis by the isolated forms of RNA polymerases I and II from Novikoff hepatoma cells were examined. RNA polymerases I and II were classified on the basis of their chromatographic behavior on DEAE-Sephadex, as well as the response of their enzymatic activities to ionic strength, the divalent metal ions Mn^{2+} and Mg^{2+}, and the toxin, α-amanitin. For both enzymes, the inhibition of *in vitro* RNA synthesis by the ATP analogs, 3'-dATP and 3'-AmTP, was competitive for ATP. The apparent K_I values for 3'-dATP for the two enzymes were similar--1.4×10^{-5} *M* for RNA polymerase I and 7×10^{-6} *M* for RNA polymerase II. In contrast, 3'-deoxyodensine appears to have a selective inhibitory effect on ribosomal RNA production in whole cells (Siev, M.; Weinberg, R.; and Penman, S. [1969], J. Cell Biol. *41*, 510). The apparent K_I values for 3'-AmTP were 5 to 6-times higher than those for 3'-dATP, indicating that the 3'-*O*-methyl analog of ATP is not as effective as an inhibitor of *in vitro* RNA synthesis as the 3'-H analog.

INTRODUCTION

The antibiotic, 3'-deoxyadenosine (cordycepin), is an analog of adenosine in which the 3'-hydroxyl group of the ribose moiety is replaced by a hydrogen atom. 3'-dA has been found to inhibit growth of certain bacteria and mammalian cells (for review see, Guarino, 1967). Experiments on cells in culture suggest the drug acts primarily as an inhibitor of the synthesis of RNA. The active form of 3'-dA in causing the inhibition of cell growth is thought to be the phosphorylated derivative--3'-deoxyadenosine 5'-triphosphate. 3'-dATP has been shown to be incorporated into RNA by certain bacterial RNA polymerases (Shigeura and Boxer, 1964). Due to the presence of a normal 3'-OH group and a 5'-triphosphate group, the analog is apparently accepted by the RNA polymerase as a normal ribonucleotide. Once incorporated, however, the 3'-dATP would act as a chain terminator, since no 3'-OH group is present for the formation of the subsequent phosphodiester bond.

When 3'-dA is administered to HeLa cells at low concentrations, the synthesis of completed 45 S ribosomal precursor RNA in the nucleolus, as well as mature 18 S and 28 S cytoplasmic ribosomal RNA, is greatly depressed (Siev et al., 1969). In addition, the nucleolar RNA molecules that are produced are considerably shorter in length than the normal 45 S ribosomal precursor molecule. The synthesis of shorter RNA molecules after 3'-dA treatment of HeLa cells is consistent with the postulated mode of action of 3'-dATP as a chain terminator in RNA synthesis. At the same concentration of 3'-dA which drastically inhibits ribosomal RNA production, little or no effect is seen on the synthesis or size of heterogenous nuclear RNA.

Eukaryotic cells from a great variety of sources have been demonstrated to contain at least three physically and biochemically distinct DNA-dependent RNA polymerases (Roeder and Rutter, 1969; for review, Jacob, 1973). The activities which most consistently appear have been classified I, II and III, on the basis of their order of elution from DEAE-Sephadex. RNA polymerase I is optimally active at low salt concentrations (ammonium sulfate less than 0.05 M), utilizes Mn^{2+} and Mg^{2+} almost equally well to satisfy its divalent metal ion requirement, and is insensitive to inhibition by the toxin, α-amanitin. This form is of nucleolar origin and has been demonstrated to be responsible for the synthesis of ribosomal RNA (Roeder and Rutter, 1970; Zylber and Penman, 1971; Reeder and Roeder, 1972). RNA polymerase II is optimally active at ammonium sulfate concentrations between 0.1 and 0.15 M, prefers Mn^{2+} as a divalent metal ion over Mg^{2+}, and can be completely inhibited by very low concentrations of α-amanitin. This RNA polymerase activity is found in the nucleoplasm and is responsible for most, if not all, heterogenous nuclear RNA synthesis (Roeder and Rutter, 1970; Zylber and Penman, 1971). The RNA polymerase III is only found in small quantities, when present, and is highly labile during purification attempts. It maintains optimal enzyme activity over a broad range of ammonium sulfate concentrations (0 to 0.2 M) and is located in the nucleoplasm. Relatively little is known of this form of RNA polymerase. Recent experiments implicate RNA polymerase III in the synthesis of ribosomal 5 S and transfer RNA (Weinman and Roeder, 1974).

Due to the existence of distinct forms of RNA polymerase for the synthesis of ribosomal and heterogenous nuclear RNA, it is reasonable to postulate that the selective inhibition of ribosomal RNA production by 3'-dA may be due to the greater sensitivity of RNA polymerase I than RNA polymerase II to the triphosphate derivative of the drug. The possibility that two distinct RNA polymerases in a single cell might vary markedly in their response to 3'-dATP was shown for the RNA polymerases present in *Pseudomonas putida* after bacteriophage gh-1 infection. The gh-1-induced RNA polymerase isolated from infected cells displayed an apparent K_I value for 3'-dATP which was 100-times lower than that of the host *P. putida* RNA polymerase (Towle *et al.*, 1974). The purpose of this research was to determine whether *in vitro* RNA synthesis by the isolated forms of RNA polymerase I and II displayed sensitivities to 3'-dATP which would explain the action of 3'-dA in whole cells. The source of cells for this study was Novikoff hepatoma cells grown in culture. Using these cells, Desrosiers (R. Desrosiers, unpublished data) has previously demonstrated that 25 μg/ml 3'-dA would inhibit ribosomal RNA synthesis drastically, but have little effect on heterogenous nuclear RNA synthesis, consistent with the results of Siev *et al.* (1969). RNA polymerases I and II were isolated and characterized from Novikoff hepatoma cells. The characterization of the RNA polymerases present in Novikoff hepatoma cells has not been previously reported. The inhibition of *in vitro* RNA synthesis by 3'-dATP for RNA polymerases I and II was found to be competitive for ATP. The apparent K_I values for 3'-dATP for the two enzymes were similar and indicate that RNA polymerase I activity is slightly less sensitive to 3'-dATP than RNA polymerase II. The relative sensitivities toward 3'-dATP of the two enzymes is, therefore, not consistent with the mode of action postulated above for the inhibition of ribosomal RNA production by 3'-dA in whole cells.

MATERIALS AND METHODS

Isolation of Nuclei

Novikoff hepatoma cells were grown in culture at 37° C as described by Desrosiers et al. (1974). When growth had reached a density of 6 to 8 x 10^5 cells/ml (mid-logrithmic phase), cells were harvested by centrifugation. All subsequent procedures were performed at 0 to 4° C. From 2 to 2.5 x 10^9 cells were used for the typical purification. Cells were washed and concentrated in Plagemann's Basal Salt Solution (Plagemann and Roth, 1969). The cells were then suspended in 40 ml of a hypotonic buffer containing 10 mM Tris•HCl, pH 7.6, 6 mM KCl, 5 mM $MgCl_2$ and allowed to swell for 10 minutes. After this time, a 10% (v/v) solution of NP-40 detergent was added to the cell suspension to a final concentration of 0.5%. The suspension was mixed briefly and the nuclei collected by centrifugation at 1,000 x g for 2 minutes. The nuclear pellet was washed twice in the hypotonic buffer. The final nuclear pellet was suspended in about 15 ml of a buffer containing 10 mM Tris•HCl, pH 7.9, 50 mM $MgCl_2$, 1 M sucrose, 1 mM dithiothreitol.

Solubilization of RNA Polymerase Activity

The solubilization and chromatography on DEAE-Sephadex of Novikoff hepatoma RNA polymerases were carried out by a modification of the procedure of Roeder and Rutter (1970). A solution of 4 M

$(NH_4)_2SO_4$, pH 8.0, was added to the nuclear suspension to give a final concentration of 0.3 M $(NH_4)_2SO_4$. The suspension at this point became very viscous indicating the nuclei had lysed. The viscous suspension was sonicated on a Biosonik sonicator at full voltage for 1 1/2 minutes (in 10 second bursts with intermittent cooling). Sonication of the nuclear suspension resulted in a large decrease in viscosity. The sonicated solution was diluted with two volumes of a buffer containing 50 mM Tris•HCl, pH 8.0, 12.5% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol and then centrifuged at 40,000 RPM in a Spinco 40 head for 1.25 hours. To the supernatant fluid from the centrifugation, 0.28 grams of $(NH_4)_2SO_4$ per ml of solution were slowly added. The solution was stirred for 30 minutes and the precipitated material collected by centrifugation at 15,000 x g for 20 minutes. This $(NH_4)_2SO_4$- precipitated material was resuspended in a small volume of buffer containing 50 mM Tris•HCl, pH 8.0, 25% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol (TGMED Buffer). The resuspended material was dialyzed for 8 hours against 1 liter of TGMED Buffer containing 0.03 M $(NH_4)_2SO_4$. After dialysis, the insoluble material present was removed by centrifugation at 15,000 x g for 20 minutes and the supernatant solution used for further purification.

Separation of RNA Polymerases I and II

A DEAE-Sephadex column (2 x 12 cm) was packed and equilibrated with TGMED Buffer containing 0.03 M $(NH_4)_2SO_4$. The enzyme solution was loaded onto the column at a rate of 0.5 ml per minute or less. The column was then washed with two column volumes of TGMED Buffer containing

0.03 M $(NH_4)_2SO_4$. All detectable RNA polymerase activity adsorbed to the DEAE-Sephadex under these conditions. The column was eluted with a linear gradient from 0.03 M to 0.6 M $(NH_4)_2SO_4$ in TGMED Buffer (total volume of 300 ml) and fractions of about 3.5 ml were collected. Samples of the column fractions were assayed in the Limiting UTP Reaction Mixture described below. Two distinct RNA polymerase activities were routinely observed (RNA polymerases I and II) and fractions constituting the majority of each RNA polymerase activity were pooled. The pooled fractions were dialyzed against 1 liter of TGED Buffer (same as TGMED Buffer except no $MgCl_2$) containing 0.03 M $(NH_4)_2SO_4$. Small phosphocellulose columns with total bed volumes of 1.5 ml were packed and equilibrated with the same buffer. The pooled dialyzed fractions were loaded on the phsophocellulose columns and the columns were washed with five column volumes of TGED Buffer with 0.03 M $(NH_4)_2SO_4$. RNA polymerase was then eluted with TGED Buffer containing 0.5 M $(NH_4)_2SO_4$ and 0.5 ml fractions were collected. Samples (5 µl) of the column fractions were assayed in the Limiting UTP Reaction Mixture. RNA polymerase activity was generally eluted in 1 to 1.5 ml of buffer with a 70 to 80% recovery of activity. At this stage, $MgCl_2$ was added to a final concentration of 5 mM and, on occasion, bovine serum albumin was added to a final concentration of 0.5 mg/ml. Final enzyme samples were dialyzed against TGMED Buffer containing 0.03 M $(NH_4)_2SO_4$ for 6 hours, divided into small aliquots, and stored at -80° C.

Assay for RNA Polymerase Activity

The standard reaction mixture for RNA polymerase I and II contained in a volume of 0.125 ml: 50 mM Tris•HCl, pH 7.9, 1.6 mM $MnCl_2$, 1 mM dithiothreitol, 160 µg/ml calf thymus DNA, 0.6 mM each of ATP, CTP, and GTP, 0.1 mM $[^3H]$UTP (1.25 µC per assay), either 50 mM $(NH_4)_2SO_4$ (RNA Polymerase I) or 100 mM $(NH_4)_2SO_4$ (RNA polymerase II) and enzyme samples. The Limiting UTP Reaction Mixture for assaying column fractions contained in 0.125 ml: 50 mM Tris•HCl, pH 8.0, 1.6 mM $MnCl_2$, 1 mM dithiothreitol, 160 µg/ml calf thymus DNA, 0.6 mM each of ATP, CTP, and GTP, 6.7×10^{-4} mM $[^3H]$UTP (1.25 µC per assay) and enzyme samples. All reactions were initiated by the addition of enzyme and incubated for 10 minutes at 37° C. Reactions were terminated by the addition of 10 µl of 0.2 M EDTA and placed in an ice bath. Samples of 0.1 ml of each reaction mixture were pipetted onto Whatman DE81 filters, which were then washed by the procedure described by Blatti *et al.* (1970). Filters were dried at 100° C for 5 minutes and counted in a scintillation fluid containing 4 grams of 2,5-bis-2-(5-*tert*-Butylbenzoxazolyl)-Thiophene per liter of toluene.

Materials

DEAE-Sephadex (A-25) was purchased from Pharmacia Fine Chemicals. NP-40 detergent was obtained from Shell. Phosphocellulose (P-11) and Whatman DE-81 filter discs (2.4 cm diameter) were from Reeve-Angel. Calf thymus DNA was purchased from P-L Biochemicals and further purified by two successive SDS-phenol extractions, followed by extensive dialysis. 3'-dATP and 3'-AmTP were the very generous gifts of R. Desrosiers and Dr. Fritz Rottman of this department.

RESULTS

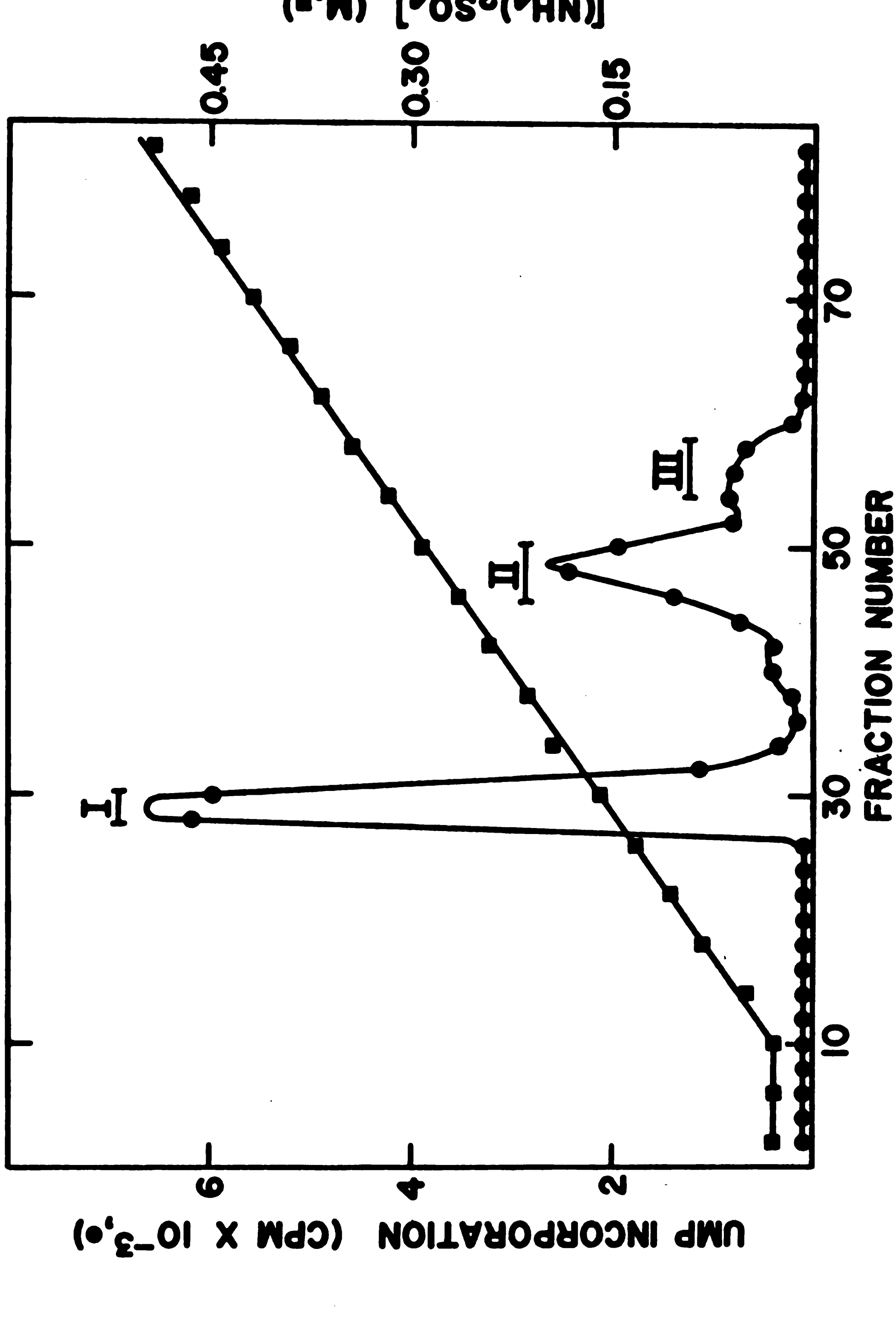
RNA Polymerase Activities in Novikoff Hepatoma Cells

A typical chromatographic separation of Novikoff hepatoma RNA polymerases on DEAE-Sephadex is shown in Figure 1. Three RNA polymerase activities were routinely detected: RNA polymerase I, which eluted at about 0.15 M $(NH_4)_2SO_4$ and constituted from 45 to 60% of the total RNA polymerase activity; RNA polymerase II, which was found at 0.28 M $(NH_4)_2SO_4$ and contained 30 to 45% of the total activity; and RNA polymerase III, which eluted at 0.33 M $(NH_4)_2SO_4$ and constituted from 5 to 10% of the activity. The third RNA polymerase activity was designated as RNA polymerase III on the basis of its insensitivity to inhibition by α-amanitin. Attempts to concentrate and further characterize this RNA polymerase activity, however, were unsuccessful, due to the small amounts present and the lability of this activity (Blatti *et al.*, 1970). Fractions with the majority of RNA polymerase activity were pooled as shown in Figure 1 and concentrated by phosphocellulose chromatography for further characterizations.

Characterizations of RNA Polymerases I and II

Certain catalytic properties of the separated RNA polymerases from Novikoff hepatoma cells were examined. The properties of RNA polymerases from diverse eukaryotic sources such as sea urchin, calf thymus, rat liver, and yeast have been found to be very similar for

Figure 1.--DEAE-Sephadex chromatography of Novikoff hepatoma nuclear RNA polymerases. Chromatography of a nuclear extract of Novikoff hepatoma cells on a DEAE-Sephadex column was performed as described in Materials and Methods. Samples (50 µl) of various fractions were assayed for RNA polymerase activity in the Limiting UTP Reaction Mixture. The concentration of $(NH_4)_2SO_4$ in various fractions was determined by conductivity measurements. The brackets indicate the fractions which were pooled for concentration by phosphocellulose chromatography.



UMP INCORPORATION (CPM X 10⁻³,●)
2
4
6
FRACTION NUMBER
10
30
50
70
[(NH4)2SO4] (M,■)
0.15
0.30
0.45
I
II
III

corresponding forms of RNA polymerase. These properties, thus, can be used as a diagnostic tool to confirm the designations of RNA polymerases I and II assigned on the basis of their order of elution from DEAE-Sephadex.

The effect of various concentrations of $(NH_4)_2SO_4$ on the activity of RNA polymerases I and II is shown in Figure 2. RNA polymerase I was found to display optimal enzyme activity at relatively low concentrations of $(NH_4)_2SO_4$, from 0.04 to 0.06 $\underline{M}$. At 0.2 $\underline{M}$ $(NH_4)_2SO_4$, the activity of RNA polymerase I was less than 10% of that seen at 0.05 $\underline{M}$ $(NH_4)_2SO_4$. RNA polymerase II, on the other hand, required higher concentrations of $(NH_4)_2SO_4$, from 0.1 to 0.15 $\underline{M}$, for optimal enzyme activity. At a concentration of 0.25 $\underline{M}$ $(NH_4)_2SO_4$, RNA polymerase II was still 50% as active as the optimal enzyme activity.

RNA polymerases I and II were both dependent on the presence of a divalent metal ion for enzyme activity. The effect of varying the concentrations of the divalent metal ions, Mn^{2+} and Mg^{2+}, on the activity of the two RNA polymerases is shown in Figure 3. Both RNA polymerases I and II show maximal enzyme activity at about 2 m$\underline{M}$ Mn^{2+} and between 4 and 10 m$\underline{M}$ Mg^{2+}. RNA polymerase I is more efficient at utilizing Mg^{2+} as a divalent metal ion than RNA polymerase II. With RNA polymerase I, the optimal enzyme activity with Mg^{2+} is about 1/2 that seen for Mn^{2+}, while for RNA polymerase II, Mg^{2+} is only utilized about 1/4 as efficiently as Mn^{2+}.

The activities of RNA polymerases I and II can be differentiated by their sensitivity to the toxin, α-amanitin. RNA polymerase II activity was found to be very sensitive to α-amanitin and was completely

Figure 2.--The effect of varying the concentration of $(NH_4)_2SO_4$ on in vitro RNA synthesis by the Novikoff hepatoma RNA polymerases I and II. Standard reaction mixtures were prepared as described in Materials and Methods except that $(NH_4)_2SO_4$ was added to the reaction mixtures at the concentrations indicated. Reactions were initiated by either the addition of RNA polymerase I (●) or RNA polymerase II (■), incubated for 10 minutes, and analyzed for RNA synthesis as described in Materials and Methods.

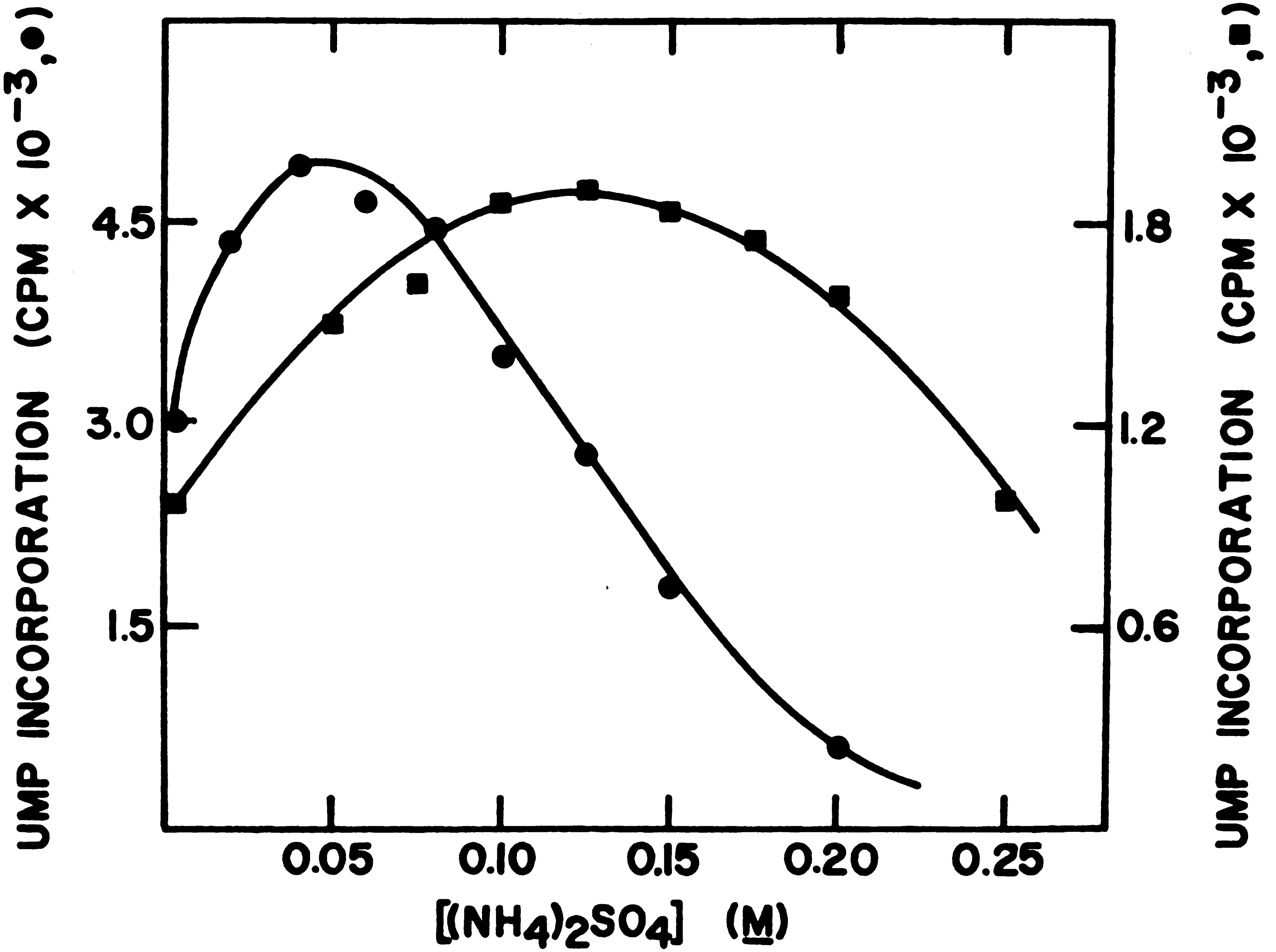
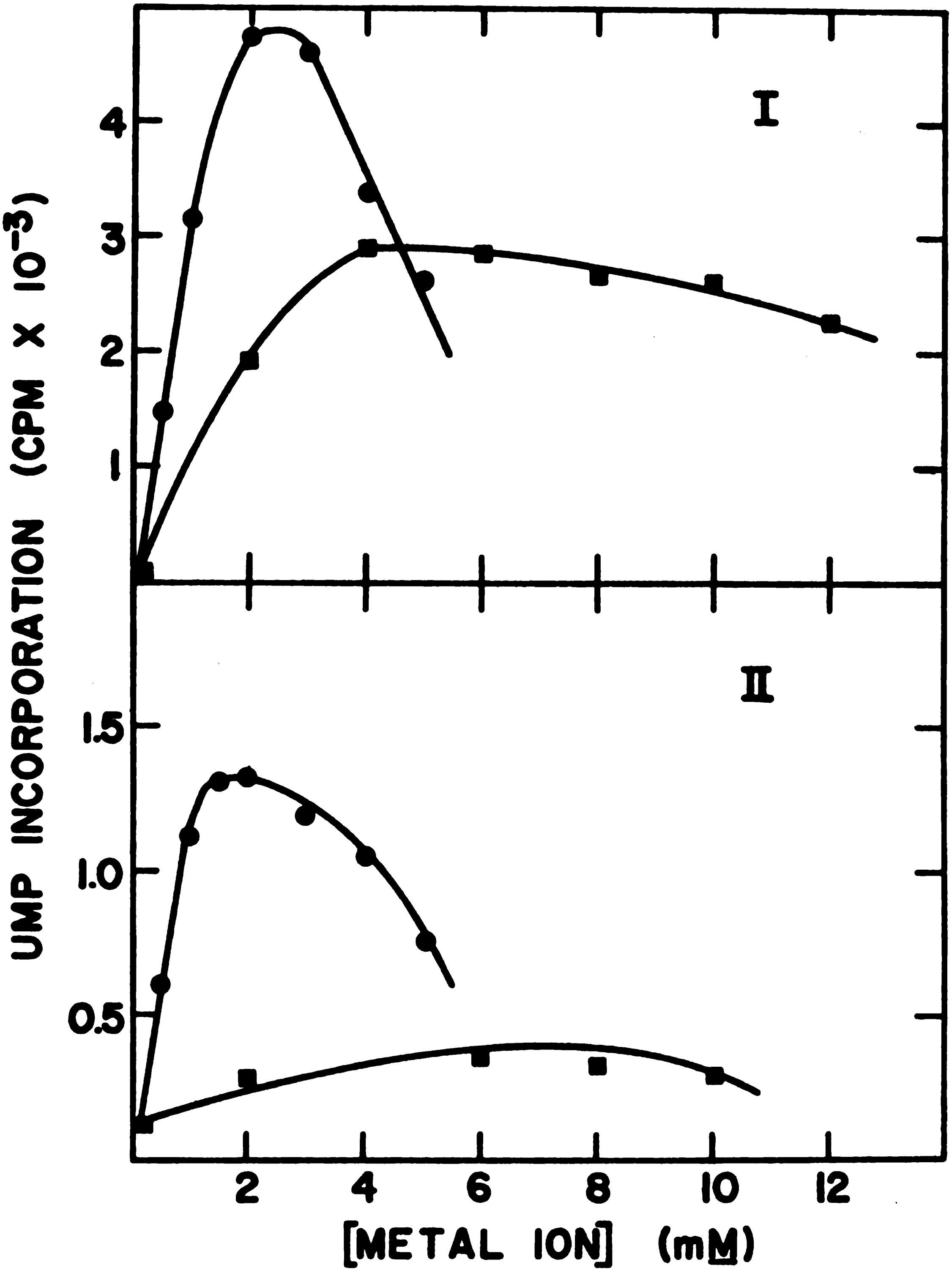

UMP INCORPORATION (CPM X 10^{-3},●)
4.5
3.0
1.5
UMP INCORPORATION (CPM X 10^{-3},■)
1.8
1.2
0.6
0.05
0.10
0.15
0.20
0.25
[(NH$_4$)$_2$SO$_4$] (M)

Figure 3.--The effect of varying the concentration of the divalent metal ion on in vitro RNA synthesis by the Novikoff hepatoma RNA polymerases I and II. Standard reaction mixtures were prepared as described in Materials and Methods except that the concentration of Mn^{2+} was varied as indicated (●) or Mn^{2+} was replaced by Mg^{2+} at the concentrations indicated (■). Reactions were initiated by either the addition of RNA polymerase I (upper diagram) or RNA polymerase II (lower diagram), incubated for 10 minutes, and analyzed for RNA synthesis as described in Materials and Methods.


I
II
UMP INCORPORATION (CPM X 10^{-3})
1
2
3
4
0.5
1.0
1.5
2
4
6
8
10
12
[METAL ION] (mM)

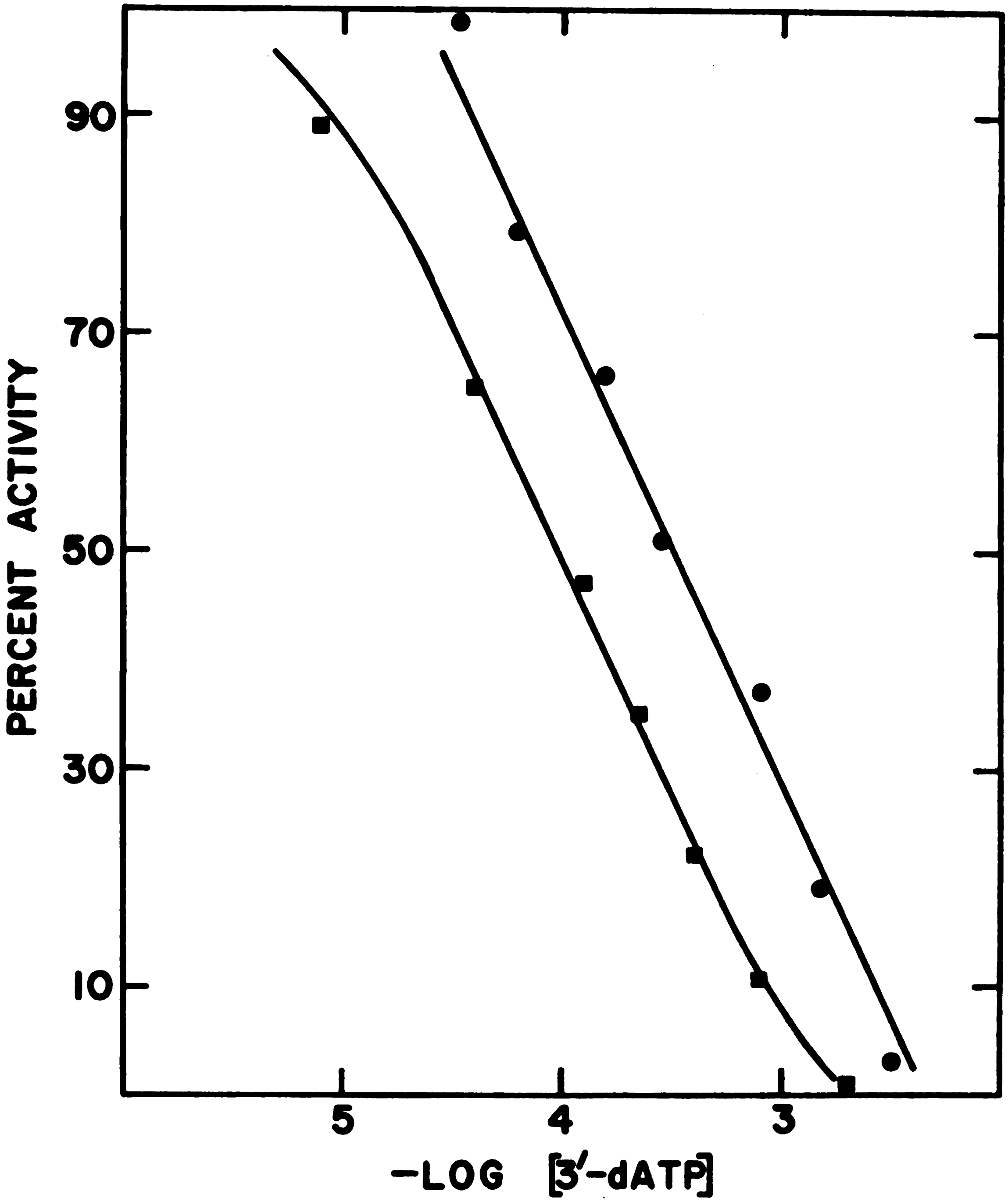
inhibited by a concentration of 3 μg/ml. RNA polymerase I activity was completely unaffected at this concentration of inhibitor.

The properties of Novikoff hepatoma RNA polymerases I and II are consistent with those reported by Roeder and Rutter for RNA polymerases I and II from rat liver or sea urchin on the basis of four criteria: the order of appearance and approximate concentration of $(NH_4)_2SO_4$ necessary for elution from DEAE-Sephadex; the concentrations of $(NH_4)_2SO_4$ necessary for optimal enzyme activity; the relative ability to use Mn^{2+} and Mg^{2+} as divalent metal ions; and the sensitivity to the inhibitor, α-amanitin. Due to the similarities of the properties of the corresponding forms, it is reasonable to assume that the Novikoff hepatoma RNA polymerase I is the nucleolar enzyme responsible for the synthesis of ribosomal RNA, while the Novikoff hepatoma RNA polymerase II is localized in the nucleoplasm and synthesizes most, if not all, of the heterogenous nuclear RNA.

Inhibition of RNA Polymerases I and II by 3'-dATP

In order to determine the relative sensitivities of RNA polymerases I and II to 3'-dATP, the effect of the inhibitor on calf thymus DNA-primed *in vitro* RNA synthesis by the two Novikoff hepatoma enzymes was examined. At the concentration of ATP present in the standard reaction mixture, 0.6 m*M*, the amount of 3'-dATP required to inhibit the RNA polymerase I activity to a given degree was about 3-times higher than the amount required to inhibit RNA polymerase II activity to the same extent (Figure 4). Inhibition of RNA polymerase I activity by 50% occurred at 0.3 m*M* 3'-dATP, while 50% inhibition of

Figure 4.--The effect of 3'-deoxyadenosine 5'-triphosphate on in vitro RNA synthesis by the Novikoff hepatoma RNA polymerases I and II. Standard reaction mixtures were prepared as described in Materials and Methods except that 3'-dATP was added to the reaction mixtures at the concentrations indicated. Reactions were initiated by either the addition of RNA polymerase I (●) or RNA polymerase II (■), incubated for 10 minutes, and analyzed for RNA synthesis as described in Materials and Methods. RNA synthesis in reactions containing 3'-dATP was expressed as the percentage of RNA synthesis in control reactions containing no 3'-dATP. In standard reaction mixtures containing no 3'-dATP, 100% activity was equivalent to 4500 CPM of UMP incorporation in 10 minutes for RNA polymerase I and 1300 CPM for RNA polymerase II.


PERCENT ACTIVITY
90
70
50
30
10
5
4
3
-LOG [3'-dATP]

RNA polymerase II occurred at 0.09 m*M* 3'-dATP. Thus, RNA polymerase I activity appeared to be slightly less sensitive to inhibition by 3'-dATP than RNA polymerase II activity.

To further study the inhibition of *in vitro* RNA synthesis by 3'-dATP, the effect of varying the concentration of ATP on initial reaction rates in absence and presence of 3'-dATP was determined. Within experimental error, 3'-dATP appeared to act as a competitive inhibitor of ATP for both RNA polymerases I and II (Figures 5 and 6). The apparent Km values for ATP for both RNA polymerases were similar--3.5×10^{-5} *M* for RNA polymerase I and 4×10^{-5} *M* for RNA polymerase II. The apparent K_I value for 3'-dATP for RNA polymerase I was calculated to be 1.4×10^{-5} *M*, while that for RNA polymerase II was 7×10^{-6} *M*. As was seen in Figure 4, RNA polymerase II appeared to be more sensitive to inhibition by 3'-dATP than RNA polymerase I. Virtually identical results were obtained when a high molecular weight preparation of Novikoff hepatoma DNA was used as template instead of calf thymus DNA (data not shown). The inhibition of *in vitro* RNA synthesis by 3'-dATP did not depend on the DNA template used. Thus, the isolated forms of RNA polymerase I and II do not possess the relative difference in sensitivity to 3'-dATP in *in vitro* RNA synthesis that could explain the selective inhibition of ribosomal RNA synthesis by 3'-dA in while cells studies.

Another structural analog of ATP is 3'-*O*-methyladenosine 5'-triphosphate (3'-AmTP). This analog is similar to 3'-dATP in that it differs from ATP only at the 3'-position of the ribose moiety. Instead of the 3'-OH group of ATP being replaced by a hydrogen atom as in

Figure 5.--The effect of varying the concentration of ATP in the absence and presence of 3'-deoxyadenosine 5'-triphosphate on in vitro RNA synthesis by Novikoff hepatoma RNA polymerase I. Standard reaction mixtures for RNA polymerase I were prepared and run as described in Materials and Methods except that the concentration of ATP was varied as indicated. Reaction mixtures also contained the following concentrations of 3'-dATP: ● , no 3'-dATP: ▲ , 9.6 x 10^{-5} M 3'-dATP; ■ , 5.6 x 10^{-4} M 3'-dATP. Reactions were terminated and analyzed for RNA synthesis as described in Materials and Methods.

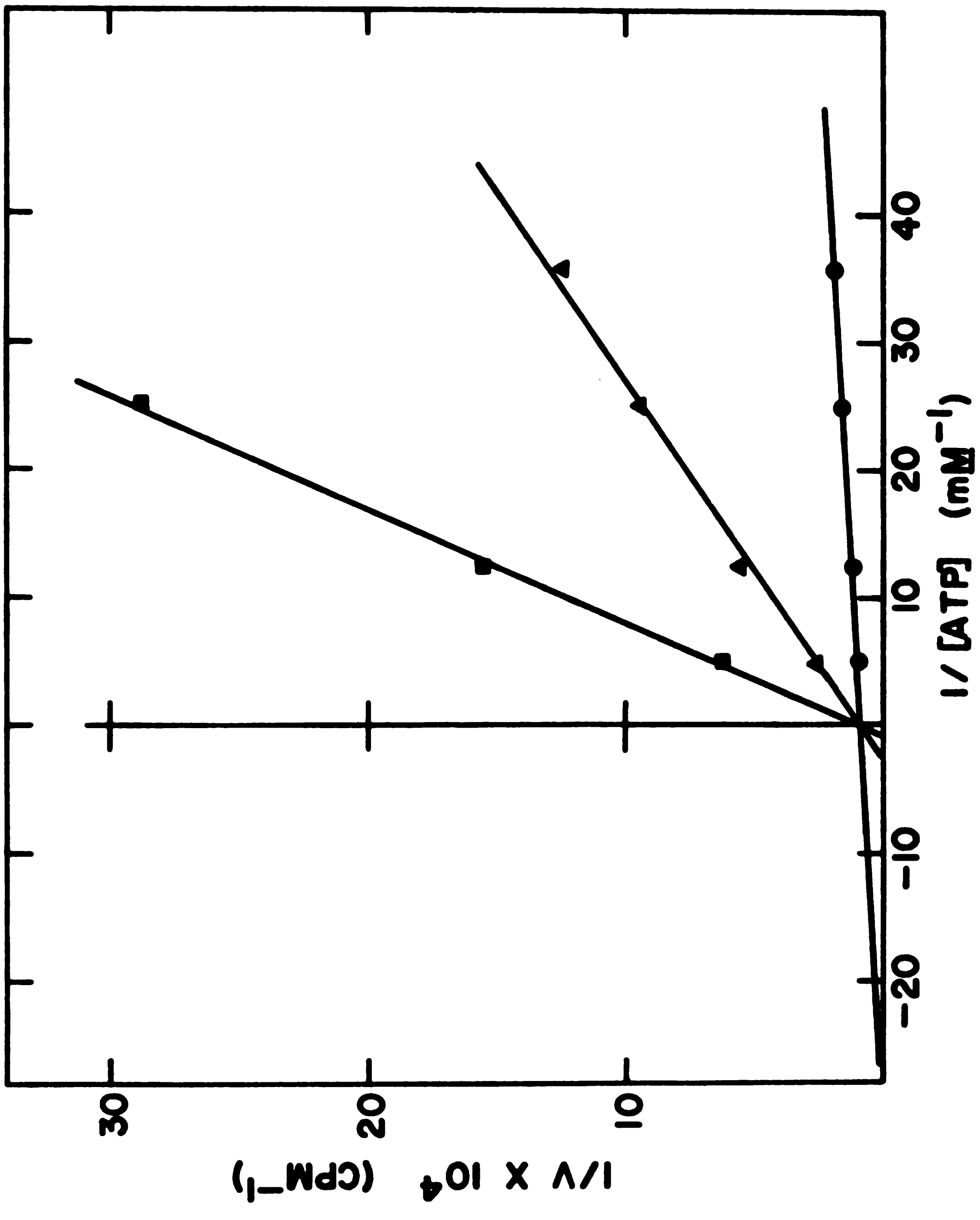
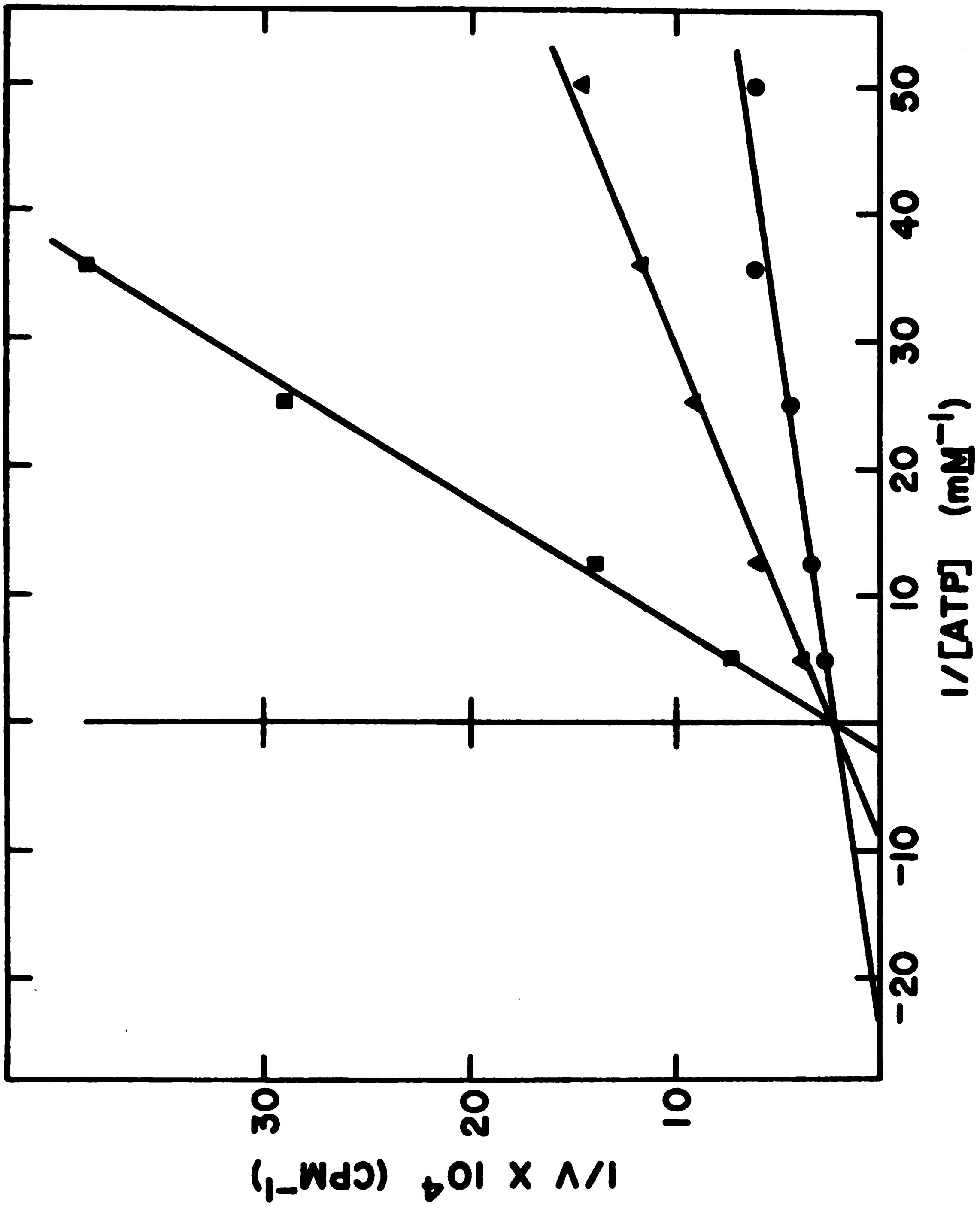

1/V X 10⁴ (CPM⁻¹)
30
20
10
1/[ATP] (mM⁻¹)
−20
−10
10
20
30
40

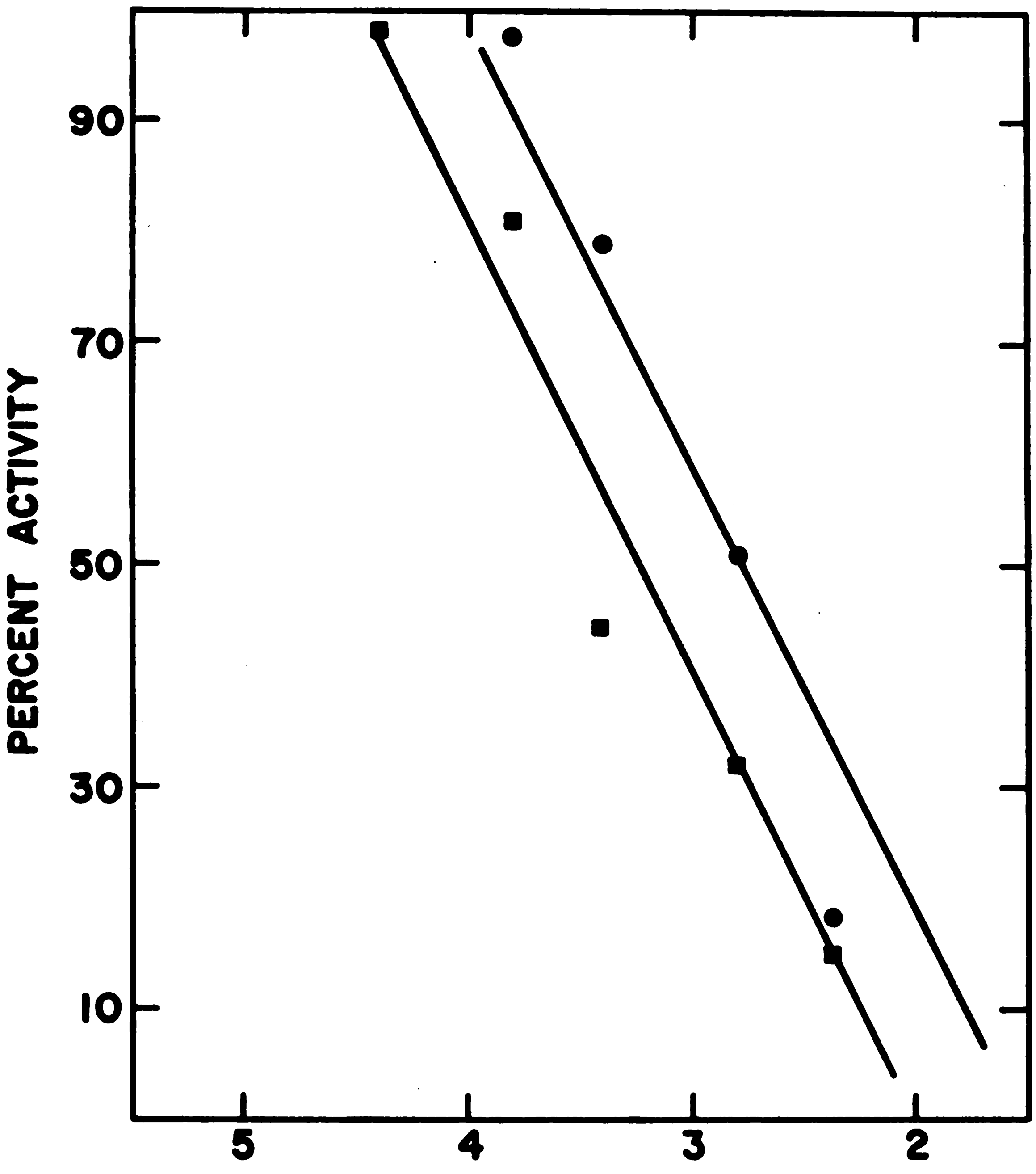
Figure 6.--The effect of varying the concentration of ATP in the absence and presence of 3'-deoxyadenosine 5'-triphosphate on in vitro RNA synthesis by Novikoff hepatoma RNA polymerase II. Standard reaction mixtures for RNA polymerase II were prepared and run as described in Materials and Methods except that the concentration of ATP was varied as indicated. Reaction mixtures also contained the following concentrations of 3'-dATP: ● , no 3'-dATP; ▲ , 1.2 x 10^{-5} M 3'-dATP; ■ , 5.6 x 10^{-5} M 3'-dATP. Reactions were terminated and analyzed for RNA synthesis as described in Materials and Methods.


1/V X 10⁴ (CPM⁻¹)
30
20
10
1/[ATP] (mM⁻¹)
-20
-10
10
20
30
40
50

3'-dATP, in 3'-AmTP it is replaced by the stereochemically bulkier O-methyl group. 3'-AmTP could conceivably act analogously to 3'-dATP. It contains the 2'-OH group necessary for identification as a ribonucleoside and the 5'-triphosphate group necessary for phosphodiester bond formation. If 3'-AmTP became incorporated into an RNA chain, however, it would act as a chain terminator.

To test whether 3'-AmTP could act as an inhibitor of *in vitro* RNA synthesis, the effect of the analog on calf thymus DNA-primed RNA synthesis by the Novikoff hepatoma RNA polymerases was tested. As can be seen in Figure 7, 3'-AmTP inhibited the activity of both Novikoff hepatoma RNA polymerases. At 0.6 mM ATP, 50% inhibition of the activity of RNA polymerase I activity occurred at 0.6 mM 3'-AmTP, while 50% inhibition of RNA polymerase II activity occurred at 1.6 mM 3'-AmTP. As seen with 3'-dATP, the inhibition of RNA polymerase II activity was about 2.5-times more sensitive to the ATP analog than that of RNA polymerase I. The inhibition by 3'-AmTP of both RNA polymerase activities was competitive for ATP (data not shown). The apparent K_I value for 3'-AmTP for RNA polymerase I was 8.8×10^{-5} M and for RNA polymerase II was 3.6×10^{-5} M. These values are about 5 to 6-times higher than the apparent K_I values for 3'-dATP for these two enzymes. Thus, the presence of the stereochemically bulkier O-methyl group results in 3'-AmTP being a less effective inhibitor of *in vitro* RNA synthesis than 3'-dATP.

Figure 7.--The effect of 3'-0-methyladenosine 5'-triphosphate on in vitro RNA synthesis by the Novikoff hepatoma RNA polymerases I and II. Standard reaction mixtures were prepared as described in Materials and Methods except that 3'-AmTP was added to the reaction mixtures at the concentrations indicated. Reactions were initiated by either the addition of RNA polymerase I (●) or RNA polymerase II (■), incubated for 10 minutes, and analyzed for RNA synthesis as described in Materials and Methods. RNA synthesis in reactions containing 3'-AmTP was expressed as the percentage of RNA synthesis in control reactions containing no 3'-AmTP. In standard reaction mixtures containing no 3'-AmTP, 100% activity was equivalent to 2300 CPM of UMP incorporation in 10 minutes for RNA polymerase I and 2400 CPM for RNA polymerase II.


PERCENT ACTIVITY
90
70
50
30
10
5
4
3
2
—LOG [3′-AmTP]

DISCUSSION

3'-Deoxyadenosine has been shown to inhibit ribosomal RNA production when present at concentrations which do not affect the synthesis of heterogenous nuclear RNA (Siev et al., 1969). A reasonable explanation for the differential effect of 3'-dA is that the enzyme responsible for the synthesis of heterogenous nuclear RNA (RNA polymerase II) is much more resistant to inhibition by 3'-dATP than the enzyme responsible for the synthesis of ribosomal RNA (RNA polymerase I). In this paper, it has been shown that in vitro RNA synthesis by RNA polymerase II is actually slightly more sensitive to 3'-dATP than is in vitro RNA synthesis by RNA polymerase I. Thus, the isolated forms of RNA polymerases I and II do not exhibit the specificity towards 3'-dATP necessary to explain the effects of 3'-dA in whole cells.

Blatti et al. (1970) have previously reported that RNA polymerases I and II isolated from calf thymus displayed similar "K_I values" for 3'-dATP. No studies showing that 3'-dA selectively inhibits ribosomal RNA synthesis in calf thymus have been performed. Furthermore, the "K_I values" reported were not true K_I values, but the concentrations of inhibitor necessary to cause 50% inhibition when the concentration of ATP was equal to the Km value for ATP. Therefore, the studies reported here for Novikoff hepatoma cells is the first

time that the effects of 3'-dA on whole cells have been coupled with accurately determined K_I values for 3'-dATP for a single cell type.

One possible explanation for the discrepancy between the effects of 3'-dA in whole cells and the relative sensitivities of the RNA polymerases towards 3'-dATP is that the isolated forms of the RNA polymerase differ from the forms of the enzyme functioning in the cell. For instance, it has been demonstrated that RNA produced _in vitro_ by the isolated forms of RNA polymerases I and II is much shorter in length than the corresponding _in vivo_ RNA products. Several protein factors which affect _in vitro_ RNA synthesis by eukaryotic RNA polymerases have been described (for example; Stein and Hausen, 1970; Lee and Dahmus, 1973). It is conceivable that such protein factors may be bound to the RNA polymerases in the nucleus as part of the transcriptional complex, but are lost during purification. The binding of such factors could possibly alter the RNA polymerase to make it more or less sensitive to 3'-dATP. Studies are currently underway on the sensitivities of RNA polymerases I and II activities in whole nuclei to 3'-dATP aimed at examining this possibility (R. Desrosiers, personal communication). Under these conditions, all factors which affect transcriptional activity of the RNA polymerases in the cell should be present and active.

It must also be considered a distinct possibility from these studies that the selective inhibition of ribosomal RNA production by 3'-dA in whole cells is not due to the differential sensitivities of RNA polymerases I and II to 3'-dATP. Numerous hypotheses concerning the mechanism of 3'-dA inhibition of ribosomal RNA production can be

envisioned, with little, if any, supportive data favoring any particular one. For instance, it is conceivable that different nucleotide pools for ribosomal and heterogenous nuclear RNA synthesis might exist. If this is so, 3'-dATP might be selectively concentrated in the nucleolar pool causing a higher molar ratio of 3'-dATP to ATP in this pool than the nucleoplasmic pool. The higher 3'-dATP to ATP ratio could cause the selective inhibition of ribosomal RNA synthesis, even though the enzymes responsible for ribosomal and heterogenous nuclear RNA synthesis do not differ drastically in their sensitivities to 3'-dATP. Another possibility is that the inhibition of mature ribosomal RNA production by 3'-dA does not occur at the level of transcription, but in the post-transcriptional processing of the 45 S ribosomal precursor molecule. If some step in the processing of the ribosomal precursor was more sensitive to 3'-dATP than RNA synthesis by either RNA polymerase I or II, it could result in the selective disruption of mature ribosomal RNA production. The inhibition of mature ribosomal RNA production by a defect in the post-transcriptional processing might also cause a decrease in the amount of 45 S ribosomal RNA present by some indirect "feedback" control or increased degradation of 45 S ribosomal RNA. The above hypotheses are obviously highly speculative and the final elucidation of the mechanism by which 3'-dA selectively inhibits ribosomal RNA production awaits further experimentation.

The inhibition by 3'-dATP of _in vitro_ RNA synthesis by the Novikoff hepatoma RNA polymerases was shown to be competitive for ATP. Another ATP analog, 3'-AmTP, which is similar to 3'-dATP in the position of ATP altered, also inhibits _in vitro_ RNA synthesis competitively for

ATP. 3'-AmTP was a less efficient inhibitor than 3'-dATP for both RNA polymerases I and II by about 5 to 6-times. Thus, the Novikoff hepatoma RNA polymerases appear to be better able to discriminate 3'-AmTP from the natural substrate ATP for binding to the active site than 3'-dATP. The presence of the larger substituent at the 3'-position of 3'-AmTP than 3'-dATP probably accounts for this difference. It is impossible to determine from the experiments reported here whether either 3'-dATP or 3'-AmTP is incorporated into RNA by the RNA polymerases I or II. The inhibition by these analogs could be entirely due to a simple competition with ATP for a single binding site. If the 3'-dATP or 3'-AmTP did become bound to the active site of the RNA polymerase, however, there is no apparent reason why incorporation into the RNA chain should not occur. The fact that the apparent K_I values of 3'-dATP and 3'-AmTP are not strikingly different than the apparent Km value for ATP for both Novikoff hepatoma RNA polymerases suggests that these enzymes do not have a rigid requirement for a hydroxyl group at the 3'-position for binding of the substrate molecule. This suggestion is rather surprising when it is considered that this particular position in the substrate molecule is the site of catalytic action of these enzyme molecules.

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SECTION III

MAREK'S DISEASE HERPESVIRUS-INDUCED DNA POLYMERASE

SUMMARY

Marek's disease is a highly contagious malignant lymphoma of chickens whose etiological agent is a herpesvirus. The mechanism of replication of Marek's disease herpesvirus (MDHV) DNA in both productively infected and tumor cells of infected chickens is of interest in establishing models for the molecular basis of this disease. As a first step towards this goal, the productive infection of duck embryo fibroblasts (DEF) by MDHV was examined. The infection of DEF by MDHV led to the induction of a novel DNA polymerase. The following is a summarization of studies on the properties of this MDHV-induced DNA polymerase.

The MDHV-induced DNA polymerase could be distinguished from the DNA polymerase activities of uninfected DEF by its chromatographic behavior on phosphocellulose, by its sedimentation coefficient, and by its catalytic properties. MDHV-induced DNA polymerase eluted from a phosphocellulose column at 0.2 M KCl. The DNA polymerase activities of uninfected DEF eluted at 0.30 M KCl and 0.45 M KCl. The sedimentation coefficient of MDHV-induced DNA polymerase, as determined by sucrose density gradient centrifugation at 0.25 M KCl, was 5.9S. The DNA polymerase activities of the nuclear fraction of uninfected DEF exhibited sedimentation coefficients of 3.1S and 7.3S, while the DNA polymerase activities of the cytoplasmic fraction had sedimentation coefficients of 3.1S and 8.0S. The 3.1S DNA polymerase activity

corresponded to the DNA polymerase activity which eluted at 0.45 M KCl from phosphocellulose. The 7.3S and 8.0S DNA polymerase activities corresponded to the activities which eluted at 0.3 M KCl. It is not clear whether the 7.3S and 8.0S DNA polymerase activities are distinct or closely-related enzymes.

Several of the properties of in vitro DNA synthesis by the MDHV-induced DNA polymerase also distinguish it from the DNA polymerase activities of uninfected DEF. The MDHV-induced DNA polymerase could not effectively utilize either poly(dA)•oligo(dT) or poly(dC)•oligo(dG) as template-primers. The DNA polymerases of uninfected DEF could utilize these template-primers. MDHV-induced DNA polymerase also could not utilize poly(rA)•oligo(dT) or poly(rC)•oligo(dG) as template-primers, or oligo(dT) as a primer, indicating it was not a polymerase of the type R-DNA polymerase, a reverse transcriptase, or a terminal nucleotidyl transferase. The in vitro synthesis of DNA by the MDHV-induced DNA polymerase was more resistant to inhibition by sulfhydryl reagents than was DNA synthesis by the 7.3S or 8.0S DNA polymerase activities. The activity of MDHV-induced DNA polymerase was inhibited by 50% by the addition of 20 mM $(NH_4)_2SO_4$ to the reaction mixture. The 3.1S, 7.3S, and 8.0S DNA polymerase activities were also inhibited by $(NH_4)_2SO_4$, but higher concentrations were necessary to give 50% inhibition. Thus, with respect to a number of its catalytic and structural properties, the MDHV-induced DNA polymerase appears to be distinct from the DNA polymerase activities of uninfected DEF.

The MDHV-induced DNA polymerase presumably plays a role in MDHV DNA replication in the productively infected DEF. It was observed that the total amount of 7.3S nuclear DNA polymerase activity in DEF increased about 2.5-times after infection by MDHV. It is possible, therefore, that this enzyme may also play a role in MDHV DNA replication. It will be of interest to determine whether MDHV-induced DNA polymerase might also play a role in replication in lymphoid tumor cells of MDHV-infected chickens. If so, this enzyme might provide a potential target for a chemotherapeutic agent for controlling Marek's disease.

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