THE UNIVERSITY OF MANITOBA

PARTIAL PURIFICATION AND PROPERTIES OF RNA POLYMERASES FROM *RHODOPSEUDOMONAS SPHAEROIDES* GROWN UNDER HETEROTROPHIC AND PHOTOSYNTHETIC CONDITIONS

BY

GARY BRUCE JACOBSON

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

RNA polymerase [ribonucleoside triphosphate: RNA nucleotidl transferase, EC 2.7.7.6] was partially purified from heterotophically and photosynthetically grown *Rhodopseudomonas sphaeroides* "L" by a combination of ultracentrifugation, ammonium sulfate fractionation and DEAE-cellulose and Sephadex G-100 chromatography. The polymerases isolated from the heterotrophic cells (H-enzyme) and photosynthetic cells (P-enzyme) were purified approximately 13- and 92-fold, respectively. When assayed with [^{3}H]-CTP, both the H and P enzymes demonstrated an absolute dependence on the presence of all four nucleoside triphosphates. The P enzyme was dependent on the presence of DNA and a divalent cation for maximal activity while the H enzyme showed significant amounts of incorporation in the absence of DNA or divalent cation. Both enzymes were able to transcribe native or denatured calf thymus DNA and native DNA extracted from heterotrophic or photosynthetic *Rps. sphaeroides* with similar efficiencies. Both the H and the P enzyme activities were stimulated by the addition of KCl at a concentration of less than 0.3 M to the reaction mixture; at higher concentrations, KCl inhibited both enzymes. Both enzymes were inhibited by low concentrations of rifampicin, the H enzyme being more sensitive than the P enzyme: the 50% inhibition values for the H and P enzymes were 2.5 and 6.0 ng rifampicin per μg protein, respectively.

The H and P RNA polymerases both exhibited potent poly A and poly U polymerase activities. These homopolymer polymerase activities displayed similar kinetics to the RNA polymerase activities. The poly A polymerase activities of both the H and the P enzymes showed an absolute dependence on DNA and a divalent cation and were inhibited by rifampicin. The addition of single nucleotides to the single-NTP (poly A-synthesizing) reaction mix-

ture caused an inhibition of from 70 to 90% depending on the nucleotide and the enzyme. The P enzyme was slightly more sensitive to the inhibitory effect of single nucleotides than the H enzyme. The H and P enzymes were able to transcribe native or denatured calf thymus DNA and DNA from heterotrophic or photosynthetic *Rps. sphaeroides* in a single-NTP reaction with similar efficiencies.

The H and P enzymes were both eluted in the void volume when subjected to chromatography on Sephadex G-200 in high salt, implying molecular weights in excess of 800000. Both enzymes exhibited multiple protein bands when electrophoresed in SDS-polyacrylamide gels. The interpretation of these results with respect to the control of gene expression in non-sulfur photosynthetic bacteria is discussed.

To my parents, Anne and Nathan Jacobson

and to my wife, Ricki

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ABBREVIATIONS

ATP	adenosine 5' - triphosphate
CTP	cytidine 5' - triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DEAE	diethylaminoethyl
EDTA	ethylenediaminetetraaceticacid
GTP	guanosine 5' - triphosphate
MBA	N,N' - methylenebisacrylamide
NTA	nitrilotriacetic acid
NTP	unspecified nucleoside 5' - triphosphate
poly A	3' → 5' polymer of adenylic acid
poly C	3' → 5' polymer of cytidylic acid
poly G	3' → 5' polymer of guanylic acid
poly U	3' → 5' polymer of uridylic acid
RNase	ribonuclease
RNA	ribonucleic acid
hn RNA	heterogenous nuclear RNA
m RNA	messenger RNA
r RNA	ribosomal RNA
t RNA	transfer RNA
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N' - tetramethylethylenediamine
TES	N - tris (Hydroxymethyl)methyl-2-aminoethane sulfonic acid
TCA	trichloroacetic acid
TRIS	tri(hydroxymethyl)aminomethane
UTP	uridine 5' - triphosphate

INTRODUCTION

INTRODUCTION

Purple non-sulfur photosynthetic bacteria modulate the synthesis of their photosynthetic pigments in response to changes in light intensity and oxygen tension (Cohen-Bazire et al, 1957; Sistrom, 1962b). Changes in the ratios of synthesis of bacteriochlorophyll and carotenoids are paralled by changes in the levels of the enzymes which synthesize these pigments. Thus extracts from cultures of Rhodopseudomonas sphaeroides grown aerobically in the dark show lower levels of δ-aminolevulinic acid (ALA) synthetase and ALA dehydrase than extracts from cultures grown anaerobically in the light (Lascelles, 1959; Bull and Lascelles, 1963). The differences in rates of synthesis of pigments and in the enzyme levels between aerobically and anaerobically grown cells must reflect differential gene expression in these cells under two different growth conditions. To elucidate the molecular mechanism of this differential gene expression requires studies on the rates of synthesis and levels of nucleic acids in these bacteria.

To date, all attempts to demonstrate detectable levels of mRNA species coding for distinct heterotrophic or photosynthetic proteins in photosynthetic bacteria have failed (Gray et al, 1964; Ferretti and Gray, 1967; Yamashita and Kamen, 1968; Witkin and Gibson, 1972b; Chow, 1976a). On the other hand, there is much indirect evidence that control of gene expression in these bacteria is effected at the level of translation; this control may be mediated by the synthesis of different populations of ribosomes in heterotrophic and photosynthetic cultures (Witkin and Gibson, 1972a; Mansour and Stachow, 1975; Chow, 1976a, b and c). However, if translational control were exerted by separate ribosome populations, the question could arise as to what controlled the synthesis of the distinct components of the two ribosome populations. This control might be exerted at the level of

transcription of rRNA. Indirect evidence that heterotrophic and photosynthetic cells of the Rhodospirillales contain differences in their rRNA has been adduced (Chow, 1976c; Lessie, 1965a; Marrs and Kaplan, 1970). Thus on the basis of the present evidence, transcriptional control of gene expression in these bacteria cannot be ruled out; the synthesis of different species of rRNA in heterotrophic and photosynthetic cells would explain why attempts to detect different species of mRNA have failed.

A logical candidate for exerting transcriptional control is RNA polymerase. This enzyme activity synthesizes all classes of RNA in the cell. RNA polymerase regulates the synthesis of these RNA's by responding to molecular signals under a variety of conditions; during balanced growth, nutrient limitation, during the transition from the vegetative to the dormant phase of growth (reviewed in Losick and Chamberlin, 1976). The aim of this study was to purify and examine the properties of RNA polymerase isolated from cultures of photosynthetic bacteria that had been grown under heterotrophic or photosynthetic conditions. It was expected that, if transcriptional control was operating, differences in the physical or biological properties of the two enzymes would be manifested. In the properties examined, the two enzymes behaved in a virtually identical fashion. Poly A polymerase activity which has been found associated with RNA polymerase from other bacteria (Chamberlin and Berg, 1962; Fox and Weiss, 1964; Stevens and Hentry, 1964) was associated with the partially purified RNA polymerases. These activities were examined and found to behave similarly under a variety of conditions. Interestingly, the RNA polymerase from both the heterotrophic and photosynthetic cells had an apparent molecular weight greater than 800 000 and showed multiple protein bands when subjected to electrophoresis in SDS polyacrylamide gels. It is concluded that if there are structural and functional differences between the two enzymes

which affect transcriptional regulation of gene expression, they are too subtle to be detected by the methods employed in this study.

HISTORICAL

HISTORICAL

The Photosynthetic Bacteria

The existence, if not the identify, of photosynthetic bacteria has been known for over two thousand years. As Kondrat'eva has written, "In 208 B.C., the Roman historian Pliny write about the red waters of Lake Vulsineus in the crater of an extinct volcano near Rome." (Kondrat'eva, 1965). Many instances of bodies of water being completely or partially colored red have been recorded since then. The agents responsible for imparting the red hue have been identified as the purple sulfur bacteria. While the purple sulfur bacteria in particular, and photosynthetic bacteria in general, can be isolated from a variety of environments, they are most abundant in stagnant bodies of water where organic material is undergoing decay and hydrogen sulfide is present. It was the famous Russian microbiologist, S. Winogradsky, who provided a physiological explanation for this correlation. He proved that the purple sulfur bacteria oxidize the hydrogen sulfide to sulfur and sulfuric acid during their metabolism (Vinogradskii, 1887). Before going further with a description of the ecology and physiology of the photosynthetic bacteria, it is appropriate to consider the current state of their taxonomy.

In the sixth edition of Bergey's Manual (van Niel, 1957, 7th edition), the photosynthetic bacteria was placed into three families in the suborder *Rhodobacteriineae*, order *Pseudomonadales*. The three families were: the *Thiorhodaceae* or purple sulfur bacteria, the *Athiorodaceae* or non-sulfur purple and brown bacteria, and the *Chlorobacteriaceae* or green sulfur bacteria. The differentiation of these families was based mainly on differences in pigment composition and sulfur metabolism. Additional consideration of the type of metabolism exhibited (photosynthetic or photo-

synthetic and heterotrophic) were also used in the classification scheme. On the basis of more intensive work with pure cultures on the metabolism, physiology and pigment and DNA base composition of the photosynthetic bacteria, this classification scheme was revised in the eighth edition of the Manual (Pfennig and Truper, 1974). A separate order, the Rhodospirillales, was created for the photosynthetic (or phototrophic) bacteria. The distinction between the three Families was kept, but the names were changed; the Thiorhodaceae were named the Chromatiaceae, the Athiorhodaceae were named the Rhodospirillaceae and the Chlorobacteriaceae were named Chlorobiaceae. Although the former names are more descriptive of the color and type of sulfur metabolism of the bacteria and are the epithets used even in current literature, the latter names are those given in the Manual, which is authoritative, and should be used.

The photosynthetic bacteria, as a group, are virtually ubiquitous. Although they can be isolated from soil, their most common habitat is water, in the form of fresh or salt water, lakes or stagnant ponds. As mentioned above, the purple sulfur bacteria, the Chromatiaceae, abound in waters that are anerobic and contain decaying organic matter and hydrogen sulfide. The related presence of both decaying organic material and hydrogen sulfide is explained by the fact that the sulfur in the decomposing protein is released as sulfate which is then reduced by being used as a terminal electron acceptor during anaerobic respiration by certain chemoorganotrophic bacteria; for example Desulphovibrio spp. Another characteristic of the purple sulfur bacteria is their tolerance of high concentrations of salt. These same properties (growth in high salt, anaerobiosis, presence of decaying organic matter and hydrogen sulfide in the environment) are also characteristic of the green sulfur bacteria, the Chlorobiaceae. There are minor differences, such as pH and sulfide concentration optima,

between the two families. The major differences between the purple and green sulfur bacteria are: the composition of the photosynthetic pigments, the morphology of the (subcellular) organelles which contain the pigments, and the method of deposition of elemental sulfur granules during metabolism. The Chromatiaceae synthesize bacteriochlorophylls a or b while the Chlorobiaceae synthesize bacteriochlorophylls of type c or d (designations of Jensen et al, 1964). In the Chromatiaceae, the (internal) photosynthetic membrane system is continuous with the cytoplasmic membrane and may exist in the form of lamellae, vesicles or tubular structures. In the Chlorobiaceae, the photosynthetic pigments are located in structures called "chlorobium vesicles" which lie under and are attached to the cytoplasmic membrane. With the exception of one genus, the Chromatiaceae deposit elemental sulfur inside the cells as an intermediate in the oxidation of sulfide. In contrast, members of the Chlorobiaceae always deposit the sulfur on the outside of the cells during oxidation of sulfide. Although the green and purple sulfur bacteria are obviously distinct facilies, they can be grouped together on the basis of similar properites and contrasted with the purple non-sulfur bacteria, the Rhodospirillaceae.

The Rhodospirillaceae, of which Rhodopseudomonas sphaeroides is a member, inhabit many of the same environments as the Chromatiaceae and the Chlorobiaceae, viz., fresh and salt water, soil and stagnant water. While the environments inhabited by the three families are the same, the metabolisms exhibited by the Rhodospirillaceae on the one hand and by the Chromatiaceae and Chlorobiaceae on the other hand are fundamentally different. The purple non-sulfur bacteria are primarily photoorganotrophs and faculative anaerobes. That is, they grow photosynthetically in the presence of simple organic molecules which they photoassimilate and/or use as electron donors for carbon dioxide reduction. Most species of the Rhodospirillaceae

can also use hydrogen gas as an electron donor for the reduction of carbon dioxide, i.e. they are also capable of growing photolithotrophically. Except for Rhodopseudomonas palustris, the purple non-sulfur bacteria cannot use thiosulfate or elemental sulfur as electron donors during carbon dioxide assimilation. They do not, therefore, form or deposit elemental sulfur during growth. Most species can grow either under anaerobic conditions in the light (phototrophic growth) or under aerobic conditions in the dark (heterotrophic growth). These last two properties-inability to use sulfur as an electron donor and facultatively anaerobic growth - distinguish the Rhodospirillaceae from the Chromatiaceae and Chlorobiaceae since the latter two are capable of using sulfur as an electron donor for carbon dioxide reduction, do deposit elemental sulfur during growth and, with the exception of one microaerophilic genus in the Chromatiaceae, are obligate anaerobes.

The photosynthetic bacteria thus comprise a group of metabolically diverse organisms. This diversity and the relative ease in obtaining large quantities of cells has incited intensive research over the last forty-five years. This research, in turn, has afforded a wealth of information on various aspects of the biology of these interesting organisms. One of these aspects, discussed at length below, is the adaptive responses of these organisms to changes in light intensity and oxygen tension.

One of the first comprehensive and quantitative studies on the kinetics of pigment formation under various conditions by photosynthetic bacteria was carried out by Cohen-Bazire and her coworkers (Cohen-Bazire et al, 1957). They, and succeeding workers, limited their studies to a few species in the Rhodospirillaceae. In contrast to the photosynthetic sulfur bacteria, the photosynthetic non-sulfur bacteria offered several technical advantages:

(1) They were faculative anaerobes, able to carry out heterotrophic

(aerobic) metabolism or photosynthetic (anaerobic) metabolism.

(ii) Most species adapted readily from one type of metabolism to the other.

(iii) They grew relatively rapidly and homogeneously (i.e. they didn't clump, thus facilitating optical density and viability measurements) in a variety of complex and synthetic media thus,

(iv) large quantities of macromolecules and organelles could be purified from the cells in a relatively short period of time.

Cohen-Bazire et al (1957) demonstrated that Rps. sphaeroides and R. rubrum adjusted their cellular contents of photosynthetic pigment (bacteriochlorophyll and caroteroids) to the prevailing light intensity and oxygen tension. Cultivation of the cells under high light intensities led to a reduction in the pigment content relative to cells cultivated under low light intensities. When the light intensity was altered in a growing culture, the cells adjusted their pigment content accordingly by either increasing (under low light intensity) or decreasing (under high light intensity) the rate of pigment synthesis until a steady-state level characteristic of the new light intensity had been achieved. Introduction of air into a photosynthetically growing culture resulted in a complete cessation of pigment synthesis but not of growth. Although light was a prerequisite for photosynthesis in these bacteria, it was not necessary (or sufficient) for pigment synthesis. Cultivation of Rps. sphaeroides under semi-anaerobic conditions in the dark led to the cells turning red. Extraction of the cells with organic solvents (e.g. methanol-acetone) showed that some carotenoids but very little bacteriochlorophyll were synthesized under these conditions.

In an attempt to explain their results, Cohen-Bazire et al (1957) hypothesized that the steady-state oxidation level of a regulatory carrier

molecule in the electron transport chain controlled the rate of synthesis of the photosynthetic pigments. They suggested that an increase in light intensity led to an increased rate of water photolysis. Water photolysis, according to van Niel (1941), resulted in the formation of oxidized and reduced products represented symbolically as (OH) and (H). Thus an increase in light intensity would lead to an increase in the steady-state level of (OH). This increase in the level of (OH) resulted in a flow of electrons towards (OH) causing a shift in the carriers to a more oxidized state. Introduction of oxygen into the cells would have the same effect since oxygen, like (OH), can act as a terminal electron acceptor. Since pigment synthesis stopped under conditions of high light intensity or aeration, the authors proposed that the ratio of oxidized: reduced carrier controlled the rate of pigment synthesis; the greater the ratio, the lower the rate of synthesis and vice-versa. To rationalize the different rates of carotenoids and bacteriochlorophyll synthesis under aerobic or dark semi-anaerobic conditions, they suggested that the two rates of synthesis must have been controlled by different critical oxidized: reduced carrier ratios.

Lascelles (1959) and Bull and Lascelles (1963) carried out experiments on growing cultures and resting cell suspensions of *Rps. sphaeroides*. They showed that the activities of two of the enzymes responsible for pyrrole synthesis changed in a manner parallel to that of the pigments. These two enzymes were: δ-aminolevulinic acid (ALA) synthetase, which catalyzes the synthesis of ALA from glycine and succinyl CoA, and ALA dehydrase, which synthesizes the monopyrrole porphobilinogen from two molecules of ALA. These two enzymes partake in a branched biosynthetic pathway which diverges after the synthesis of protoporphyrin; one branch leads to the synthesis of heme and of the cytochromes while the other branch leads to the synthesis of bacteriochlorophyll. Lascelles (1959) showed that the activities of these two enzymes increased in cultures undergoing adaptation to form bacterio-

chlorophyll. Also the activities of these enzymes in cell-free extracts of bacteria grown photosynthetically were four to five times higher than in extracts of bacteria grown heterotrophically. Using inhibitors of protein and nucleic acid synthesis, such as p-fluorophenylalanine, 8-azaguanine, 5-fluorouracil and ethionine, they showed that, in cultures incubated in the dark under low oxygen tension or incubated in the light anaerobically, the synthesis of bacteriochlorophyll and of ALA synthetase was inhibited. The results implied that the synthesis of photosynthetic pigments was dependent upon continued protein synthesis.

Sistrom (1962 a), in studies on growing cultures of Rps. sphaeroides, inhibited protein synthesis in the cultures by adding chloromycetin or by sulfur starvation. His results showed that bacteriochlorophyll and carotenoid synthesis could not proceed in the absence of protein synthesis. He also found that there was negligible protein turnover (as measured by the release of [^{14}C]-phenylalanine from acid-precipitable material) after inhibition of protein synthesis. Sistrom cautioned against interpreting these experiments and those of Lascelles (1959) as proving that the inhibition of pigment synthesis was due to the inability of the cultures to form the enzymes necessary for pigment synthesis. In both sets of experiments, the cultures had been growing for several hours under photosynthetic conditions before the inhibition of protein synthesis and therefore must have contained an adequate level of the requisite enzymes. On the other hand, Bull and Lascelles (1963) measured the decline in ALA synthetase activity in cultures which had been allowed to attain a high content of the enzyme. They then stopped growth by the addition of 8-azaguanine, 5-fluorouracil or p-fluorophenylalanine. They found that after the addition of the inhibitors the ALA synthetase activity dropped to 25-50% of its original activity. They concluded that the anti-metabolites affected the

enzyme activity or another factor concerned with pigment synthesis.

Despite the insights into the biosynthetic pathways of pigment synthesis and the relationships between light intensity, oxygen tension and pigment synthesis that were gained, it was obvious that the above experimental approaches were inadequate for answering the fundamental molecular biological problems concerning the control of gene expression that were posed by these bacteria. Interest shifted, then,to the nucleic acid metabolism of these organisms during adaptation to photosynthetic or heterotrophic conditions; particularly to the detection of specific classes of mRNA.

Lessie (1965 b) showed that in photosynthetically growing cultures of Rps. sphaeroides that had been subjected to an increase in light intensity, and thus had stopped synthesizing bacteriochlorophyll, the rate of total RNA synthesis suddenly increased. Conversely, bacteria subjected to a decrease in light intensity appeared to stop synthesizing RNA. The RNA synthesis was measured by withdrawing aliquots of the culture at timed intervals and measuring them for their orcinol-reactive material (Schneider, 1945). Labeling experiments showed that during a period when RNA synthesis had appeared to cease, incorporation of [^{14}C]-uracil into TCA-precipitable material increased, indicating that actual synthesis was occurring. Pulse-chase experiments showed that an unstable RNA fraction was rapidly synthesized. This rapidly-labeled RNA showed a heterogenous size distribution on sucrose gradients. Thus he showed that, in response to conditions which cause an increase in pigment synthesis and an apparent cessation of net RNA synthesis, Rps. sphaeroides nevertheless synthesized an RNA fraction which had some of the properties of mRNA.

Since the quantity of structural and catalytic proteins specifically concerned with photosynthetic metabolism increased in bacteria transferred

from dark aerobic to light anaerobic conditions, it was logical to assume that the concentrations of mRNA molecules coding for these proteins also increased. In an attempt to detect changes in the compositionof rapidly-labeled RNA, Gray _et al_ (1964) used the techniques of base composition analysis. They examined the composition of total, ribosomal and soluble RNA in wild-type cells of _Rps_. _sphaeroides_ that had been grown under aerobic or anaerobic conditions. They also attempted to find differences in RNA base composition of aerobically and anaerobically grown cells of a catalase-constitutive mutant of _Rps_. _sphaeroides_. The base compositions of the RNA's extracted from these cells, grown under a variety of conditions, were striking only in their lack of variance. It should be mentioned that they did not fractionate the RNA beyond isolating "soluble" and "ribosomal" RNA. Furthermore, their "rapidly-labeled" RNA had been labeled for five minutes, which may have been much longer than the half-life of the average mRNA specie.

In a similar study, Ferretti and Gray (1967) examined the nucleotide composition of RNA's extracted from a culture of _Rps_. _sphaeroides_ undergoing a transition from dark-aerobic to light-anaerobic conditions. Again no discernible difference was evident in the base composition of the RNA's isolated at different times during adaptation. They concluded that if there were changes in the mRNA population, they were undetectable by the methods employed.

In a later study, Gray (1967) examined the synthesis of DNA, RNA and protein in _Rps_. _sphaeroides_ undergoing a transition from dark-aerobic to light-anaerobic conditions. He found no change in the cellular levels of these macromolecules during the transition period although there were large fluctuations in the incorporation rates of their precursors which he attributed to the restricted energy supply available during adaptation. He

also attempted to measure changes specifically in the mRNA levels by labeling RNA with [^{14}C]-uracil (for five minutes), isolating it and hybridizing it to cellular DNA and calculating the per cent of radioactivity present in RNase-resistant hybrids. He found a small but reproducible abrupt increase in the percentage of resistant hybrids immediately after transfer to light anaerobic conditions (from 2.8 to 3.1% of the total radioactivity in the hybridization mixture).

The technique of hybridization competition, which detects differences in base sequences of nucleic acids rather than differences in base composition, was employed by various groups in an attempt to detect differences in mRNA classes between dark-aerobic and light-anaerobic cells of R. rubrum (Yamashita and Kamen, 1968; Chow, 1976a) of Rps. sphaeroides (Witkin and Gibson, 1972b). In addition to chromosomal DNA, Witkin and Gibson (1972b) also tested RNA species for hybridization to satellite (extrachromosomal) DNA (Gibson and Niedermann, 1970). No differences in the abilities of unlabeled RNA from either aerobically or anaerobically grown cells to compete with labeled RNA for sites on DNA from aerobically or anaerobically grown cells could be detected. It appeared that there were no specific mRNA species synthesized by photosynthetic bacteria when adapting to different culture conditions. In a later report, Yamashita and Kamen (1969) noted that light exerted a stimulatory effect on uracil incorporation in cultures of R. rubrum transferred from the dark. From pulse-chase and sucrose gradient centrifugation experiments, they concluded that this light-stimulated uracil incorporation may have represented mRNA synthesis associated with chromatophore and bacteriochlorophyll synthesis. However, no evidence was adduced that unequivocably demonstrated the presence of such mRNA.

Witkin and Gibson (1972a) attempted to detect differences in mRNA populations between heterotrophic and photosynthetic cultures of Rps. sphaeroides

by examining the rates and extents of degradationof mRNA. The cultures were labeled for thirty seconds with [^{3}H]-uracil and then rifampicin and cold uracil were added to inhibit specifically any further initiation of RNA synthesis. The decrease in acid-precipitable radioactivity was followed. They found that the unstable RNA from steady-state aerobic cultures had a different half-life and extent of degradation from the unstable RNA of a steady-state anaerobic culture. Similar differences were noted between cultures undergoing aerobic to anaerobic transitions and those undergoing anaerobic to aerobic transitions. They also found that addition of puromycin or chloramphenicol to the cultures affected the extents of degradation. They concluded that a difference in the ribosome populations of heterotrophic and photosynthetic cultures existed and that an "air-sensitive component" was associated with the heterotrophic but not the photosynthetic cells.

Pursuing this line of experimentation, Chow (1976a) studied the degradation of pulse-labeled RNA in aerobic and anaerobic cultures of R. rubrum. He found that pulse-labeled RNA from aerobic but not from anaerobic cultures underwent a secondary degradation about twenty minutes after the addition of rifampicin and cold uracil. On the basis of these and other experiments on rRNA instability after rifampicin treatment (Yuan and Shen 1975, Chow, 1976a), he concluded that the observed secondary degradation was due to the breakdown of rRNA, not mRNA and that therefore the ribosomes of heterotrophic cells were less stable than those of photosynthetic cells.

To study these differences further, Chow developed an in vitro protein synthesizing system for extracts of heterotrophic and photosynthetic R. rubrum. In the relatively crude S-30 system, (which was obtained by centrifuging a clarified cell extract at 30,000 x g to obtain a crude ribosomal pellet, and a supernatant fluid as a source of soluble factors) the

heterotrophic and photosynthetic protein synthesizing systems showed different responses to polyamines, $MgCl_2$, and biotin (Chow, 1976b). In the more refined S-100 system (Chow, 1976c), (which was obtained by centrifuging the S-30 fraction at 60,000 x g and then centrifuging the S-60 fraction at 100 000 x g) various combinations of ribosomes and S-100 fractions from heterotrophic (H) or photosynthetic (P) cell extracts were tested for activity. The HS-100 fraction obtained from stationary-phase cultures gave very low activityywhen tested in combination with ribosomes from either H or P extracts of R. rubrum in logarithmic or stationary phase of growth. Similar low activity was obtained when HS-100 was tested with E. coli ribosomes. All other combinations of ribosomes and supernatant fluids resulted in much higher incorporation. When HS-100 was added to a homologous system of P ribosomes and PS-100, a significant inhibitory effect was observed, with 50% inhibition occurring with about 10 μg of HS-100 protein. Extensive physiocochemical studies on this HS-100 fraction revealed that both inhibitor and activator activities of protein synthesis were associated with it. The activator activity was concluded to be due to a small molecular weight RNA molecules which was associated with the H ribosomes in their native state. The inhibitor activity was concluded to be due to an oligoribonucleotide or a very short sequence of double-stranded RNA which hypothetically arose from the activator molecule, perhaps by cleavage. In addition to these and other functional differences, structural differences between the H and P ribosomes and S-100 fractions were also demonstrated by analysis of their proteins on SDS and urea polyacrylamide gels.

Structural differences between the ribosomal proteins of the 29S subunit of aerobically and anaerobically grown Rps. palustris have been demonstrated by Mansour and Stachow (1975). From their data they concluded that Rps. palustris possessed two distinct ribosomal populations, one for aerobic

growth conditions and one for anaerobic growth conditions. Furthermore, they showed that during an anaerobic to aerobic transition, the "anaerobic ribosome population" appeared to be degraded before the "aerobic ribosome population" was synthesized. Heterotrophic cells of Rps. sphaeroides have been shown to lack mature 23S rRNA (Lessie, 1965a, Marrs and Kaplan, 1970), a normal component of the 50S ribosomal subunit in all other procaryotic organisms.

It is clear that the evidence to date is strongly in favor of some mechanism of translational control operating in photosynthetic bacteria. On the other hand, there has also been much evidence, of a negative variety, against transcriptional control playing a role in gene expression. This evidence has consisted of the failure to detect species of mRNA specific for photosynthetic or heterotrophic cultures. This type of evidence, of course, does not prove that transcriptional control does not operate in photosynthetic bacteria. Although the data presented in this thesis do not show any significant functional or structural differences between the RNA polymerases of heterotrophic and photosynthetic cells of Rsp. sphaeroides, recently Chow (1977) has found that hetertrophic cells of R. rubrum contained three chromatographically distinct species of RNA polymerase activity. In addition to their RNA polymerase activities, these partially purified enzymes had a poly A polymerase activity associated with them. These poly A polymerase activities demonstrated different kinetics and different responses to Mg++ concentration than their associated RNA polymerase activities. Further studies on these enzymes may yet uncover a mechanism of transcriptional control in these bacteria.

The combined approach of genetics and biochemistry to problems in microbiology has always proved more fruitful than either discipline alone. Recently an agent which transfers segments of the chromosome of Rps. cap-

sulata has been found in culture filtrates of the bacteria (Marrs, 1974; Solioz *et al*, 1975). Using these gene transfer agents (GTA), Yen and Marrs (1976) have constructed a preliminary map of the genes for carotenoid and bacteriochlorophyll synthesis. Mutants in a wide range of functions, especially photosynthetic functions, are relatively easy to obtain in *Rhodopseudomonas* species (Griffiths and Stanier, 1956; Yen and Marrs, 1976). The use of these mutants combined with theGTA recombination system should facilitate the elucidation of the control of gene expression in photosynthetic bacteria.

RNA POLYMERASE

Introduction

In bacteria, the enzyme that mediates the transcription of genetic information from DNA to RNA is DNA-directed RNA polymerase (ribonucleoside triphosphate: RNA nucleotidyltransferase, EC 2.7.7.6), commonly referred to as RNA polymerase. This enzyme catalyzes the synthesis of a single-stranded RNA molecule from its nucleoside triphosphate precursors, ATP, GTP, CTP and UTP. The central role of RNA polymerase in the regulation of gene exprssion has made it the object of intensive research over the past fifteen years. This research has led to a broad understanding of the coarse and fine mechanisms of control that the bacterial cell employs to regulate and coordinate its macromolecular syntheses.

The first reports of an enzyme activity able to incorporate a labeled ribonucleotide into an acid-insoluble RNA-like polymer were by Grunberg-Mango and Ochoa (1955) and Grunberg-Manago _et al_ (1955). The enzyme, present in extracts of _Azotobacter vinelandii_, was named polynucleotide phosphorylase. It was originally believed that this enzyme was involved in the flow of genetic information from DNA ultimately to protein. However, the lack of requirement by the enzyme for a DNA template, the freely reversible nature of the reaction and the fact that the polymer product bore no resemblence to cellular RNA cast doubt on this belief. The idea that polynucleotide phosphorylase was responsible for the biosynthesis of RNA was discarded in 1959 when Weiss and Gladstone reported the presence of an enzyme activity in rat liver nuclei capable of synthesizing RNA from [^{32}P]-labeled CTP and the three other ribonucleoside triphosphates. This activity was sensitive to DNase, and therefore template dependent, and showed optimal activity in the presence of all four nucleoside triphosphates. In contrast, polynucleo-

tide phosphorylase showed no template dependence and could synthesize an acid-insoluble polyribonucleotide in the presence of one, two, three, or four nucleoside diphosphates (Grunberg-Mango et al, 1955, 1956). Later, Weiss was able to demonstrate the presence of a DNA-dependent RNA polymerase activity in extracts from the bacterium Micrococcus luteum (formerly M. lysodeikticus; Weiss and Nakamoto, 1961). Other laboratories subsequently reported the presence of an RNA polymerase activity in extracts of other organisms (Hurwitz et al. 1960; Stevens, 1960; Huang et al, 1960; Chamberlin and Berg, 1962; Furth et al, 1962; Stevens and Henry, 1964). RNA polymerase was shown to catalyze a reaction formally analogous to the original DNA polymerase discovered by Kornberg (Kornberg et al, 1956) which utilized a DNA template and all four deoxyribonucleoside triphosphates to form DNA strands complementary to the template strands. It is now known that RNA polymerase in its various forms in procaryotic and eucaryotic cells is responsible for the synthesis of all cellular RNA: hnRNA, mRNA, tRNA and rRNA. In addition much evidence has been adduced recently for the presence of a unique RNA polymerase activity in bacterial and animal cells infected with very small DNA viruses. This RNA polymerase apparently synthesizes an oligonucleotide RNA primer complementary to a template DNA strand immediately prior to a round of DNA synthesis. It is currently thought that synthesis of this oligonucleotide primer may be a prerequsite to the initiatio rof DNA synthesis in all organisms (see Kornberg, 1976).

Studies on the molecular mechanism of the RNA polymerase reaction and its regulation obviously require large amounts of highly purified enzyme. There have been several published procedures for the purificaton of RNA polymerase from a vareity of sources. Some of these procedures and the problems attending them will be briefly disucssed.

Until a few years ago, the method of choice for obtaining highly

active preparations of RNA polymerase in relatively high yield has been that of Chamberlin and Berg (1962). Using E. coli, they lysed the cells by grinding with glass beads in a Waring Blendor. The nucleic acids were precipitated by the addition of streptomycin sulfate and the polymerase was then precipitated with protamine sulfate. Ammonium sulfate fractionation and DEAE-cellulose chromatography completed the purification. The streptomycin and protamine sulfates, being natural products, were inherently subject to variability in their preparation and their use sometimes led to considerable losses in polymerase activity.

To avoid the use of streptomycin and protamine, Babinet (1967) employed a phase partition system. In this procedure nucleic acids were removed by repeated partitioning between polyethylene glycol and dextran. While this method avoided the use of streptomycin and protamine, the phase partitioning was time consuming and required the centrifugation and dialysis of large volumes of solution especially if attempted on a large scale.

In order to streamline the purification procedure and obtain purer enzyme, Burgess (1969 a) devised a procedure which involved a mild DNase treatment of an extract of E. coli. In addition to partially reducing the viscosity of the extract, the DNase degraded enough DNA to release the polymerase from the ternary complex (DNA:RNA:polymerase) in which form it was likely to be sedimented with ribosomes and other cellular debris. This procedure eliminated the necessity for streptomycin and protamine or phase partitioning. Reverse ammonium sulfate fractionation, DEAE-cellulose and phosphocellulose chromatography followed by agarose gel filtration chromatography constituted the remainder of the purification steps. The phosphocellulose chromatography step removed the sigma subunit from the holoenzyme (Burgess et al, 1969). In order to prepare holoenzyme Burgess (1969 a) substituted alternate centrifugations in low and high salt agarose columns

instead of the phosphocellulose step. These additional sizing procedures resulted in enzyme which was greater than 98% pure and which showed only four subunits (α, β, β', and σ) upon electrophoresis in SDS polyacrylamide gels.

As purer preparations of RNA polymerase holo- and core enzyme became available, the complexity of the different phases of the reaction catalyzed by the enzyme became apparent. The presence of even trace levels of contaminating nucleic acids, proteases, nucleases or enzymes that mimicked the activity of RNA polymerase became less acceptable. Thus Chamberlin (1974) criticized Burgess' procedure for its use of DNase and the large number of sizing steps.

In 1968, Alberts and co-workers described the preparation and use of DNA-cellulose columns for studying DNA-binding proteins. In 1970, Zillig and co-workers described the use of a synthetic polymer, Polymin P (a polyethyleneimine), for the precipitation of nucleic acids and RNA polymerase from extracts of _E. coli_. The synthetic nature of this quarternary amine compound made it less subject to variability than streptomycin and protamine. The nucleic acids and polymerase were easily separated by differential elution from the precipitate with buffers of increasing ionic strength.

Recently, Burgess and Jendrisak (1975) have combined these two methods with others into a rapid large-scale purification procedure for _E. coli_ RNA polymerase. This improved method employed lysozyme and SDS to lyse the bacteria, shearing of the DNA in a Waring Blendor to reduce viscosity followed by Polymin P precipitation and elution similar to that described by Zillig _et al_ (1970). Chromatography on DNA-cellulose followed by chromatography on a high-salt agarose column completed the procedure. The yield was about 45% and the enzyme was purified about 800-fold from the activity

in the crude extract. The Polymin P precipitation and elution achieved a significant purification prior to the subsequent steps and should find use in the purification of RNA polymerase from other organisms. In fact, Jendrisak and Burgess (1975) employed Polymin P in a modification of the above procedure to purify RNA polymerase II from wheat germ. Their method resulted in an impressive purification of 4000-fold and a yield of about 60%.

Structure of RNA Polymerase

RNA polymerase from *E. coli* has been characterized to a greater extent than other bacterial RNA polymerases so the following discussion will be limited mainly to the *E. coli* enzyme.

E. coli RNA polymerase is an oligomeric protein which can be resolved into four major subunits by electrophoresis in denaturing gels or by gel chromatography in a denaturing buffer. The denaturant is usually SDS or urea. The subunits are referred to as: β'(165,000), β (155,000), σ (86,000) and α (39,000) where the numbers in parentheses refer to the approximate molecular weight (Burgess, 1969 b). Functional polymerase consists of two types of oligomers: the holoenzyme, which has the stoichiometry 2α, β, β' and σ, and the core enzyme which has the stoichiometry 2α, β, β'. The sigma subunit can be removed from the holoenzyme by chromatography on phosphocellulose (Burgess *et al*, 1969). Core enzymes can transcribe native calf thymus DNA almost as efficiently as holoenzyme. However, the ability of holoenzyme to transcribe native T4 phage DNA is virtually abolished when sigma is removed. Addition of sigma back to core enzymes restores its ability to transcribe T4 DNA efficiently (Burgess *et al*, 1969). On the basis of these results and the results of other experiments, Travers and Burgess (1969) proposed that sigma was required for the initiation of RNA synthesis at correct promoter sites. They suggested that transcription in the absence of sigma was due to mistakes in initiation. Finally, they proposed that sigma functioned only to select the proper initiation sites for the polymerase molecule; once initiation of the proper site had been accomplished, sigma was released from the enzyme-DNA complex and could be reused by another core polymerase (Travers and Burgess, 1969).

The resolution of the function of sigma led to a perplexing realization, namely: since sigma could be lost from the enzyme by a relatively

mild purification step, it was possible that other loosely bound "factors", necessary for transcriptional specificity, could also be lost from the *in vivo* form of the enzyme during purification. On the other hand, purified factors which increased RNA synthesis when added to *in vitro* assays with core or holoenzyme would not necessarily constitute an intrinsic component of the polymerase. For example, when *B. subtilis* was infected with phage SP01, a host protein named delta was found associated with the RNA polymerase in addition to phage-specified polypeptides. Delta appeared to alter the transcriptional specificity of the polymerase (Pero, Nelson and Fox, 1975). In most phage infections, phage-specified proteins have been found associated with bacterial RNA polymerase where they apparently play a role in enabling host polymerase to initiate synthesis at correct promoter sites on phage DNA (Losick and Pero, 1976). Thus the problem is a semantic as well as a biochemical one. It is difficult, as Chamberlin (1976) has pointed out, to decide whether a modified polymerase has had its specificity for transcription altered or simply lost.

Molecular weight determinations of purified core or holoenzyme are complicated by the fact that the enzyme aggregates at low ionic strengths (< 0.1). Detectable aggregation can even occur at an ionic strength of 0.2. The high salt concentrations, necessary for assuring dissociation of the aggregates into single promoters, complicate molecular weight determinations in analytical ultracentrifuges due to the substantial solvent corrections which are necessary (Chamberlin, 1974). In addition, there is some inevitable breakdown of holoenzyme into core enzyme and sigma. According to Chamberlin (1976), *E. coli* polymerase has an $S_{20,W}$ value of 12.6 at ionic strengths above 0.28; its calculated molecular weight is 383,000. This is in good agreement with the value of 390,000 $\pm$ 10% which was calculated from adding up the molecular weights of the α, β and β' subunits (in

the ratio 2:1:1) obtained from gel electrophoresis experiments (Burgess, 1969 b; Berg and Chamberlin, 1970). The moelcular weight of holoenzyme obtained from these gel electrophoresis experiments was 480,000 ± 10%.

The subunit patterns of RNA polymerase obtained from other bacteria are generally similar to the subunit pattern of *E. coli* RNA polymerase (Table I). The RNA polymerases from *Bacillus spp*. have similar subunits to the *E. coli* polymerase, however the molecular weight of the *Bacillus* subunit that appears to correspond to the *E. coli* sigma is much lower (Maia *et al*, 1971; Losick *et al*, 1970; and Hermoso *et al*, 1972).

Catalytic Properties

Purified bacterial RNA polymerase has been shown to be able to catalyze three distinct types of polymerization reaction:

(i) the template-dependent synthesis of a complementary polyribonucleotide.

(ii) the template-dependent synthesis of homopolymers or reiterated copolymers.

(iii) the template-independent synthesis of [poly (A): poly (U)] or poly (IC).

The first reaction is characteristic of RNA polymerase activity *in vivo*. It has been the most widely studied of the reactions catalyzed by RNA polymerase. A recent review by Chamberlin (1976), summarizes the current state of knowledge about this important reaction.

The second reaction was discovered during early studies of RNA polymerase. That the reaction catalyzed by the enzyme was nucleotide-dependent was shown by appropriate controls from which one, two or three unlabeled nucleotides were omitted from the reaction mixture. Surprisingly, omission of three nucleotides sometimes resulted in a greater amount of incorporation

Table I

Subunit Structure of RNA Polymerase From Various Organisms[a]

Organism	β	β' (MW x 10^{-3})	σ	α	Reference
Escherichia coli	165	155	95	39	Burgess (1969 b)
Bacillus subtilis	155	154	56	43	Avila *et al*, (1971)
Pseudomonas putida	165	155	98	44	Johnson *et al*, (1971)
Caulobacter crescentus	165	155	101	44	Bendis and Shapiro (1973)
Acinetobacter calcoaceticus	150	np[b]	np	37	Kleppe and Kleppe (1976)
Rps. sphaeroides	~160[c]	~160[c]	95	39	This study

[a] modified from Burgess (1976).

[b] not present or not separated on gels.

[c] co-migrated with *E. coli* $\beta + \beta'$ which were not clearly separated.

than when all four nucleotides were present (Chamberlin and Berg, 1962; Fox and Weiss, 1964; Stevens and Henry, 1964). A detailed study of this phenomenon was undertaken and a possible mechanism for the reaction was proposed (Chamberlin and Berg, 1964). It was shown that purified preparations of RNA polymerase could catalyze the polymerization of (labeled) ATP into polyriboadenylic acid. The reaction was dependent on the presence of a single-stranded DNA template and the length of the product was found to be 5 to 10 times greater than the length of the dTMP sequence that served as the template. Low (micromolar) concentrations of other ribonucleoside triphosphates could inhibit the polymerization reaction. Chamberlin and Berg (1964) proposed that slippage of the growing poly A chain from the dTMP residues continually exposed the ultimate dTMP residue in the sequence and that this led to a reiterative synthesis of poly A. The proposed mechanism for the high efficiency inhibition by micromolar concentrations of NTP's was based on studies with defined synthetic templates (dI, dC, dT, dAT polymers) and apurinic acid. They concluded that high efficiency inhibition only occurred if a nucleotide complementary to the inhibiting nucleotide was present adjacent to the poly T seuqence. Thus, if the template sequence was ...TTTTC..., only GTP would efficiently inhibit the synthesis of poly A. While the *in vivo* function, if any, of this synthesis is still obscure, it is known that there are at least two forms of *E. coli* RNA polymerase holoenzyme synthesized *in vivo* (Travers and Buckland, 1973; Fukuda *et al*, 1974) and that one of these forms is solely responsible for the unprimed synthesis of [poly (A)] [poly (U)] (see below) and the DNA-primed synthesis of poly (A) (Iwakura *et al*, 1974).

The significance of the third reaction is also unclear. In the presence of Mn++, ATP and UTP and in the absence of detectable template, purified *E. coli* RNA polymerase will synthesize a homopolymer duplex, [poly (A)] :

[poly (U)] . The reaction has a lag period of from 30 minutes to 2 hours depending on enzyme and substrate concentration (Smith et al, 1967; Mehrotra and Khorana, 1965). In Micrococcus luteus, a similar activity is present (Fox et al, 1963). In Azotobacter vinelandii and E. coli, purified polymerase catalyzed the unprimed synthesis of a poly (IC) copolymer from rITP and rCTP; rGTP did not substitute for rITP. Similar to the [poly (A)]:[poly (U)] reaction, the poly (IC) reaction had an absolute requirement for Mn^{++} and took place only after a lag period of abouy 15 to 30 minutes (Krakow and Karstadt, 1967).

Rifampicin Interaction with RNA Polymerase

Rifampicin is a chemical derivative of rifamycin SV, an antibiotic synthesized as a byproduct of fermentation by cultures of Streptomyces mediterranei (Margalith and Pagani, 1961). Rifampicin has found wide-spread use in the study of RNA synthesis due to the fact that it specifically inhibits RNA chain initiation although this belief has recently been challenged (Johnston and McClure, 1976; Kessler and Hartmann, 1977). In addition, mutants which are resistant to rifampicin have been isolated and in most of the cases the resistance has been shown to be due to an altered RNA polymerase (reviewed by Riva and Silvestri, 1972). By means of electrophoresis and ultracentrifugation experiments, Zillig and co-workers were able to establish that rifampicin binds to the β subunits which have different electrophoretic mobilities from wild-type (sensitive) β subunits (Zillig et al, 1970; Heil and Zillig, 1970).

The filter-binding assay developed by Hinkle and Chamberlin (1972) has been used to study the kinetics of rifampicin inhibition and the precise point in the sequence of initiation events at which rifampicin inhibits. Other techniques for measuring rifampicin inhibition kinetics include

fluorescence quenching titration (Bähr _et al_, 1976) and the binding of free rifampicin to a suspension of dextran-coated charcoal (Wehrli _et al_, 1976). It has been established that rifampicin does not inhibit sigma-dependent recognition of initiation sites (Hinkle _et al_, 1972; Bordier, 1974) and that the binding between the drug and enzyme is reversible and non-covalent (Wehrli _et al_, 1976). The various kinetic and thermodynamic constants of the association and dissociation reactions have also been determined (Bähr _et al_, 1976; Wehrli _et al_, 1976). However, the mechanisms of binding has proved to be very complex and as yet no explicit model of the binding of the drug to the enzyme has been proposed.

RNA Polymerase and the Control of RNA Synthesis

Regulation during transcription occurs during all phases of the catalytic process. Regulatory mechanisms involving antitermination or modification of polymerase were discovered during attempts to investigate the mechanism of RNA synthesis during normal or balanced growth. The irreversible commitment of bacteria towards phage production and the responses of the transcription machinery to nutrient limitation were experimental systems that have been examined in the hope of inferring ideas about the regulation of transcription in uninfected bacteria during nutrient sufficiency. However, the regulationof RNA synthesis in response to drastic or mild changes in environmental composition is itself of obvious importance and is discussed in the following paragraphs.

Certain facets of RNA synthesis regulation have been supported by so much experimental evidence that they are now elements of "dogma" in molecular biology. Thus the operon model of Jacob and Monod (1961) is now an established fact of transcriptional regulation of gene expression in procaryotes. Their model of contigous regulatory and structural genes

comprising an operon has been supported by a wealth of experimental evidence.

The control mechanisms of RNA synthesis in individual operons may be termed "fine" control; each operon responds uniquely and separately to changes in different metabolite concentrations. In addition to this fine control, bacteria have been found to exert coordinate control of the overall rates of stable and certain unstable (messenger) RNA synthesis. This level of control may be termed "coarse" control (Travers, 1971). The classic example of coarse control of RNA synthesis is the stringent and relaxed control of transcription in *E. coli* (Sands and Roberts, 1952). When met^- mutants of *E. coli* were starved for methionine, protein synthesis, as expected, was curtailed but in addition net RNA synthesis also decreased. Since the cells had been allowed to grow before starvation and had therefore accumulated functional polymerase, it appeared that an active inhibition of RNA synthesis had occurred. Another formal possibility was that an increase in the rate of degradation of RNA or polymerase had occurred. Presumably though, this latter possibility would have had to have been due to increased protease activity or synthesis. Since protein synthesis had been inhibited, the latter possibility was unlikely and the former one was explored. This reduction in RNA synthesis in response to an environmental stress (deprivation of an essential amino acid) was dubbed the "stringent response". It is now known that this stringency is due to the action of the *rel* gene product which acts at the ribosome to synthesize guanosine tetraphosphate, ppGpp. This tetraphosphate accumulates after amino acid starvation in rel+ wild type cells but not in rel^- mutants. In rel^- mutants, RNA synthesis is not reduced when protein synthesis is curtailed by amino acid starvation (reviewed by Edlin and Broda, 1968; Ryan and Borek, 1971). During the stringent response RNA synthesis is inhibited in a non-coordinated manner. It was found, for example, that the synthesis of certain

species of mRNA continued unabated while the synthesis of ribosomal and transfer RNA was drastically reduced (Morris and Kjelgaard, 1968; Stubbs and Hall, 1968). In other systems, the synthesis of mRNA was reduced upon imposition of stringent conditions (Gallant et al, 1971). The stringent response can also be invoked in both rel^+ and rel^- strains by shifting the culture from a rich to a poor medium, such as from nutreint broth to minimal-salts glucose. Such shift experiments have demonstrated that a greater fractionof the mRNA population is subject to stringent control in bacteria growing in a nutritionally rich medium than in a nutritionally poor one (Lazzarini and Dahlberg, 1971; Stamato and Pettijohn, 1971; Donini, 1972).

What is the molecular mechanism(s) that operates to limit RNA synthesis during environmental stress? Just as they have in the mechanisms of fine control, small molecular weight effector molecules and configurational changes in the DNA template and RNA polymerase have been implicated. The promoter sites for RNA polymerase binding can undergo a transition from a closed to an open conformation in a reaction which occurs over a narrow temperature range (Travers et al, 1973). The polymerase, as mentioned in preceeding sections, can undergo conformational changes induced both by changes in ionic strength and interaction with regulatory proteins. One model (Travers, 1974) proposes that RNA polymerase holoenzyme can exist in either of two conformational states. $(E\sigma)_m$ and $(E\sigma)_s$ which are in equilibrium. The $(E\sigma)_m$ state is favored at high ionic strength while at low ionic strength, neither state is favored. In addition, the protein synthesis elongation factor TuTs - formerly termed psi_r factor (Travers et al, 1971; Blumenthal et al, 1972) - is thought to stabilize the $(E\sigma)_s$ state. Finally, $(E\sigma)_m$ is thought to initiate RNA synthesis preferentially at mRNA and open rRNA promoters. In contrast, $(E\sigma)_s$ is thought to initiate at tRNA and closed rRNA promoters. Travers and Buckland (1973) have adduced indirect

evidence for the existence of such polymerase conformations and for their promoter specificities, in crude extracts of *E. coli*. *In vitro*, ppGpp has been shown to influence the conformational state and promoter specificity of RNA polymerase. Travers *et al*, (1973) have shown that ppGpp abolished the enhancement, by TuTs, or transcription from the closed rRNA promoter. However, ppGpp had no effect on transcription from the open rRNA promoter in the presence of TuTs. Thus it appears that there is a link between translation and transcription. During a stringent response, the protein-synthesizing machinery of rel^+ cells produces ppGpp which, in concert with TuTs interacts with holoenzyme changing its conformation and effecting a selective curtailment of stable RNA synthesis. In addition, initiation at certain mRNA promoters is also curtailed. It is thought that these mRNA's may code for so-called "luxury" proteins. In contrast, the synthesis of message for other maintenance proteins such as the ribosomal proteins is not measurably affected by the imposition of stringent conditions.

In addition to the reversible nature of the fine and coarse control mechanisms, RNA synthesis is strictly regulated in bacteria infected with bacteriophages and in sporulating bacteria undergoing a transition from the vegetative to the dormant phase of growth.. In the majority of the systems studied, host polymerase transcribed phage-specific polypeptides which acted to inhibit host sigma factors and to modify host polymerase (e.g. by phosphorylation or adenylation of subunits). Alternatively, the phage proteins associated with host polymerase to selectively alter the transcription to the reading of phage-specific genes (reviewed by Losick and Pero, 1976). In other cases, host polymerase is inactivated and phage-induced polymerases are synthesized. An example of this is the infection of *E. coli* with T3 or T7 phage (Schweiger and Herrick, 1974).

The importance of RNA polymerase in regulating gene expression in

response to so many diverse situations might suggest that the synthesis of the polymerase polypeptides is constitutive. However, if, in addition to all other maintenance proteins, the synthesis of RNA polymerase itself were regulated (negatively or positively), this might provide a more efficient and versatile means of regulating transcription. In fact, there is now convincing evidence for this idea. Scaife (1976) has reviewed recent evidence which suggests that the synthesis of the α, β and β' subunits may be under autogenous control. Thus rafampicin, a known inhibitor of chain initiation was found, surprisingly, to increase the rate of $\beta + \beta'$ synthesis (Hayward et al, 1973). In lysogenic strains, whose prophages carried rpoBC (the genes for the synthesis of the β and β' subunits) gene insertions, induction, which caused the synthesis of hundreds of copies of rpoBC genes, resulted in only a two-fold increase in $\beta + \beta'$ synthesis over the synthesis in uninfected cells carrying only one copy of these genes (Kirschbaum, 1973). Moreover, in a poorly suppressed strain carrying a non-polar amber mutation in the rpoB gene, normal levels of the β subunit were synthesized (Glass et al, 1975). Thus, some mechanism must operate to control the level of the RNA polymerase subunits. That the β subunit may be involved in the regulation of the rpo operon was suggested by results of experiments with two strains carrying amber rpoB mutations. When the mutation was suppressed, a temperature-sensitive phenotype was expressed which decreased both β and β' subunit synthesis at the non-permissive temperature (Khesin et al, 1976). Scaife (1976) proposed that the regulatory protein of the rpo operon was an oligomer containing the subunits of RNA polymerase and perhaps other peptides as well. This complex is thought to regulate the synthesis of an intermediate regulatory factor which is necessary for the transcription of the rpo genes. The synthesis of RNA polymerase itself appears to be regulated by a two-step process of autogenous control.

MATERIALS AND METHODS

Organism

The organism used throughout this study was *Rhodopseudomonas sphaeroides* strain "L" (wild type) which was obtained from Dr. B. Marrs (St. Louis University School of Medicine, Missouri).

Medium and Buffers

The medium was a modification of the semisynthetic medium of Lascelles (1956) with the following composition:

l-Glutamic acid	1.47 g
dl-Malic acid	2.70 g
KH_2PO_4	0.50 g
K_2HPO_4	0.50 g
$(NH_4)_2HPO_4$	0.80 g
$MgSO_4 \cdot 7H_2O$	0.20 g
$CaCl_2 \cdot 2H_2O$	53.00 mg
$MnSO_4 \cdot H_2O$	0.85 mg
$FeSO_4 \cdot 7H_2O$	2.78 mg
Na_3 Nitrilotriacetic acid	.28 g
Nicotinic acid	1.0 mg
Thiamine HCl	1.0 mmg
Biotin	10.0 μg
Yeast Extract	2.0 g
Distilled, deionized water	up to 1000.0 ml

The pH was adjusted to 6.8 with a saturated solution of sodium hydroxide. This medium is referred to in this thesis as the "GMY" (glutamate-malate-yeast extract) medium. Solid agar plates were prepared by mixing equal volumes separately autoclaved, double-strength GMY medium and Bacto Agar solution, because if the agar was included in the medium during

autoclaving, the medium turned yellowish-brown upon solidification. The final concentration of agar was 1.1 - 1.2% (w/v).

The buffers used for extraction and purification of the enzymes had the following compositions:

Extraction Buffer

50 mM Tris HCl, pH 7.5

10 mM $MgSO_4 \cdot 7H_2O$

200 mM KCl

0.1 mM dithiothreitol

0.1 mM EDTA

5% (v/v) glycerol

Chromatography Buffer ("CB")

50 mM Tris-HCl

0.1 mM dithiothretiol

0.1 mM EDTA

5% (v/v) glycerol

Growth Conditions

Rhodopseudomonas sphaeroides was purified by repeatedly streaking single bacterial colonies on GMY plates. Stock cultures were prepared by growing cells in 10 ml of liquid GMY medium until late logarithmic phase of growth and the cells were then pelleted by centrifugation and resuspended in 2-3 ml of sterile GMY medium containing 40% (v/v) glycerol. The concentrated bacterial suspension was stored in a one-dram screw-cap vial at -20°C. Under these conditions *Rps. sphaeroides* retained its viability for at least eighteen months.

Rps. sphaeroides was routinely subcultured in the photosynthetic mode of growth, i.e., under light and anaerobic conditions, because these condi-

tions inhibited the growth of possibly contaminating microorganisms and _Rps. sphaeroides_ adapted much more quickly when transferred from photosynthetic to heterotrophic conditions than when transferred from heterotrophic to photosynthetic growth.

Routine stock cultures were prepared by inoculating a colony with a sterile loop from a GMY plate into a one-dram vial that was approximately two-thirds full of GMY medium containing 0.65% (w/v) agar. The vial was tightly capped and placed in a Conviron Model E7 incubator equipped with fluorescent lighting. Growth was allowed to proceed for two to three days at 28°C; then the vial was stored in the dark at 4°C.

For large-scale growth of the organism, the following procedure was used: a loopful of bacteria was inoculated into a two-dram vial completely filled with GMY medium; the vial was tightly capped and incubated in the Conviron incubator at 28°C. Growth was allowed to proceed until the cells reached late logarithmic to early stationary phase as judged by the production of bright green to dirty brown pigments. The culture was then used as an inoculum (0.3%, v/v) for Roux bottles filled with about 600 ml of GMY medium which were similarly incubated until the cells had again reached late logarithmic or early stationary phase (about 48 hours), The Roux bottles were used to inoculate 10 or 15 liter glass carboys at a 6% (v/v) inoculum for either heterotrophic or photosynthetic growth. The carboys were equipped with a gas inlet, connected to two spargers and a gas outlet.

To obtain heterotrophic cells, the cultures were grown in aluminum foil-wrapped carboys with vigorous aeration in a 28°C incubation room. To obtain photosynthetic cells, the carboys were incubated in the fully-illuminated Conviron incubator at 28°C under semi-anaerobic conditions which were achieved by passing a gas mixture of 95% N_2 - 5% CO_2 for 5 to 10 minutes through the culture immediately after inoculation.

Growth Curve

Growth curves were constructed by following the increase in optical density of the cultures. For heterotrophic cultures, Rps. sphaeroides was grown in 15 ml of GMY medium in a 300 ml Klett flask wrapped with aluminum foil on a rotary shaker at 28°C. The optical density was measured at time intervals with a Klett-Summerson Photoelectric Colorimeter equipped with a Corning No. 66 glass filter. Cell suspensions with optical densities greater than 100 Klett units were diluted with sterile GMY medium before being read. Six ml samples were removed at time intervals from the photosynthetic cultures in Roux bottles (see "Growth Conditions") for optical density determination by the same method described above.

Harvesting of Cells

Cells were harvested at the early stationary phase of growth by centrifugation in a Sharples steam-driven centrifuge at 40 $lbs/in.^2$ and a flow rate of 200 ml/min. The packed bacteria were washed once in a buffer which contained 10 mM Tris-HCl, pH 7.8, 60 mM NH_4Cl, 10 mM $M_2SO_4 \cdot 7H_2O$ and 6 mM 2-mercaptoethanol. The washed bacteria were centrifuged in a Sorvall RC2-B refrigerated centrifuge at 25,000 x g for 30 min, weighed and stored at -80°C. The yield of heterotrophic cells was 4 to 6 grams per liter and that of photosynthetic cells was 1.5 to 2 grams per liter.

Enzyme Purification

Enzymes were purified at 4°C by the method of Burgess (1969 a) with slight modifications. Unless otherwise specified, the procedures for extracting and purifying RNA polymerase from Rps. sphaeroides grown heterotrophically or photosynthetically were identical. For brevity, the following terms will be used: P cells - bacteria grown photosynthetically;

P enzyme - RNA polymerase extracted from P cells; H cells - bacteria grown heterotrophically; H enzyme - RNA polymerase extracted from H cells.

The frozen cell pellets (about 170 g) were allowed to thaw partially at room temperature for 20 to 30 minutes, broken manually into small chunks and transferred into a pre-cooled Waring Blendor jar. Then, 175 ml of an extraction buffer (see "Medium and Buffers") and 1.5 to 2.5 times by weight of glass beads were added. The mixture was homogenized at low speed for 5 minutes and at high speed for 10 minutes. Since the jar was not equipped with a water jacket, it was necessary to plunge the jar periodically into an ice-water bath and rotate it to prevent overheating during the homogenization process.

The extract was then poured into a beaker and deoxyribonuclease I was added, with stirring, to a final concentration of 4 μ g/ml. The suspension was allowed to sit for 30 minutes during which time the broken beads had settled. The supernatant fluid was decanted into another beaker and the remaining glass beads were washed once with a small volume of the same extraction buffer and allowed to settle. These two supernatant fluids were pooled and centrifuged at 12,000 x g for 20 minutes. The final supernatant fluid from this centrifugation step was referred to as the "crude extract" (Fraction I). A small aliquot from this and all subsequent fractions was saved for protein and enzyme activity measurements. Fraction I was centrifuged again at 115,000 x g in a 60 Ti rotor for 2 hours in a Beckman Model L Ultracentrifuge. This step removed most of the ribosomes, chromatophores, and membranes, leaving a clear amber supernatant fluid (Fraction II).

To Fraction II, solid ammonium sulfate was slowly added, with constant sitrring, to a final concentration of 35% saturation. The pH was prevented from dropping by adding 0.05 ml of 1N sodium hydroxide per gram of ammonium sulfate added. The 35% saturated solution was gently stirred for at least

30 minutes and then centrifuged at 27,000 x g for 30 minutes and the pellet was discarded. The relatively high speed and long time interval for centrifugation were necessary to produce firm pellets and to ensure a clean fractionation.

The supernatant fluid from the 35% ammonium sulfate fractionation step (Fraction III) was then further fractionated by the addition of solid ammonium sulfate to 45% saturation. This solution was stirred and centrifuged as above. The supernatant fluid was discarded and the pellet was resuspended in 10 ml of CB ("Medium and Buffers") and dialyzed against 150 volumes of the same buffer. The dialyzed sample (Fraction IV) was adsorbed onto a column of DEAE-cellulose (2.5 cm x 26 cm) which had been equilibrated with CB.

The DEAE-cellulose column was first washed with one column volume of CB and proteins were then eluted by washing the column in a batchwise fashion with solutions of CB that contained increasing concentrations of KCl. Five ml fractions were collected automatically with an LKB 7000 Fraction Collector. For purification of the H enzyme, the column was washed successively with CB + 0.2 M KCl, CB + 0.3 M KCl and CB + 0.4 M KCl. The H enzyme was eluted from the column at CB + 0.3 M KCl ("Results", Fig. 3). From these results it was believed that the RNA polymerase could be desorbed from the column at an ionic strength of less than 0.3 M KCl, conceivably leaving other proteins still adsorbed to the column. Consequently, the compositions of the elution buffers were slightly changed for purification of the P enzyme; the column was washed with CB + 0.2 M KCl, CB + 0.25 M KCl, and CB + 0.3 M KCl ("Results", Fig. 2). Those fractions containing enzyme activity were pooled and concentrated by the addition of solid ammonium sulfate to 65% saturation (Fraction V). The solution was stirred for 30 minutes and then centrifuged at 27,000 x g for 20 minutes. The pellet

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was redissolved in a small volume of CB + 0.4 M KCl and stored at -80°C.

Fraction V was thawed and dialyzed against 100 volumes of CB + 0.4 M KCl. After dialysis, the sample was layered onto the surface of a column of Sephadex G-200 (2.5 cm x 30 cm for the H enzyme, 0.9 cm x 60 cm for the P enzyme) which had previously been equilibrated with CB + 0.4 M KCl. The enzyme was eluted with the same buffer and 5 ml fractions were collected as described for the DEAE-cellulose chromatography. The fractions containing most of the enzyme activity were pooled and concentrated by adding solid ammonium sulfate to 65% saturation with gentle stirring for 30 min. and then centrifuged at 27,000 x g for 20 minutes. For the H enzyme, the pellet obtained was resuspended in a small volume of CB + 0.4 M KCl, divided into several small aliquots, and stored at -80°C. For the P enzyme, the pellet was resuspended in a small volume of CB + 0.4 M KCl, an equal volume of glycerol was added, and the enzyme was stored at -20°C. For latter experiments, because of the inconvenience of having to dialyze out the glycerol before each enzyme assay, the P enzyme sample was dialyzed against CB 0.4 M KCl, divided into several small aliquots and frozen at -80°C. Under these conditions, enzyme from both P and H cells remained active for at least seven weeks. The enzyme purified by Sephadex G-200 chromatography is referred to as Fraction VI.

Preparation of DNA

Rps. sphaeroides DNA was purified from H or P cells essentially by the method of Marmur (1961). Calf thymus DNA was denatured by boiling for 15 minutes followed by rapid cooling in an ice-water-salt bath.

Protein Determination

Protein was determined by the method of Lowry *et al*, (1951) with

crystalline lysozyme as the standard.

RNA Polymerase Assay (4-NTP Reaction)

The standard RNA polymerase assay reaction mixture (0.1 ml) contained the following reagents: 50 mM TES-NaOH, pH 8.0, 1.5 mM $MnSO_4$, 0.5 mM of each of the sodium salts of unlabeled ATP, GTP, and CTP, and ^3H-labeled UTP (10 mCi/mmole), 50 μg/ml each of native and denatured calf thymus (CT) DNA, 5 mM putrescine, 5 mM 2-mercaptoethanol, 100 mM ammonium sulfate and 20 to 80 μg of enzyme protein. The reactionmixture was incubated for 50 min at 30°C and then spotted onto two rectangular pieces of Whatman #1 filter paper (1.8 cm x 2.5 cm) which were washed batchwise by the following procedure: 10 minutes in 10% TCA containing 1% sodium pyrophosphate, 10 minutes in 5% TCA containing 0.5% sodium pyrophosphate (twice), 5 minutes in 95% ethyl alcohol and finally 5 minutes in acetone. The filters were dried by a blow dryer, placed in plastic scintillation vials containing 10 ml of Scinti-Verse scintillation fluid and counted in a Beckman LS-230 liquid scintillation counter. One unit of enzyme activity is defined as that amount of enzyme that incorporates one nmole of labeled ribonucleotide in 50 minutes at 30°C under the standard assay conditions.

To correct for background counts, which were due to non-specific adsorption of labeled nucleotides to the filter papter, a blank was included in every experiment described in this thesis. The blank contained the same reagents as the test assay except that the enzyme was omitted. The background counts, which varied from 200 to 1000 CPM, were subtracted from the test assay counts.

Homopolymer Polymerase Assay (single NTP reaction)

The assay procedure for the formation of homopolymers (poly G, poly C,

poly U, or poly A) was identical to the procedure used for the formation of RNA ("RNA Polymerase Assay"). The reaction mixture contained a single labeled NTP at a concentration of 0.5 mM and a specific radioactivity of 10 mCi/mmole. The other components of the reaction mixture are specified in the tables below.

SDS Polyacrylamide Gel Electrophoresis

Electrophoresis of various enzyme fractions was carried out in polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) essentially by the method of Weber and Osborn (1969). Solutions of the following compositions were used:

1. Sample Buffer

SDS	1 g
2-mercaptoethanol	1 ml (of a 14.3 M solution)
0.02 M phosphate buffer, pH 7.0	100 ml

2. Gel Buffer

SDS	2 g
$NaH_2PO_4 \cdot H_2O$	8.82 g
Na_2HPO_4	20.45 g
distilled, deionized water	1000 ml

3. Acrylamide Gel Solution

acrylamide	22.2 g
MBA	0.6 g
distilled, deionized water	100 ml

4. Running Buffer

1 part of gel buffer and 1 part of water

The gels (7.5% acrylamide) were polymerized by the addition of a freshly made solution of ammonium persulfate (final concentration 0.2%

w/v) and TEMED (final concentration 0.17%, (v/v) in glass tubes with an inner diameter of 6 mm. The length of the gels was approximately 9.5 cm. A small amount of water was carefully layered over the surface of the gels to prevent the formation of a meniscus.

The enzyme samples (10-50 μg of protein) were mixed with an equal volume of sample buffer and heated to 100°C for 5 minutes. The mixture was cooled and one drop of a 40% (w/v) sucrose solution and 3 μl of 0.1% (w/v) bromophenol blue solution, as tracking dye, were added. The cathode and anode chambers were filled with running buffer and the samples were carefully layered onto the surfaces of the gels. Electrophoresis was carried out at 1.5 mA per gel for 16 hours followed by 5 mA per gel for 8 hours. By this time the tracking dye had travelled about three-quarters the length of the gel. Gels were removed from the tubes by riming with a steel needle through which distilled water was squirted. The length of the gels and the distance travelled by the trailing and leading edges of the tracking dye were recorded. The gels were immersed overnight in a solution of 12.5% TCA, and stained for 1.5 hours in a solution of 0.2% Coomassie Brilliant Blue in 45% water - 45% ethyl alcohol - 10% glacial acetic acid in a Bio Rad Diffusion Destainer (Bio Rad Laboratories, Richmond, California) for 15.5 hours. The length of the gels and the positions of the protein bands were recorded. Using these values as well as those of the gel length before destaining and the dye distance, the relative mobilities of the protein bands could be calculated (Wever and Osborn, 1969). Consequently, the molecular weights of the protein species were estimated by comparing their relative mobiliteis with those of the standard molecular weight markers, such as: bovine serum albumin (MW = 67,000), ovalbumin (MW = 45,000), myoglobin (MW = 17,000), and horse hear cytochrome C (MW = 12,400). Commercial samples of <u>Escherichia</u>

coli RNA polymerase (Sigma) were also electrophoresed along with our enzyme samples for a qualitative comparison of their enzyme subunits.

Chemicals

The following chemicals were purchased from the Sigma Chemical Company: l-glutamic acid, dl-malic acid, calf thymus DNA type I, the sodium salts of unlabeled GTP, CTP, ATP and UTP, serum albumin (bovine), lysozyme (egg white), nicotinic acid, thiamine hydrochloride, biotin, dithiothreitol (Cleland's reagent), 2-mercaptoethanol, DNase I, RNase A (pancreatic ribonuclease), RNase T (*Aspergillus oryzae*), RNA polymerase (*E. coli*), DEAE-cellulose, TES, TRIS, putrescine, rifampicin.

The following radioisotope-labeled compounds were purchased from New England Nuclear Corp. All these compounds had a specific radioactivity greater than 20 Ci/mmole except for GTP which had a specific radioactivity of 5 - 10 Ci/mmole:

CTP	5 -[^{3}H],	tetrasodium salt
ATP	2,8 -[^{3}H],	tetrasodium salt
GTP	8 -[^{3}H],	tetrasodium salt
UTP	5 -[^{3}H],	tetrasodium salt

The following chemicals were purchased from various companies which are listed in parentheses: acrylamide, MBA, TEMED (Eastman Kodak Co.), ammonium sulfate, enzyme grade (Schwarz-Mann div. of Becton, Brickinson and Co.), Scinti-Verse (Fisher Scientific Co.), Sephadex G-200 (Pharmacia Chemical Co.), Yeast Extract (Difco Laboratoreis), horse heart cytochrome C, ovalbumin, bovine serum albumin, sperm whale myoglobin (Mann Research Laboratories).

All other chemicals were reagent grade or better.

RESULTS

Growth Curves

Figure 1 shows the typical growth curves of *Rps. sphaeroides* grown either heterotrophically or photosynthetically. Under our conditions, *Rps. sphaeroides* always grew faster under heterotrophic than under photosynthetic conditions. The mean generation times for heterotrophic and photosynthetic growth were about 2.7 and 4.1 hours, respectively. By midlogarithmic phase, the cells were heavily pigmented; the heterotrophic cells were red and the photosynthetic cells were greenish-brown.

Enzyme Purification

RNA polymerase was purified from about 170 g of heterotrophic or photosynthetic *Rps. sphaeroides* harvested in the late exponential growth phase by the methods described in "Materials and Methods". The enzyme purification results obtained under the standard 4-NTP assay conditions are summarized in Tables II and III and described in detail in the following paragraphs.

(a) Crude extract and high-speed centrifugation supernatants fractions I and II:

There was a three-and-a-half fold difference between the amount of protein extracted from the H and P cells (1260 vs. 4400 mg). Although less protein was extracted from the H cells, the total enzyme activity in this extract was higher than that in the extract from the P cells (13335 vs. 8756 units). The specific activity of the enzyme in the H cell extract was 5-fold higher than that in the P cell extract. Possible reasons for these differences are presented in the Discussion.

(b) Ammonium sulfate fractionation, fractions III and IV:

In going from the first to the second ammonium sulfate fractionation steps, both the H and P enzyme samples showed a considerable decrease in total enzyme activity (from about 9700 to 300 units). This drop in enzyme

FIGURE 1. Growth curves of *Rhodopseudomonas sphaeroides* under heterotrophic or photosynthetic growth conditions.

(Δ——Δ) photosynthetic growth

(⊙——⊙) heterotrophic growth

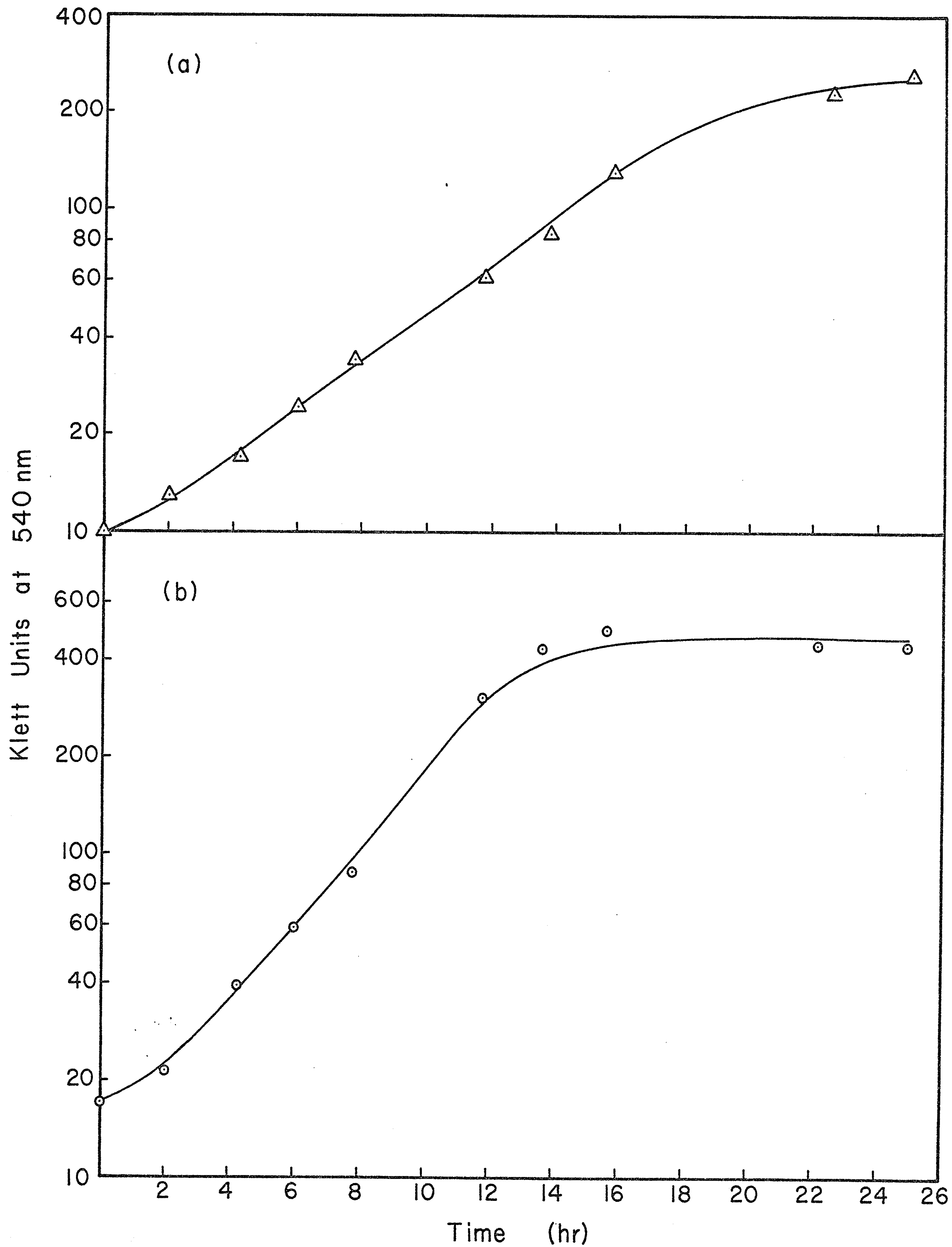

(a)
(b)
Klett Units at 540 nm
Time (hr)
400
200
100
80
60
40
20
10
600
2
4
6
8
10
12
14
16
18
20
22
24
26

Table II

A Summary of the Purification of RNA Polymerase from Heterotrophically Grown *Rps. sphaeroides* (a)

Fraction	Step	Volume (ml)	Total Protein (mg)	Total Activity (units)(b)	Specific Activity(c)	Purification (fold)	Yield (%)
I	1. Crude Extract	200	1260	13335	10.5	1.0	100
II	2. High-speed Centrifugation	170	493	13535	27.7	2.6	102
III	3. 1st Ammonium Sulfate (0-0.35)	181	277	9747	35.2	3.3	73
IV	4. 2nd Ammonium Sulfate (0.35-0.45)	13.6	49.2	321	6.5	0.6	2
V	5. Pooled DEAE-cellulose	22	ND(d)	2290	ND	ND	ND
VI	6. Pooled Sephadex G-200	19.4	4.5	617	137	13	5

(a) Enzyme was extracted from 170 g (wet weight) of late log cells and assayed under the standard 4-NTP conditions.

(b) 1 unit = 1 nmole [^{3}H]-UMP incorporated in 50 min at 30°C.

(c) specific activity - units/mg protein.

(d) Not determined.

Table III

A Summary of the Purification of RNA Polymerase From Photosynthetically Grown Rps. sphaeroides [a]

Fraction	Step	Volume (ml)	Total Protein (mg)	Total Activity (units)[b]	Specific Activity[c]	Purification (fold)	Yield (%)
I	1. Crude Extract	176	4400	8756	2.0	1.0	100
II	2. High-speed centrifugation	134	1595	7561	4.7	2.4	86
III	3. 1st Ammonium sulfate (0-0.35)	145	885	9790	11.1	5.6	112
IV	4. 2nd Ammonium sulfate (0.35-0.45)	12.8	74	354	4.8	2.4	4
V	5. Pooled DEAE-cellulose	31.8	25	3210	128	64	37
VI	6. Pooled Sephadex G-200	8.85	8	1470	184	92	17

(a) Enzyme was extracted from 170 g (wet weight) of late log cells and assayed under the standard 4-NTP conditions.

(b) 1 unit = 1 nmole [^{3}H]-UMP incorporated.

(c) specific activity = units/mg protein.

activity was obviously an artifact because in the following purification steps (DEAE-cellulose and Sephadex G-200) higher total activity was regained. It is believed that this apparent loss was caused by our preparation method - the 45% ammonium sulfate pellet was dissolved in a volume less than one-tenth the volume of the extract from the previous step. It is conceivable that this concentration of enzyme caused an aggregation to occur which led to partial inactivation. Another possibility is that the ammonium sulfate in the pellet inhibited the enzyme activity in the assay (Tables VI and VII).

(c) DEAE-cellulose fraction, fraction V:

The H and P enzymes, partially purified by ammonium sulfate fractionation (Fraction IV) were then subjected to ion-exchange chromatography on a DEAE-cellulose column (2.5 cm x 2.6 cm). Figure 2 shows a typical elution profile of the P enzyme from the column (which turned brown for about 40% of its length during adsorption of the enzyme). Three distinct UV-adsorption peaks at 280 nm were readily visible at fraction numbers 13-17, 38-43, and 54-59 (Figure 2). All of the RNA polymerase activity, tested by the standard 4-NTP assay, was present in the last peak. The activity-containing samples were pooled, concentrated by ammonium sulfate precipitation (65% saturation) and frozen. Enzyme Fraction IV from heterotrophic cells was similarly treated to prepare its Fraction V (Fig. 3).

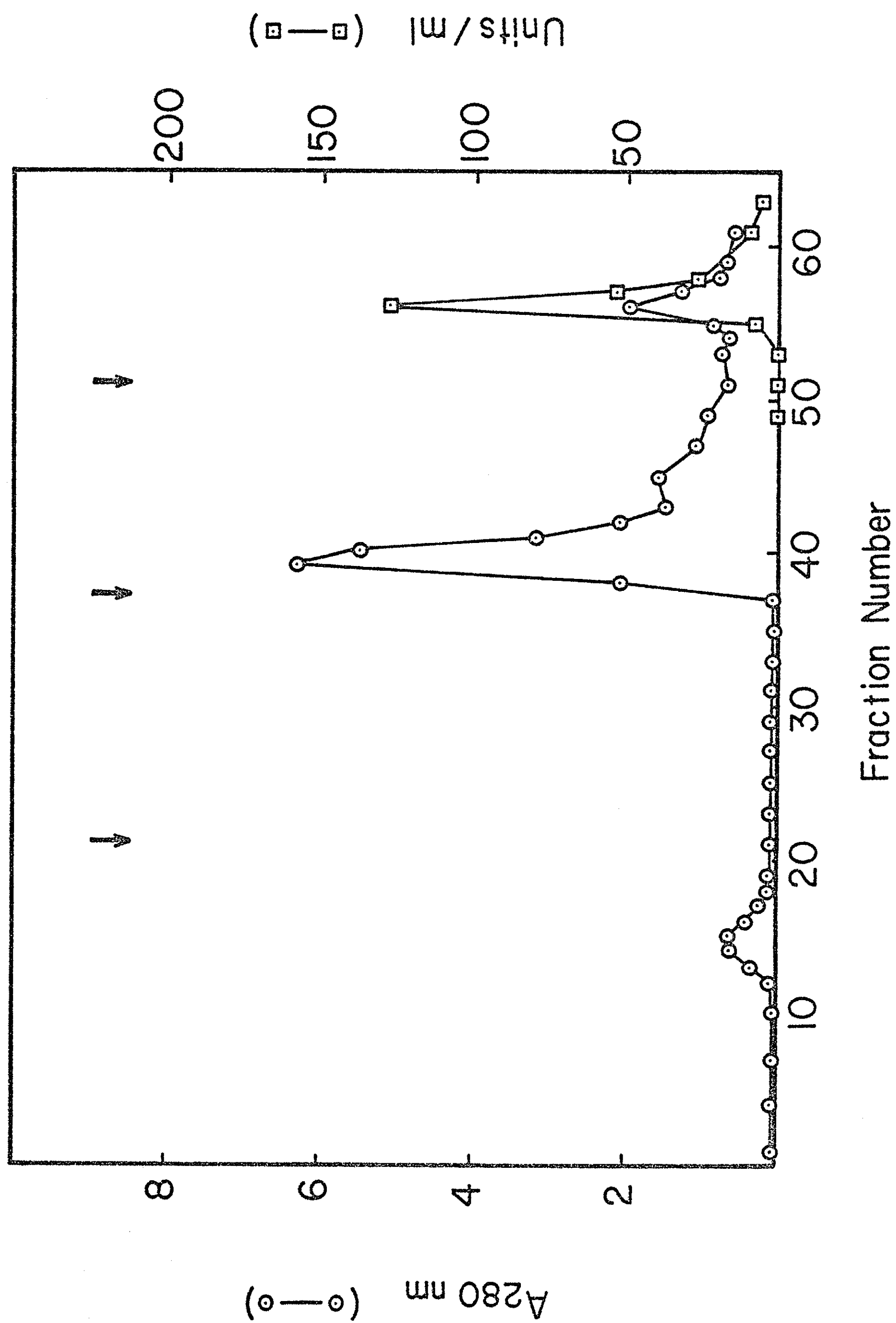
(d) Sephadex G-200 fraction, fraction VI:

Fraction V was thawed and dialyzed against CB + 0.4 M CKl then subjected to chromatography on a column of Sephadex G-200 (2.5 cm x 30 cm for the H enzyme, 0.9 x 60 cm for the P enzyme, "Materials and Methods"). Figure 4 shows a typical elution profile of the H enzyme from the column. One distinct UV-absorbing peak was visible which emerged in the void volume (fractions 4-7) along with a trailing shoulder (fractions 8-12).

FIGURE 2. DEAE-Cellulose column profiles of photosynthetic Rps. sphaeroides enzyme. Dialyzed ammonium sulfate Fraction IV was applied to a DEAE-cellulose column (2.5 cm x 26 cm) and eluted as described in "Materials and Methods". The arrows indicate the fraction number at which the ionic strength of the influent buffer was changed ("Materials and Methods").

(O——O) Absorbance at 280 nm.

(□——□) Enzyme activity.



Fraction Number
10
20
30
40
50
60
A280 nm (○—○)
2
4
6
8
Units / ml (□—□)
50
100
150
200

FIGURE 3. DEAE-cellulose column profile of heterotrophic _Rps. sphaeroides_ enzyme. Dialyzed ammonium sulfate Fraction IV was applied to DEAE-cellulose column (2.5 cm x 26 cm) and eluted as described in "Materials and Methods". The arrows indicate the fraction number at which the ionic strength of the influent buffer was changed ("Materials and Methods").

(⊙——⊙) Absorbance at 280 nm.

(⊡——⊡) Enzyme activity.

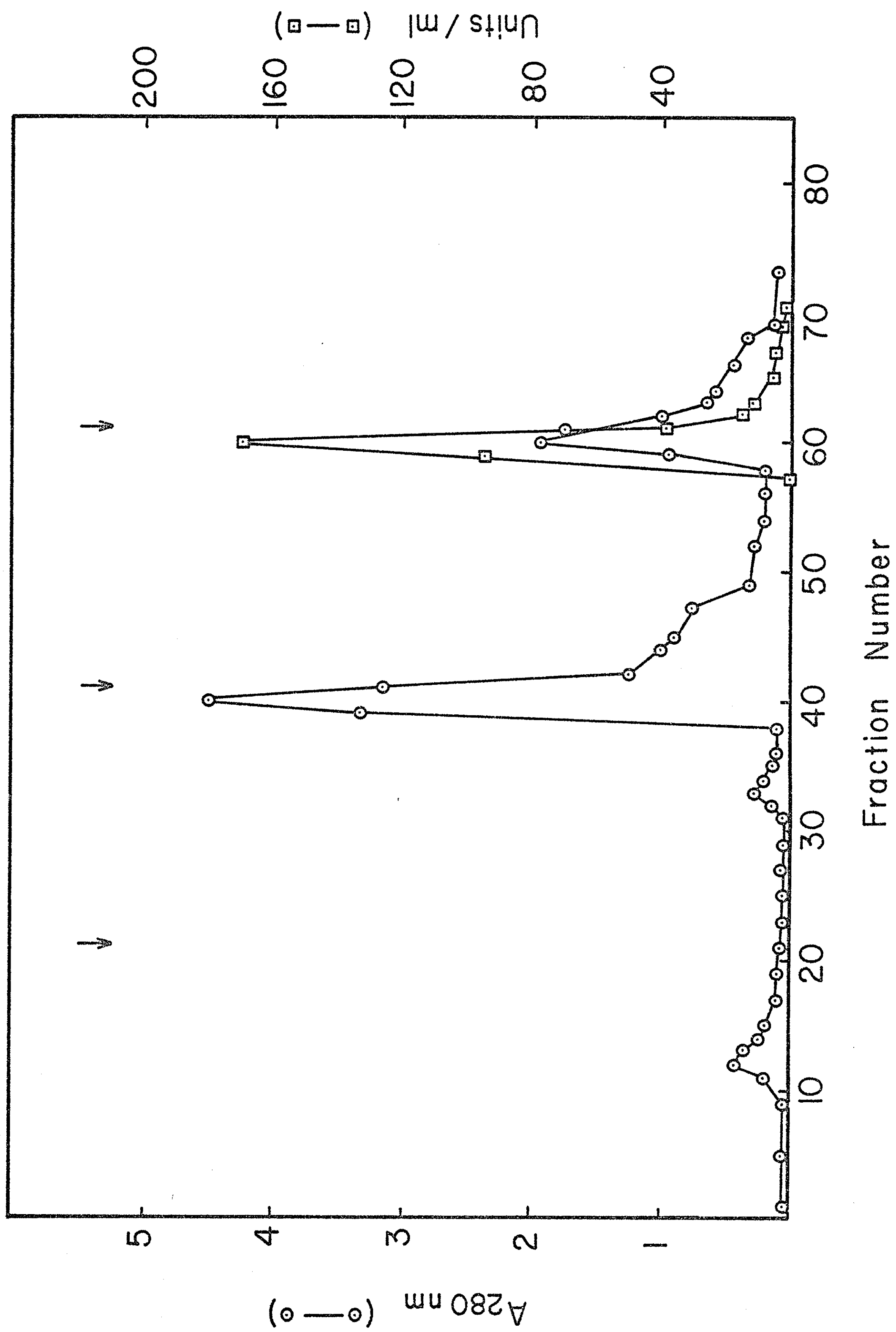

Fraction Number
$A_{280\,nm}$ (⊙—⊙)
Units / ml (⊡—⊡)
10
20
30
40
50
60
70
80
1
2
3
4
5
40
80
120
160
200

FIGURE 4. Sephadex column profile of heterotrophic *Rps. sphaeroides* enzyme. Enzyme Fraction V was applied to a Sephadex G-200 column (2.5 cm x 30 cm) and eluted as described in "Materials and Methods".

(⊙——⊙) Absorbance at 280 nm.

(⊡——⊡) Enzyme activity.

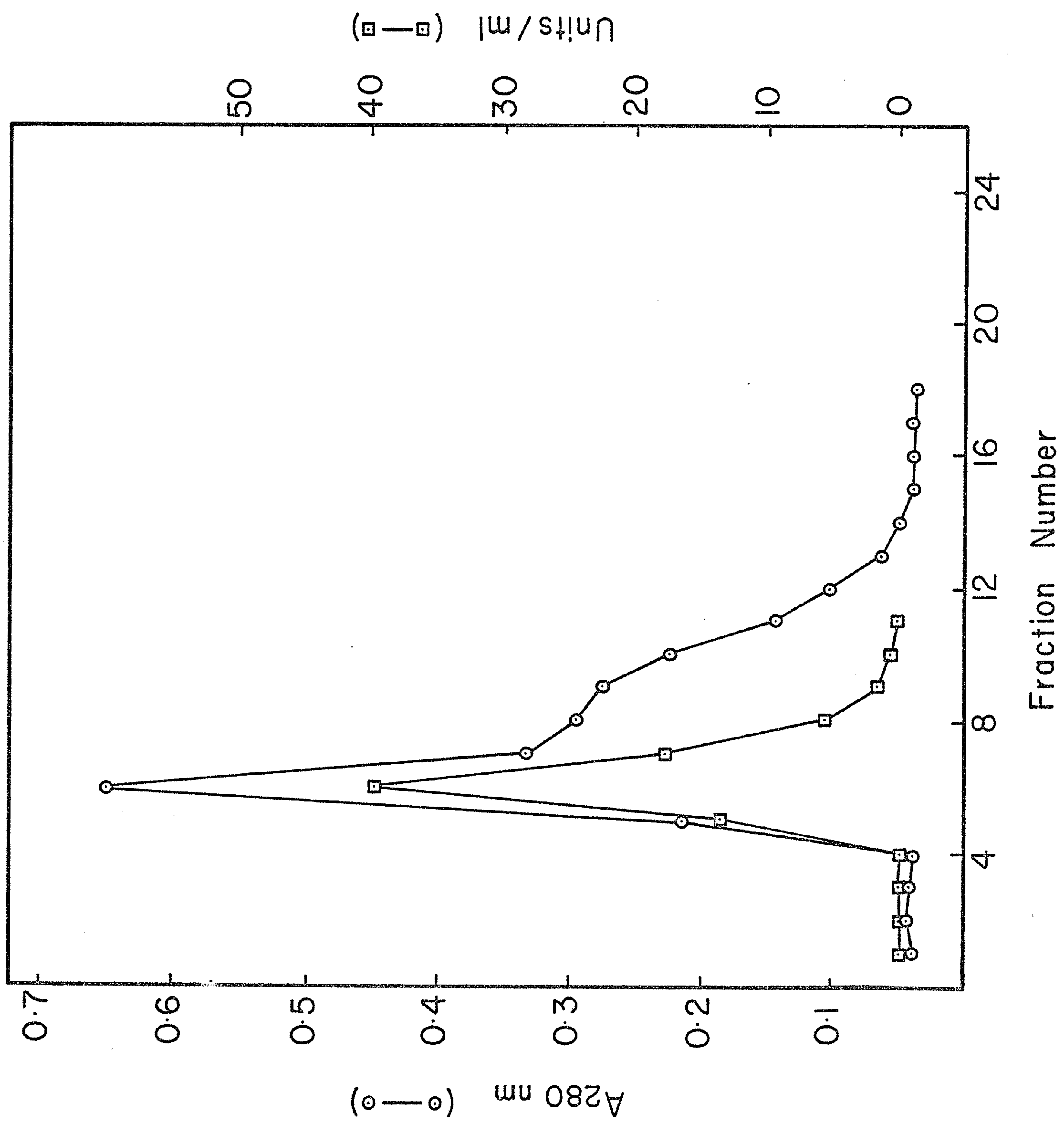
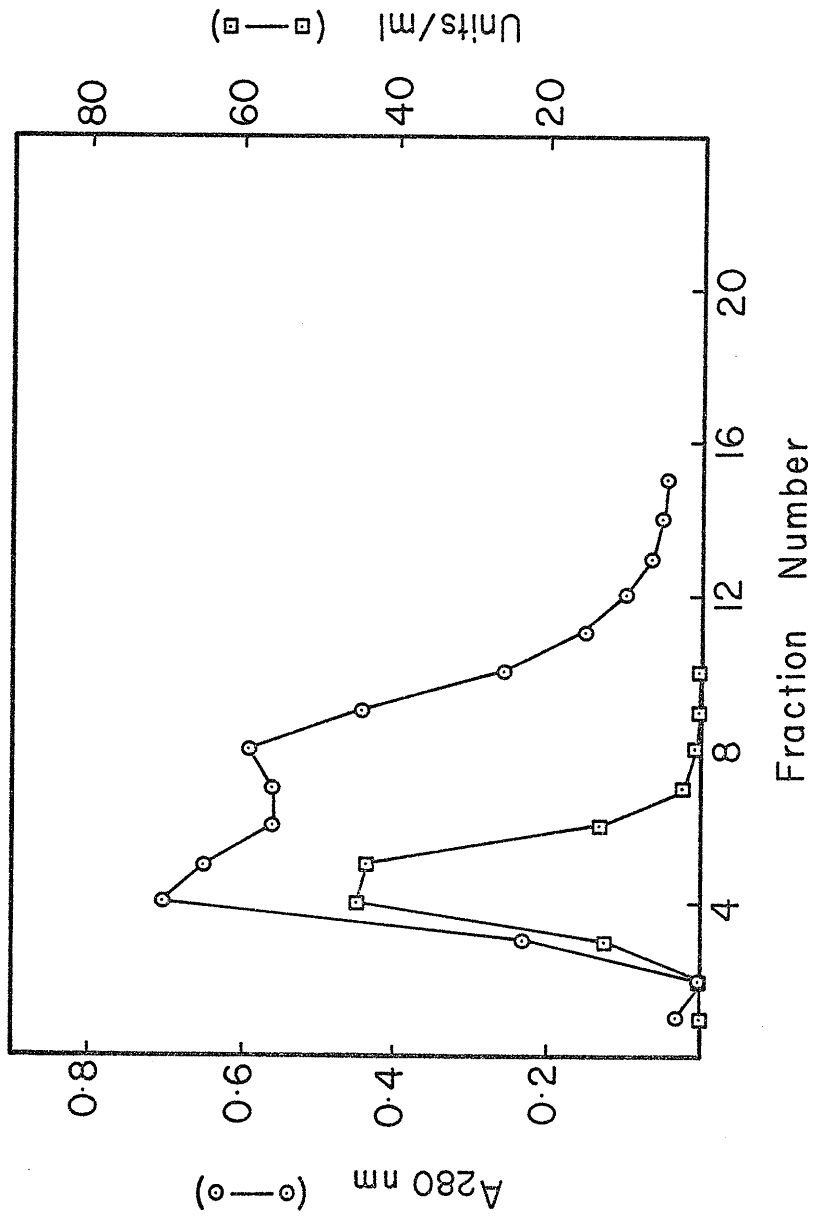

A280 nm (⊙—⊙)
0·1
0·2
0·3
0·4
0·5
0·6
0·7
Units / ml (⊡—⊡)
0
10
20
30
40
50
Fraction Number
4
8
12
16
20
24

FIGURE 5. Sephadex column profile of photosynthetic *Rps. sphaeroides* enzyme. Enzyme Fraction V was applied to a Sephadex G-200 column (0.9 cm x 60 cm) and eluted as described in "Materials and Methods".

(O——O) Absorbance at 280 nm.

(□——□) Enzyme activity.



$A_{280\,nm}$ (⊙—⊙)
0·8
0·6
0·4
0·2
Units/ml (⊡—⊡)
80
60
40
20
4
8
12
16
20
Fraction Number

Most of the RNA polymerase activity, was present in the peak while a small amount was associated with the shoulder. It was believed that the trailing shoulder could be separated further from the peak by use of a longer column. Accordingly, the P-enzyme Fraction V was chromatographed on a longer column (60 cm) and its elution profile is shown in Fig. 5. Two overlapping UV-absorbing peaks were visible at fraction numbers 3-6 and 7-11. The RNA polymerase activity was present solely in the first peak.

(3) Enzyme activity in Fractions I-VI assayed in the presence or absence of added DNA template or unlabeled NTP's.

In addition to the results presented in Tables II and III, three sets of control experiments had also been carried out for all 6 enzyme fractions in the following reaction mixtures:

(i) -DNA-NTP's -the reaction mixture contained neither added calf thymus DNA nor unlabeled NTP's.

(ii) -DNA + NTP's -the reaction mixture contained added unlabeled NTP's but no calf thymus DNA.

(iii) +DNA - NTP's -the reaction mixture contained added calf thymus DNA but no unlabeled NTP's.

The results are presented in Table IV. Fraction I-VI showed negligible activity when assayed under the first (-DNA -NTP's) control conditions. An unexpected result was obtained in the second (-DNA +NTP's) control experiment. Fractions I-IV showed almost the same activity in this (second) control reaction mixture as in the standard reaction mixture, i.e., +DNA +NTP's. Fractions V and VI, however, showed negligible activity in the second control. Two possible explanations can be offered for the activity present in this second control in Fractions I-IV:

(i) The DNase treatment under our conditions was insufficient to digest all the bacterial DNA and therefore this residual DNA was able to

act as a template for the enzyme or

(ii) The enzyme activity observed was not a true RNA polymerase activity but was due to some contaminating enzymes which could incorporate a single species of nucleotides into an acid-insoluble polymer in the absence of DNA, such as polynucleotide phosphorylase (Grunberg-Manago et al, 1955, polyphosphate kinase (Kornberg et al, 1956) or homopolymer polymerases (Chamberlin and Berg, 1962, 1964). The first explanation is preferred because of the results discussed in "physico-chemical properties" (section f, below, and Table V). The A_{280}/A_{260} ratios suggested the presence of a significant quantity of nucleic acid in Fractions I-IV while there was evidently less nucleic acid in Fractions V and VI (Table V).

Another unexpected result was obtained when Fractions I-VI were treated under the third control conditions (+DNA -NTP's). Fractions I-IV and Fraction V (heterotrophic) showed negligible activity in this control while Fractions V (photosynthetic) showed an activity that was about 80% that of the standard reaction mixture (+DNA +NTP's). Both the H and P Fraction VI enzymes showed higher activity in the third control than in the standard reaction mixture. For the H enzyme, the activity in this third control was about 170% of the activity in the standard reaction mixture, while for the P enzyme, the activity of the control was 140% that of the standard reaction. Since labeled UTP was the only nucleotide present in this control, it appeared that a homopolymer polymerase activity had been co-purified with the RNA polymerase activity. This activity was examined in more detail and is discussed in a later section ("Homopolymer Synthesis").

(f) Physico-chemical properties of enzyme Fractions I-VI:

The optical density of all 6 Rps. sphaeroides enzyme fractions (H and P) was measured at 260 and 280 nm and their A_{280}/A_{260} ratios are presented in Table V. As mentioned earlier ("Growth Curves"), both the H and P cells

Table IV

Rhodopseudomonas sphaeroides RNA polymerase activity assayed under various conditions (a)

	HETEROTROPHIC (units)				PHOTOSYNTHETIC (units)			
Fractions	+DNA -NTP's	-DNA -NTP's	-DNA +NTP's	+DNA -NTP's	+DNA +NTP's	-DNA -NTP's	-DNA +NTP's	-DNA +NTP's
I	13,300	657	13,100	777	8,760	1,080	7,310	1,220
II	13,600	583	13,100	500	7,560	687	7,120	740
III	4,750	1,120	13,000	780	9,790	461	9,700	477
IV	321	5	200	10	354	39	377	41
V	2,290	56	296	151	3,210	17	94	2,570
VI	617	5	0	1,050	1,470	49	52	2,120

(a) Enzymes were prepared by the same method described in Tables II and III. Their activities were measured in a standard 4-NTP assay mixture with (+) or without (-) calf thymus DNA or 3 unlabeled NTP's.

Table V

Physico-chemical Properties of H and P Enzyme Fractions

Fraction	Color	A_{280}/A_{260}	Enzyme Subunits*
H			
I	dark amber	0.80	N.D.
II	dark amber	0.68	N.D.
III	amber	0.61	N.D.
IV	dark amber	1.17	α β β' σ
V	light amber	N.D.	N.D.
VI	clear	1.35	α β β' σ
P			
I	dark green	0.86	N.D.
II	dark amber	0.77	N.D.
III	amber	0.68	N.D.
IV	dark amber	1.10	α β β' σ
V	light amber	1.20	α β β' σ
VI	faint amber	1.18	α β β' σ

* ND = not determined.

α, β, β', σ - refers to the presence of polypeptides that co-migrated with commercial *E. coli* RNA polymerase on SDS-polyacrylamide gels.

were pigmented, however the pigments did not interfere with either A_{280}/A_{260} ratios or protein determination (Chow, 1976 c) and were completely removed during the high-speed centrifugation step. The A_{280}/A_{260} ratios of the first three fractions were considerably less than 1, while the ratios for the last three fractions were greater than 1. These observations imply that significant quantities of nucleic acid were present in Fractions I-III but were reduced during the last three stages of purification. These results suggest that the enzyme activity observed in the above-mentioned second control assay (-DNA + NTP's) in Fraction I-IV was probably due to the presence of endogenous bacterial DNA.

Various fractions of the Rps. sphaeroides H and P enzymes were also subjected to polyacrylamide gel electrophoresis in SDS along with a commercial sample of E. coli RNA polymerase (Sigma). The results are presented in Fig. 6 and Table V. The E. coli sample displayed at least 6 protein bands three of which were identified as the (β + β'), σ and α subunits by comparing their relative mobilities with those of known molecular weight proteins. The remaining three protein bands were presumably some "inert" proteins added to the E. coli sample for stabilizationof the enzyme activity, e.g., bovine serum albumin (Burgess, 1969 a). Protein bands with similar mobilities as the E. coli RNA polymerase (β + β', σ and α) subunits were also present in the Rps. sphaeroides enzyme Fraction VI and V and VI. In addition to these subunits, the H and P Fraction VI enzymes showed a number of faint minor bands which are, at present, believed to be contaminating proteins, although the possibility that they actually represent the subunit structure of the RNA polymerase Rps. sphaeroides cannot be totally eliminated since the elution profiles of the enzymes from the Sephadex columns suggest that the molecular weight of the Rps. sphaeroides enzymes are in excess of 800 000. This value is approximately twice the molecular weight reported for other procaryotic

FIGURE 6. Photographs of SDS-polyacrylamide gels. The *Rps. sphaeroides* H and P enzymes from various stages of purification were subjected to electrophroesis in 7.5% SDS polyacrylamide gels and the gels were stained for protein as described in "Materials and Methods".

A: Fraction IV, P enzyme.

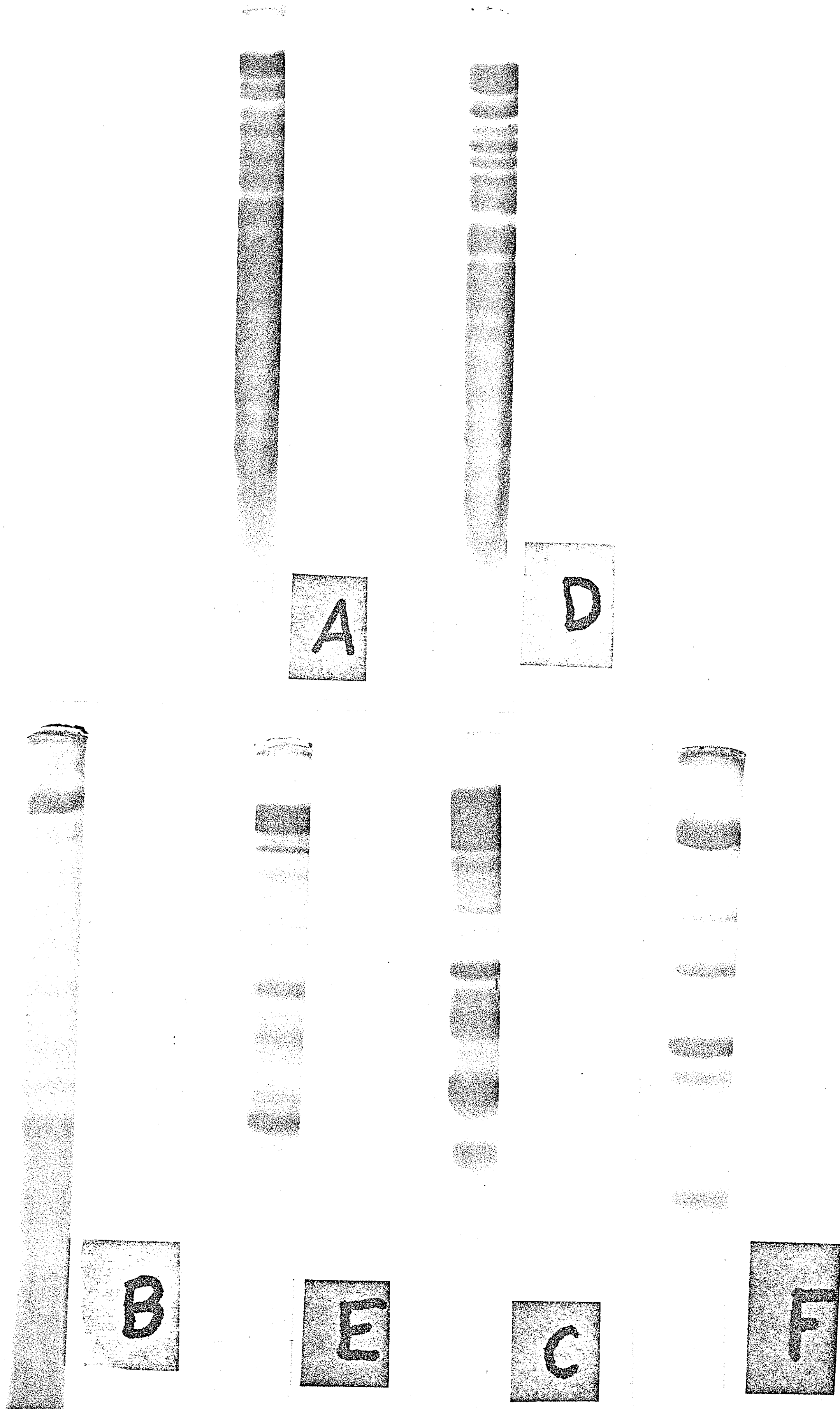
D: Fraction IV, H enzyme.

B: Fraction V, P enzyme.

E: Fraction VI, H enzyme.

C: Fraction VI, P enzyme.

F: Commercial *E. coli* RNA polymerase (Sigma).


A
D
B
E
C
F

RNA polymerases (see "Historical"). The overall pattern of protein bands for the H and P enzymes (Fraction VI) is very similar (Fig. 6).

Time Course Study of RNA Synthesis

Figure 7 shows the kinetics of RNA synthesis by the P and H (Fraction VI) enzymes. The reactions catalyzed by both enzymes proceeded without a detectable lag period and the reaction was essentially complete after one hour. No endogenous nuclease activity was evident.

Requirements of the RNA Polymerase (4-NTP) Reaction

Enzyme Fraction VI (Sephadex fraction) was used for this and all succeeding experiments unless otherwise specified. Tables VI and VII list the requirements for RNA synthesis for the P and H enzymes respectively. (a) P enzyme - required added DNA template and a divalent cation for full activity. Omission of DNA (or addition of DNase) or Mn^{++} reduced the amount of $[^3H]$-UMP incorporated to less than 10% (Table VI). Magnesium was able to substitute for Mn^{++} (see below and Fig. 1). The reaction catalyzed by the P enzyme apparently did not have an absolute requirement for all four nucleotides. Omission of a single unlabeled nucleotide resulted in a residual incorporation ranging from about 25 to 50% of that incorporated in the complete system. Furthermore, omission of all three unlabeled resulted in a higher amount of $[^3H]$-UMP incorporation than that in the complete system. These results will be discussed in detail in a later section ("Incorporation in the Absence of One or More Nucleotides"). Omission of putrescine, ammonium sulfate or 2-mercaptoethanol either stimulated or had no effect on the reaction. These compounds, however, did show a stimulatory effect in preliminary experiments where enzyme Fractions II, III, or IV were employed and were therefore included in the complete

Table VI

Requirements for RNA Synthesis: The H Enzyme

Components	nmoles of $[^3H]$-UMP incorporated per mg protein	percent of complete
complete system [(a)]	23.8	100
minus DNA	6.6	28
minus Mn^{++}	5.4	23
minus GTP	17.0	71.4
minus CTP	16.2	68.1
minus ATP	12.2	51.2
minus GTP, CTP, ATP	27.1	114
minus putrescine	24.0	101
minus $(NH_4)_2SO_4$	89.8	377
minus 2-mercaptoethanol	34.1	143
plus RNase T_1 (175 units)	4.6	19
plus DNase I (50 μg/ml)	6.6	28

(a) The complete system contained: 50 nM TES-NaOH, pH 8.0, 1.5 mM $MnSO_4$, 0.5 mM of each of the sodium salts of unlabeled ATP, GTP and CTP and ^3H-labeled UTP (10 mCi/nmol), 50 μg/ml each of native and denatured calf thymus (CT) DNA, 5 mM putrescine, 5 mM 2-mercaptoethanol, 100 mM $(NH_4)_2SO_4$ and 19.6 μg of H enzyme protein.

Table VII

Requirements for RNA Synthesis: The P Enzyme

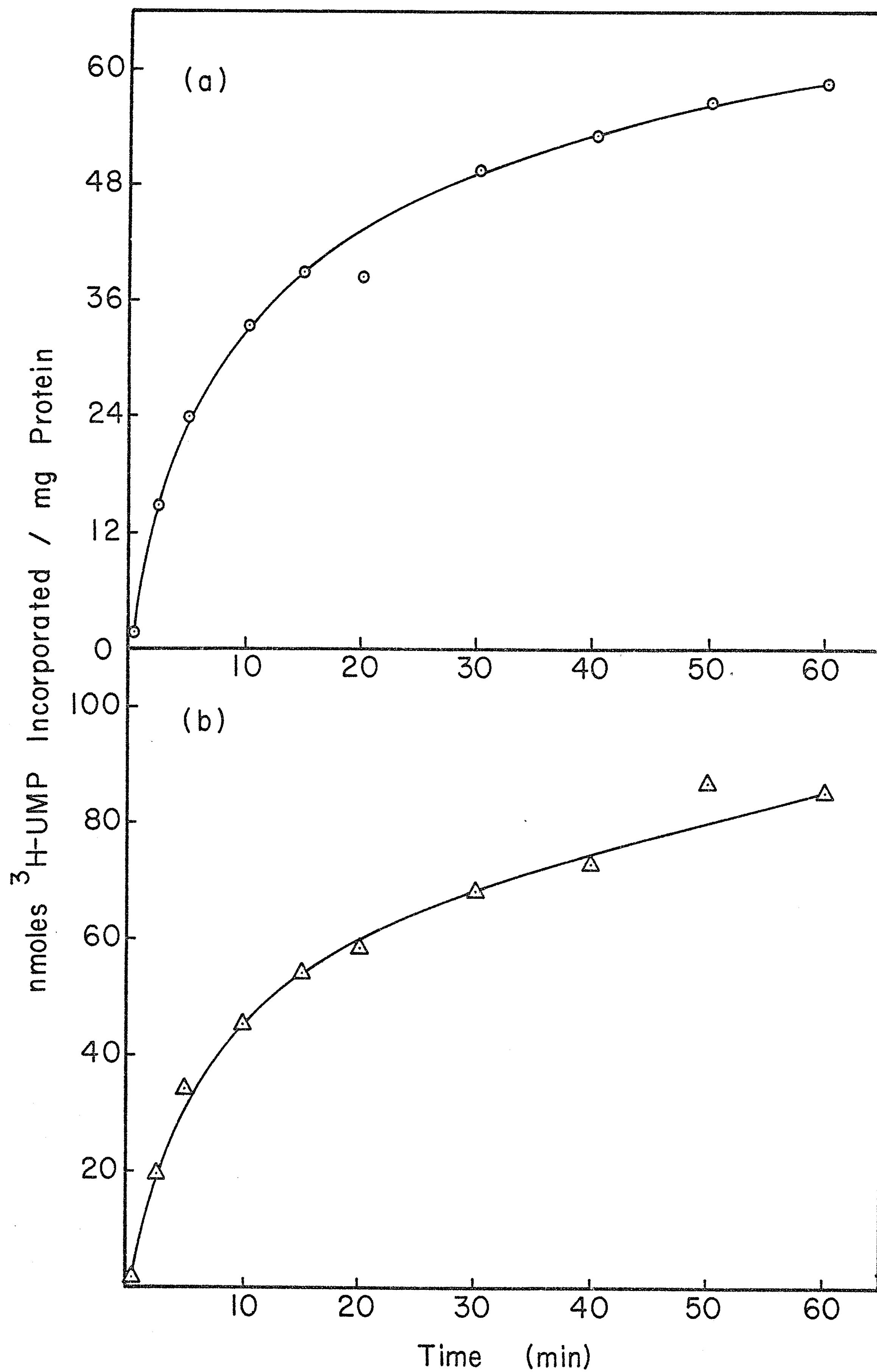
Components	nmoles of [^{3}H]-UMP incorporated per mg protein	percent of complete
complete system [a]	56.5	100
minus DNA	4.3	7.6
minus Mn^{++}	1.0	1.8
minus GTP	28.7	50.8
minus CTP	13.7	24.2
minus ATP	20.6	36.5
minus GTP, CTP, ATP	92.6	164
minus putrescine	62.7	111
minus $(NH_4)_2SO_4$	94.6	167
minus 2-mercaptoethanol	57.1	101
plus RNase T_1 (175 units)	4.0	7.1
plus DNase I (50 μg/ml)	3.6	6.4

[a] The complete system contained: 50 mM TES-NaOH, pH 8.0, 1.5 mM $MnSO_4$, 0.5 mM of each of the sodium salts of unlabeled ATP, GTP and CTP, and ^{3}H-labeled UTP (10 mCi/mmole), 50 μg/ml each of native and denatured calf thymus (CT) DNA, 5 mM putrescine, 5 mM 2-mercaptoethanol, 100 mM $(NH_4)_2SO_4$ and 9.8 μg of P enzyme protein.

FIGURE 7. Time course of RNA synthesis by enzyme Fraction VI of heterotrophic or photosynthetic *Rps. sphaeroides*. The reaction was carried out under the standard RNA polymerase assay conditions.

(⊙——⊙) Heterotrophic enzyme.

(△——△) Photosynthetic enzyme.


(a)
(b)
60
48
36
24
12
0
100
80
60
40
20
10
20
30
40
50
60
nmoles ³H-UMP Incorporated / mg Protein
Time (min)

assay system for calculation of enzyme activity recovery as presented in Tables II and III. Addition of RNase T_1 to the reaction mixture resulted in a 93% reduction of incorporation, suggesting that the product of the reaction was RNA in nature.

(b) H enzyme - the pattern of DNA, divalent ion and nucleotide requirements as well as the sensitivity to DNase and RNase of the H enzyme were similar to those of the P enzyme. However, a number of minor differences were noticed. In the absence of DNA or Mn^{++}, the H enzyme consistantly incorporated more [^{3}H]-UMP than the P enzyme (compare lines 2, 3 and 13 in Tables VI and VII). When a single unlabeled nucleotide was omitted, the residual enzyme activity in the H reaction was greater than that of the P reaction (50-70% vs. 25-50%), and omission of all three unlabeled nucleotides in the H enzyme reaction mixture also resulted in a greater incorporation. Similar to the P enzyme, the H enzyme did not require putrescine, ammonium sulfate or 2-mercaptoethanol. In fact, ammonium sulfate inhibited the H enzyme activity to a greater extent than the P enzyme activity. The reasons for this difference are not clear. Another difference between these two enzymes is in the sensitivity of their product(s) to RNase T_1. As mentioned above, RNase T_1 rendered 93% of the P enzyme product acid-soluble whereas this nuclease was able to digest only about 80% of the H enzyme product.

A detailed study on the nature and concentration of the required components (DNA, Mn^{++}, etc.) had also been carried out and the results are discussed in the following paragraphs.

Incorporation in the Absence of One or More Nucleotides

Both the H and P enzymes catalyzed a significant amount of [^{3}H]-UMP incorporation in the absence of single nucleotides (Tables VI and VII, lines 4-6). These results are surprising since almost all other procaryo-

tic polymerases incorporated negligible amounts of a labeled nucleotide when a single species of nucleotide was omitted from the reaction mixture (Stevens, 1964; Chamberlin and Berg, 1962; Fox and Weiss, 1964; Johnson _et al_, 1971; Furth _et al_, 1962). Purified RNA polymerase from _E. coli_ or _M. lysodoiklicus_ can catalyze the synthesis of a homopolymer, such as poly adenylic or polyuridylic acid, in the presence of one, two or three nucleotides (Chamberlin and Berg, 1964; Stevens, 1964; Fox and Weiss, 1964). It was believed, therefore, that the residual synthesis catalyzed by the P enzyme in the absence of single nucleotides was due to the synthesis of homopolymer (polyuridylic acid in this case) and not RNA. Accordingly, the products, of the reactions carried out with single nucleotides omitted, were subjected to degradation by specific nucleases. The data (not shown) suggested that the product of the reactions was not RNA and probably was polyU. When CTP was used as the labeled nucleotide in a 4-NTP reaction, and single NTP's were omitted, the reaction was seen to be absolutely dependent on the presence of all 4NTP's (Table VIII).

Both the H and P enzymes catalyzed a larger amount of [^{3}H]-UMP incorporation into acid-insoluble polymer in the presence of only one nucleotide than in the presence of all 4 nucleotides (Tables VI and VII, cf lines 1 and 7). This was due to the phenomenon of homopolymer synthesis mentioned above and is discussed in greater detail in a later section, ("Homopolymer Synthesis").

Enzymatic and Alkaline Digestion of RNA Polymerase Product

To show directly that the product of the reaction catalyzed by _Rps. sphaeroides_ RNA polymerase was RNA, the product was subjected to digestion by specific nucleases and KOH. The results, shown for the P enzyme, are listed in Table IX. As can be seen, the product was completely resistant

Table VIII

Effect of Omission of Nucleotides on the Reaction

Catalyzed by the P Enzyme

Omission	nmoles of (^{3}H) CMP incorporated/mg protein	Percent of Complete
Complete System*	266	100
minus GTP	20	7.5
minus ATP	13	4.9
minus UTP	11	4.1
minus GTP, ATP, UTP	37	14

* The complete system contained: 50 mM TES-NaOH, pH 8.0, 2 mM $MnSO_4$, 150 μg/ml native calf thymus DNA, all four nucleotides at 0.5 mM and (^{3}H) CTP at a specific radioactivity of 10 mCi/nmole.

Table IX

Effects of Nucleases and Alkali on the Product Synthesized by the P Enzyme (4-NTP reaction)

Addition	nmoles of $[^3H]$-UMP incorporated per mg protein	%
none [(a)]	335	100
DNase I (50 μg/ml) [(b)]	426	127
RNase T_1 (175 units) [(b)]	74	22
RNase A (50 μg/ml) [(b)]	26	7.8
KOH (0.5 N, 37°C/18 hr)	25	7.5

[(a)] The complete system contained: 50 mM TES-NaOH, pH 8.0, 2 mM $MnSO_4$, 150 μg/ml of native CT DNA, 0.5 mM each of ATP, CTP, GTP and UTP; UTP was labeled to a specific radioactivity of 10 mCi/mmole.

[(b)] The enzymes were added in a volue of 5 μl to the complete system after an initial incubation of 50 min at 30°C, and incubation was continued for a further 65 minutes.

to DNase I and was sensitive to pancreatic RNase (RNase A), RNase T_1 and KOH.

DNA Template

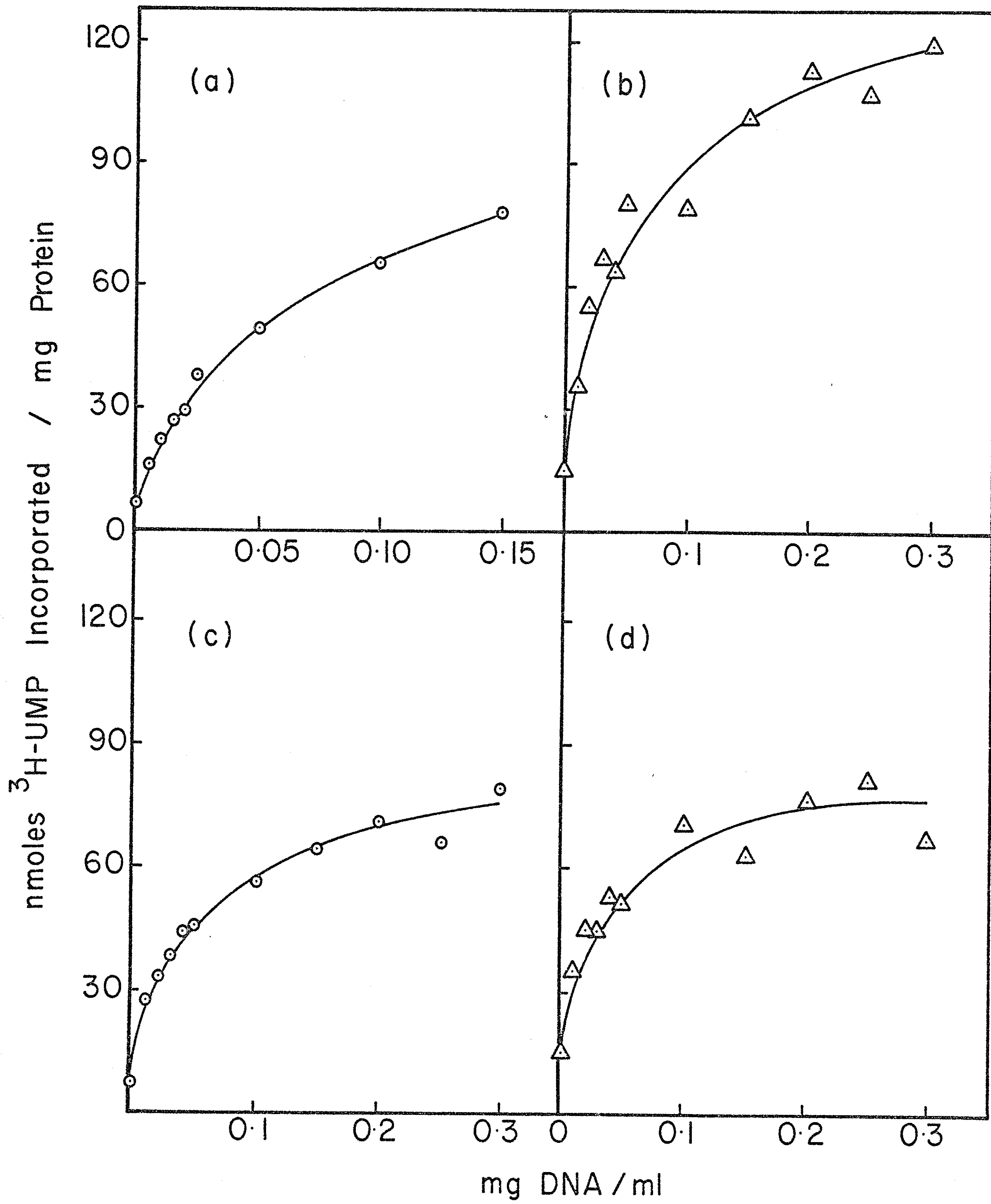
The effect of concentration and nature of DNA template on Rps. sphaeroides RNA polymerase activity (Fr. VI) was studied in a 4-NTP reaction mixture. Both P and H enzymes showed a low, but significant level of activity in the absence of DNA, indicating the presence of a small amount of polynucleotide phosphorylase in the enzyme preparation (Fig. 8). The addition of either single-or double-stranded calf thymus DNA greatly stimulated the amount of [^{3}H]-UMP incorporation (Fig. 8). No significant differences existed between the doubled-stranded and single-stranded DNA for the H enzyme activity (Figs. 8a and 8c) whereas the P enzyme showed a definite preference for double-stranded DNA (Figs. 8b and 8d).

Since one of the main goals of this study was to examine possible differences between the P and H RNA polymerases, in the hope of assigning a role to the enzymes in transcriptional control of gene expression, it was of obvious interest to test the template activity of the DNA's isolated from heterotrophic and photosynthetic Rps. sphaeroides cells. Only the native form of Rps. sphaeroides DNA was used and the results are shown in Figure 9. When H-DNA was used as a template, the H enzyme incorporated more [^{3}H]-UMP than when using P-DNA (Figs. 9a and 9b). On the other hand, the P enzyme showed a similar activity with DNA extracted from either H cells or P cells. In comparison among the double-stranded P and H Rps. sphaeroides and single- and double-stranded calf thymus DNA, the H enzyme was able to transcribe all four templates equally well. In contrast, the P enzyme transcribed double-stranded CT DNA approximately 60% better than the DNA from either H or P Rps. sphaeroides cells (calculated from the

FIGURE 8. Effect of different concentrations of native and denatured calf thymus DNA on enzyme Fraction VI of heterotrophic or photosynthetic *Rps. sphaeroides*. Enzyme activity was measured in the standard RNA polymerase reaction mixture with the indicated amount of double-stranded (a and b) or single-stranded calf thymus DNA. Putrescine, ammonium sulfate and 2-mercaptoethanol were omitted and $MnSO_4$ was 2 mM.

(⊙——⊙) Heterotrophic enzyme.

(△——△) Photosynthetic enzyme.

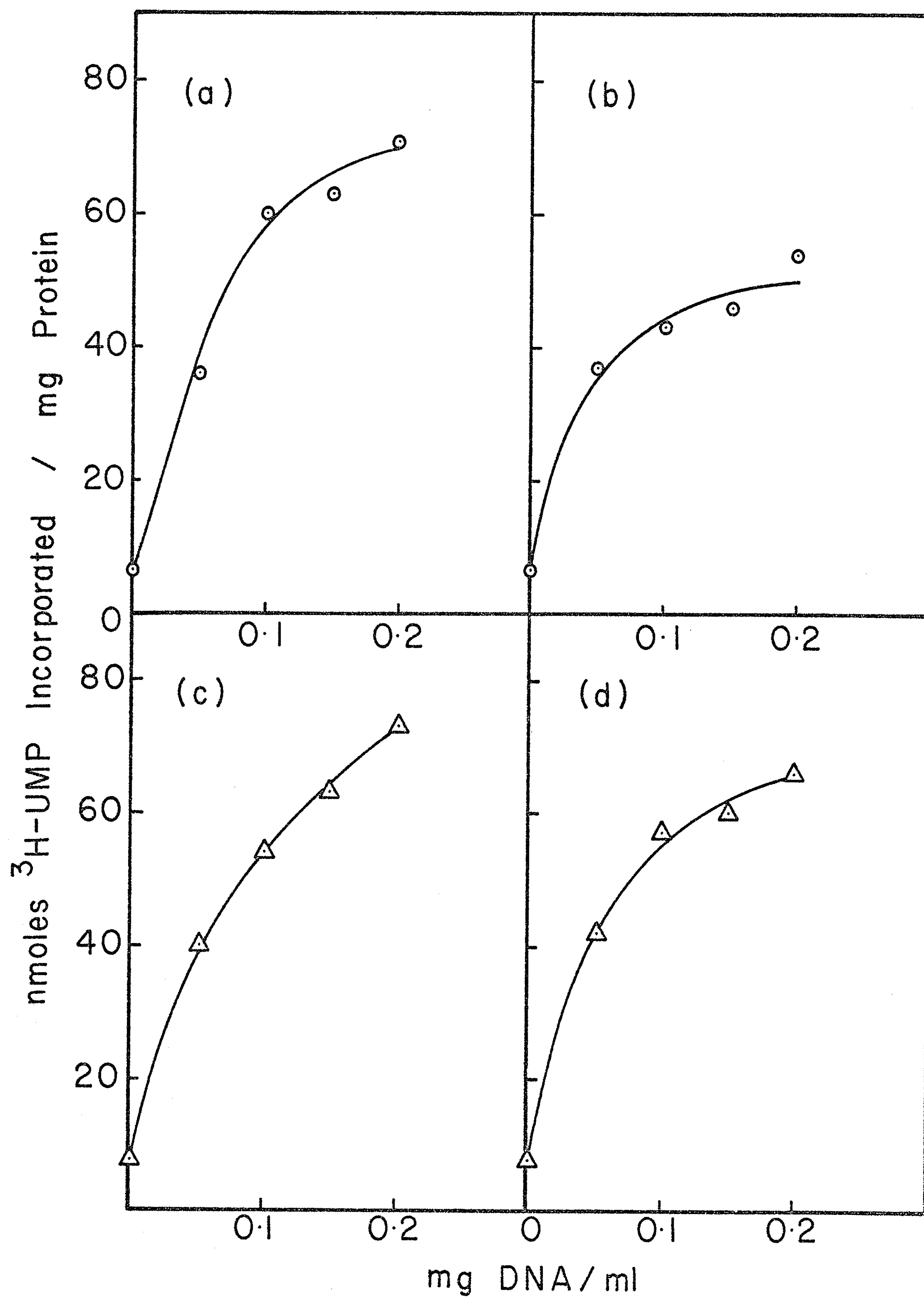


120
90
60
30
0
(a)
(b)
(c)
(d)
0·05
0·10
0·15
0·1
0·2
0·3
0
nmoles ^{3}H-UMP Incorporated / mg Protein
mg DNA / ml

FIGURE 9. Effect of different concentrations of native heterotrophic or photosynthetic Rps. sphaeroides DNA on enzyme Fraction VI of heterotrophic or photosynthetic Rps. sphaeroides. Enzyme activity was measured in the standard RNA polymerase reaction mixture with the indicated amount of heterotrophic (a and c) or photosynthetic (b and d) native DNA. Putrescine, ammonium sulfate, and 2-mercaptoethanol were omitted and $MnSO_4$ was 2 mM.

(⊙——⊙) Heterotrophic enzyme.

(△—△) Photosynthetic enzyme.



80
60
40
20
0
(a)
(b)
(c)
(d)
0·1
0·2
80
60
40
20
0
nmoles ^{3}H-UMP Incorporated / mg Protein
mg DNA/ml

amount of incorporation in the presence of 0.2 mg/ml of DNA, cf. Figs. 9 and 10). A similar results has been reported from Acinetobacter calcoaceticus RNA polymerase (Kleppe and Kleppe, 1976) which has a more than two-fold greater efficiency with CT DNA than with the DNA from A. calcoaceticus at concentrations of DNA above 25 μg/ml. Similarly, RNA polymerase isolated from Pseudomonas putida (Johnson et al, 1971) can also transcribe (native) CT DNA much more efficiently than P. putida DNA.

Effect of Mn^{++} and Mg^{++}

Like all other RNA polymerases than have been studied, Rps. sphaeroides enzymes require a divalent cation for maximum activity. This requirement has already been illustrated in Tables VI and VII. To probe this result further, various concentrations of Mn^{++} and Mg^{++} were tested for their effects on the activity of the H and P enzymes (Fig. 10).

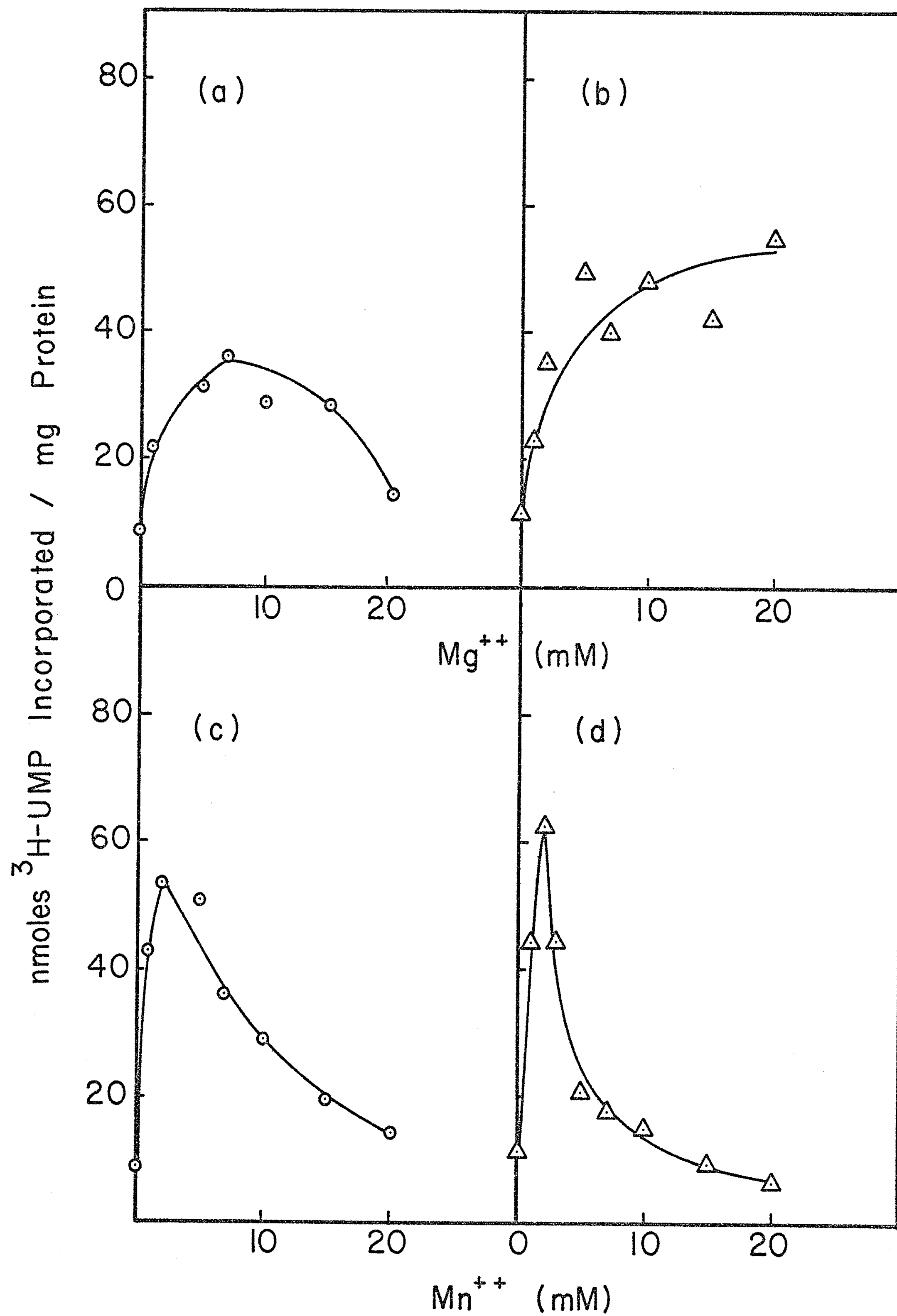
Both enzymes have a narrow optimum concentration range for Mn^{++}, with maximum activity at 2 mM (Figs. 10 c and 10 d). Magnesium can substitute, although to a lesser extent, for Mn^{++} and the optimum concentration range for both enzymes is broad. The H enzyme displays maximum activity at 7 mM Mg^{++}, at which concentration the enzyme activity is approximately 67% of that observed at 2 mM Mn^{++} (Compare Figs. 10 c and 10 a). No obvious optimum Mg^{++} concentration existed for the P enzyme in the concentration range tested (Fig. 10 b). At 20 mM Mg^{++}, the P enzyme activity was about 90% of the activity obtained when 2 mM Mn^{++} was used.

These results are quite similar to those reported by Kleppe and Kleppe (1976) for A. calcoaceticus RNA polymerase. Their results also show a narrow optimum range for Mn^{++} with a maximum at 3 mM and a broad optimum range for Mg^{++} with a maximum at about 10 mM; at 20 mM Mg^{++}, the activity is about 90% of the maximum. Similar results (narrow Mn^{++} optimum, maximum

FIGURE 10. Effect of different concentrations of $MgCl_2$ and $MnCl_2$ on enzyme Fraction VI of heterotrophic or photosynthetic _Rps. sphaeroides_. Enzyme activity was measured in the standard RNA polymerase reaction mixture with the indicated amount of $MgCl_2$ (a and b) or $MnCl_2$ (c and d) and with putrescine, ammonium sulfate and 2-mercaptoethanol omitted (Tables IV and V).

(O——O) Heterotrophic enzyme.

(△—△) Photosynthetic enzyme.


(a)
(b)
(c)
(d)
nmoles ^{3}H-UMP Incorporated / mg Protein
Mg^{++} (mM)
Mn^{++} (mM)
0
20
40
60
80
10
20

at about 2 mM Mn^{++}, broad Mg^{++} optimum) have also been reported for *E. coli* polymerase by Chamberlin and Berg (1962), Stevens and Henry (1964) and Furth *et al*, (1962) and for *Micrococcus lysodeikticus* polymerase by Fox and Weiss (1964).

KCl Effect

Ionic strength has profound effects on the transcription process ("Historical"); therefore, it was of interest to test the effect of increasing ionic strength on the P and H enzyme activities again, to ascertain whether or not there were any differences. Figures 11c and 11b show the effect of KCl on the amount of ^{3}H-UMP incorporation catalyzed by the H and P enzymes, respectively. Potassium chloride was not absolutely required for the enzyme activity but stimulated incorporation for both enzymes. Maximum stimulation of incorporation was obtained at 0.2 M KCl for the H enzyme and at 0.1 M KCl for the P enzyme. At 0.3 M KCl the transcriptional activity of both enzymes was strongly inhibited.

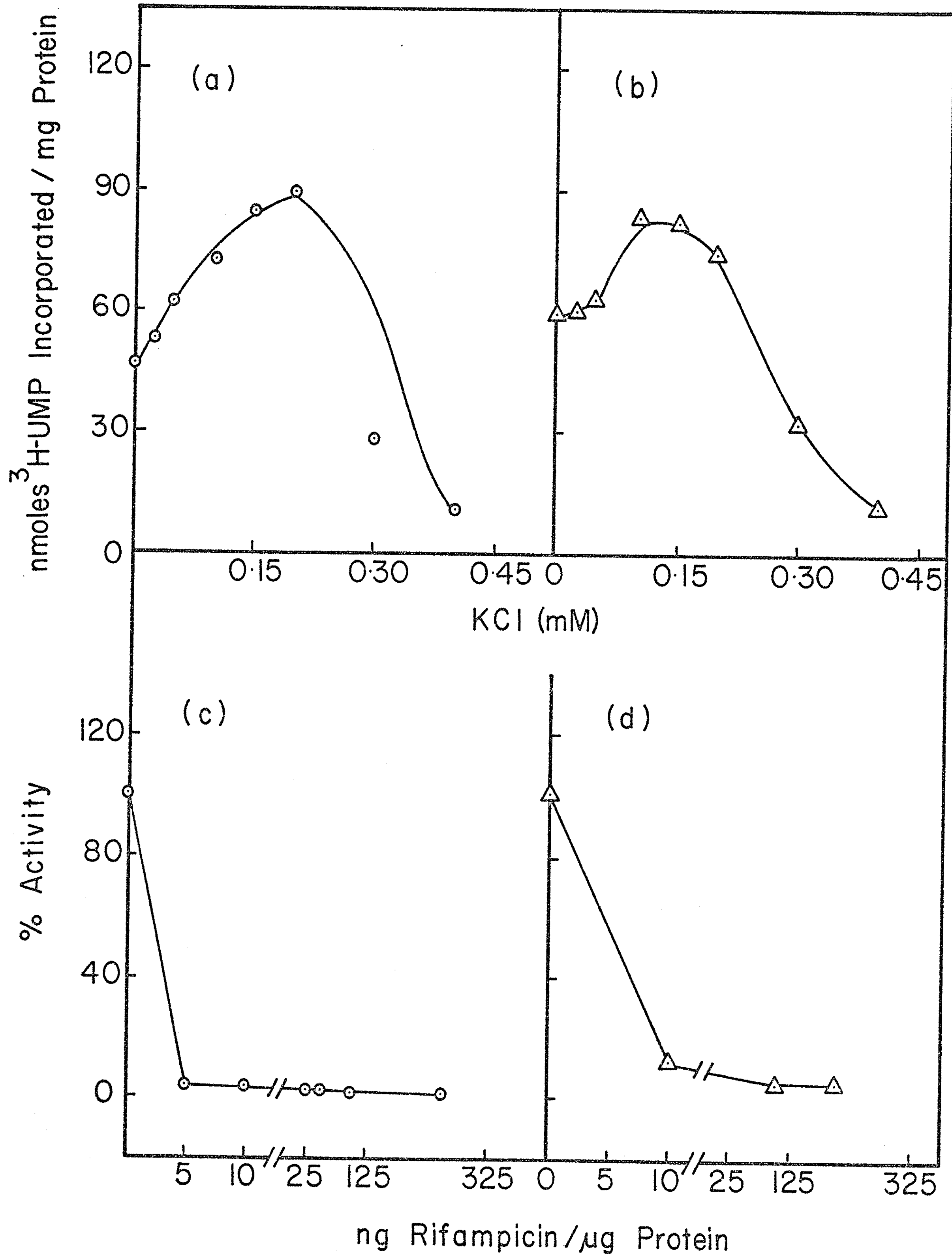
Rifampicin Effect

The inhibitory effects of rifampicin on the activity of the H and P enzymes are shown in Figs. 11c and 11d. The P enzyme was slightly more resistant to rifampicin than the H enzyme; to inhibit 50% of the enzyme activity required 2.5 and 6 mg of rifampicin per μg protein for the H and P enzymes respectively. These values are apparently much higher than the concentration of rifampicin needed to cause a 50% inhibition of *E. coli* RNA polymerase activity (Wehrli *et al*, 1968 a, b), however differences in enzyme purity render a direct comparison difficult.

FIGURE 11. Effects of KCl and rifampicin on enzyme Fraction VI of heterotrophic or photosynthetic Rps. sphaeroides. Enzyme activity was measured in a modified RNA polymerase reaction mixture with the indicated amount of KCl (a and b) or rifampicin (c and d). In addition to the NTP's and TES, the reaction mixture contained 2 mM $MnSO_4$, 150 μg/ml of native calf thymus DNA and either no (a and b) or 5 mM 2-mercaptoethanol (c and d). 100% activity was 127 nmoles/mg protein for the heterotrophic enzyme and 115 nmoles/mg protein for the photosynthetic enzyme.

(⊙——⊙) Heterotrophic enzyme.

(△—△) Photosynthetic enzyme.


(a)
(b)
nmoles ^{3}H-UMP Incorporated / mg Protein
120
90
60
30
0
0·15
0·30
0·45
0
0·15
0·30
0·45
KCl (mM)
(c)
(d)
% Activity
120
80
40
0
5
10
25
125
325
0
5
10
25
125
325
ng Rifampicin/µg Protein

Homopolymer Formation

From Tables VI and VII, it can be seen that the omission of three unlabeled nucleotides from the assay mixture resulted in a greater amount of [^{3}H]-UMP incorporation than when all four nucleotides were present. In an attempt to investigate this phenomenon further, a single NTP reaction ("Materials and Methods") were carried out in addition to the 4-NTP reactions, and the results are listed in Table X. Both the P and H *Rps. sphaeroides* enzymes were capable of incorporating low but significant amounts of GTP and CTP into acid-insoluble homopolymers. In both cases, more GTP or CTP was incorporated by the same enzymes in the presence of all four nucleotides than when only the labeled nucleotide was present (lines 1-4, Table X). The situation was the opposite for UTP and ATP. When UTP was the sole nucleotide in the reaction mixture, the H enzyme catalyzed 60% more incorporation than when all four nucleotides were present. For the P enzyme, the value was 18%. When labeled ATP was tested, the values were much higher. The H enzyme catalyzed 11 times more incorporation into homopolymer than into RNA while the P enzyme incorporated 15 times more ATP into homopolymer than into RNA. Because of these high values, and because of a similar phenomenon reported in other bacterial systems, such as *E. coli* (Chamberlin and Berg, 1964; Stevens, 1964) and *Micrococcus lysodeikticus* (Fox and Weiss, 1964), further studies were conducted on the polyadenylate-forming properties of the *Rps. sphaeroides* enzymes.

Time Course of Poly A Synthesis

Figure 12 illustrates the kinetics of poly A synthesis by the H and P enzymes. As in the case of RNA synthesis, the poly A reactions catalyzed by either enzyme proceeded without a detectable lag period. The reaction catalyzed by the H enzyme was linear for approximately 5 minutes while the

Table X

Homopolymer Formation by H and P Enzymes

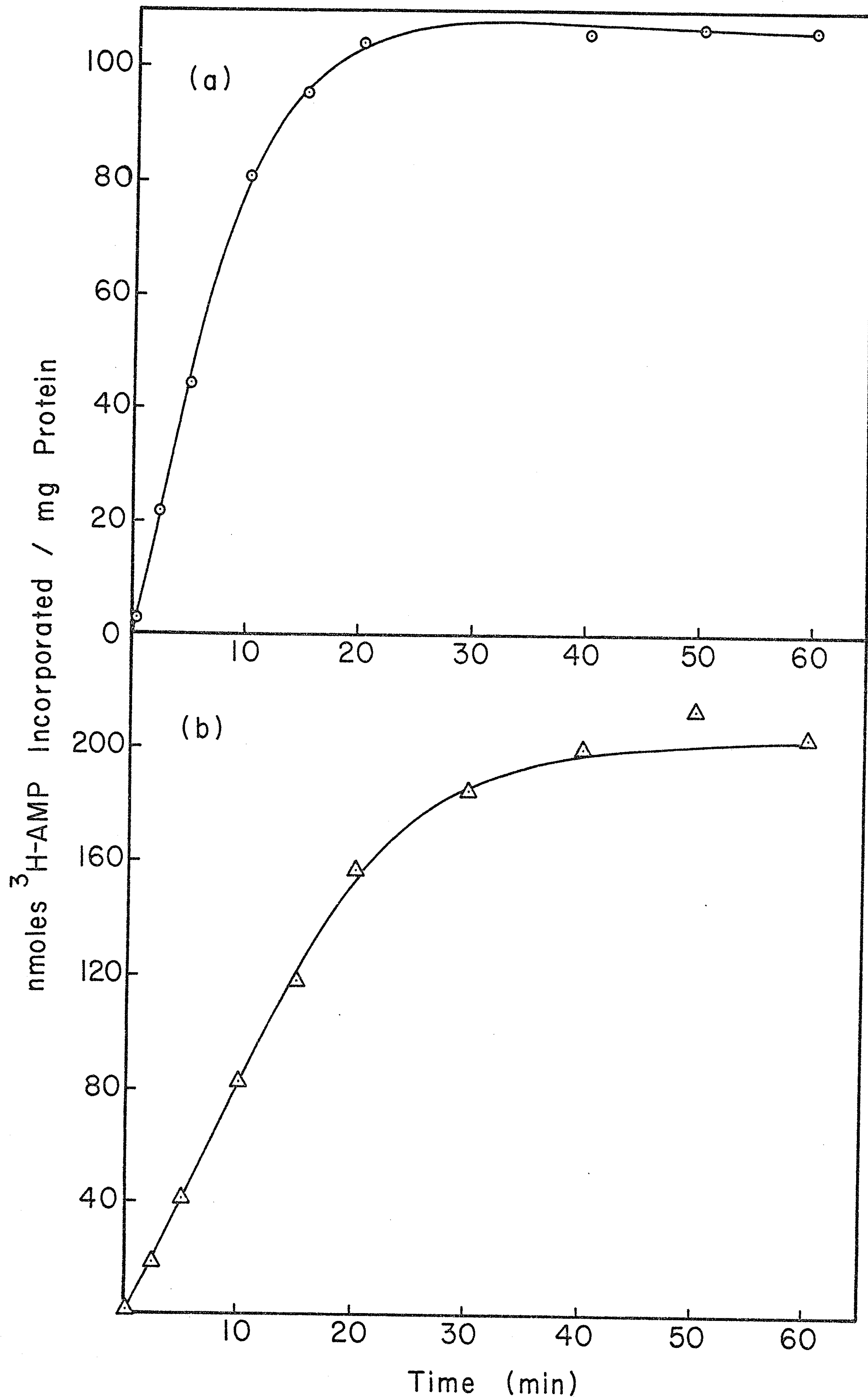
Nucleotides Added [a]	Nmoles of NMP Incorporated Per Mg Protein	
	H Enzyme	P Enzyme
[^{3}H]-GTP, CTP, ATP, UTP	81	118
[^{3}H]-GTP	12	21
[^{3}H]-CTP, GTP, ATP, UTP	147	238
[^{3}H]-CTP	23	30
[^{3}H]-UTP, GTP, CTP, ATP	59	95
[^{3}H]-UTP	93	112
[^{3}H]-ATP, GTP, CTP, UTP	38	52
[^{3}H]-ATP	406	803

[a] Each reaction mixture contained: 50 mM TES-NaOH, pH 8.0, 150 μg/ml each of native and denatured CT DNA, 0.5 mM of the indicated NTP's. The labeled triphosphate was present in each case at a specific activity of 10 mCi/mmole. The assay contained either 9.8 μg of P enzyme or 19.6 μg of H enzyme (Fraction VI).

FIGURE 12. Time course of poly A synthesis by enzyme Fraction VI of heterotrophic or photosynthetic *Rps. sphaeroides*. The reaction was carried out under the standard Homopolymer polymerase assay conditions.

(⊙——⊙) Heterotrophic enzyme.

(△—△) Photosynthetic enzyme.


(a)
100
80
60
40
20
0
10
20
30
40
50
60
(b)
200
160
120
80
40
10
20
30
40
50
60
nmoles ³H-AMP Incorporated / mg Protein
Time (min)

reaction catalyzed by the P enzyme proceeded at a linear rate for at least 15 minutes. For both enzymes, the reaction was essentially complete in 40 minutes.

Requirements for Poly A Synthesis

The effects of altering various components of the reaction mixture on the synthesis of poly A were investigated and the results are presented in Table XI. Both enzymes responded in a remarkably similar manner to alterations in the reaction mixture. Under the conditions employed in this experiment, both the H and P enzymes incorporated 16-18 times more AMP into poly A than into RNA. As was the case for RNA polymerase activity, the poly A polymerase activity showed an absolute dependence on the presence of a DNA template and a divalent cation. Although the enzymes could use double-stranded DNA as a template, they preferred single-stranded DNA. Both activities were inhibited by 97% in the presence of 50 μg/ml of rifampicin (333 ng/μg protein for the H enzyme and 250 ng/μg protein for the P enzyme).

These results (dependence on template and divalent cation, sensitivity to rifampicin) suggest that the enzyme activity for homopolymer synthesis was probably associated with that of the RNA polymerase and was not a separate contaminating enzyme.

Enzymatic and Alkali Digestion of the Poly A Product

To verify that the product of the single-NTP reaction was in fact a polyribonucleotide (in this case poly A), the product was subjected to digestion by specific nucleases and KOH. The results using the P enzyme, (Table XII) showed that the product of the reaction was completely resistant to the action of DNase I, RNase T_1, and pancreatic RNase and was completely susceptible to alkaline hydrolysis. It can be concluded, there-

fore, that the product is not DNA, contains neither pyrimidines nor GMP and that the A residues are incorporated into a structure which is likely held together by phosphodiester internucleotide linkages, i.e., that is polyadenylic acid.

Template Effect

The effect of various DNA templates on the synthesis of poly A by the H and P enzymes was tested and the results are illustrated in Fig. 13. No obvious preference for double-stranded CT DNA, DNA from H cells or DNA from P cells was demonstrated by either the H or the P enzymes.

Table XI

Effects of Various Reagents on the Synthesis of Polyadenylic Acid by RNA Polymerase

Component	H Enzyme		P Enzyme	
	nmoles $[^3H]$-AMP incorporated per mg protein	%	nmoles ^3H -AMP incorporated per mg protein	%
complete system (a)	580	100	808	100
" " + 3NTP's (0.5 nM each)	32	5.5	50	6.2
" " - (ss + ds) DNA	12	2.1	10	1.2
" " - ss DNA	475	81.9	488	60.4
" " - ds DNA	594	102	825	102
" " - Mn^{++}	30	5.2	33	4.1
" " - Mn^{++} + Mg^{++} (b)	266	45.9	350	43.3
" " + Rif (50 μ g/ml)	16	2.8	22	2.7
" " + GTP (0.5 mM)	167	28.8	160	19.8
" " + CTP (0.5 mM)	78	13	79	9.8
" " + UTP (0.5 mM)	156	26.9	156	19.3

(a) The complete system contained: 50 mM TES-NaOH, pH 8.0, 0.5 mM ATP (specific radioactivity 10 mCi/mmole, 2 mM $MnSO_4$, 150 μ g/ml each of native (ds) and denatured (ss) DNA.
(b) Mg^{++} was added to a final concentration of 7 mM for the H enzyme and to a final concentration of 10 mM for the P enzyme.

Table XII

Effects of Nucleases and Alkali on the Product Synthesized by the P Enzyme (single NTP reaction)

Addition	nmoles of $[^3H]$-AMP incorporated per mg protein	%
none [(a)]	431	100
DNase I (50 μg/ml) [(b)]	529	123
RNase T_1 (175 units) [(b)]	563	131
RNase A (50 μg/ml) [(b)]	518	120
KOH (0.5 N 37°C/18 hr)	6	1

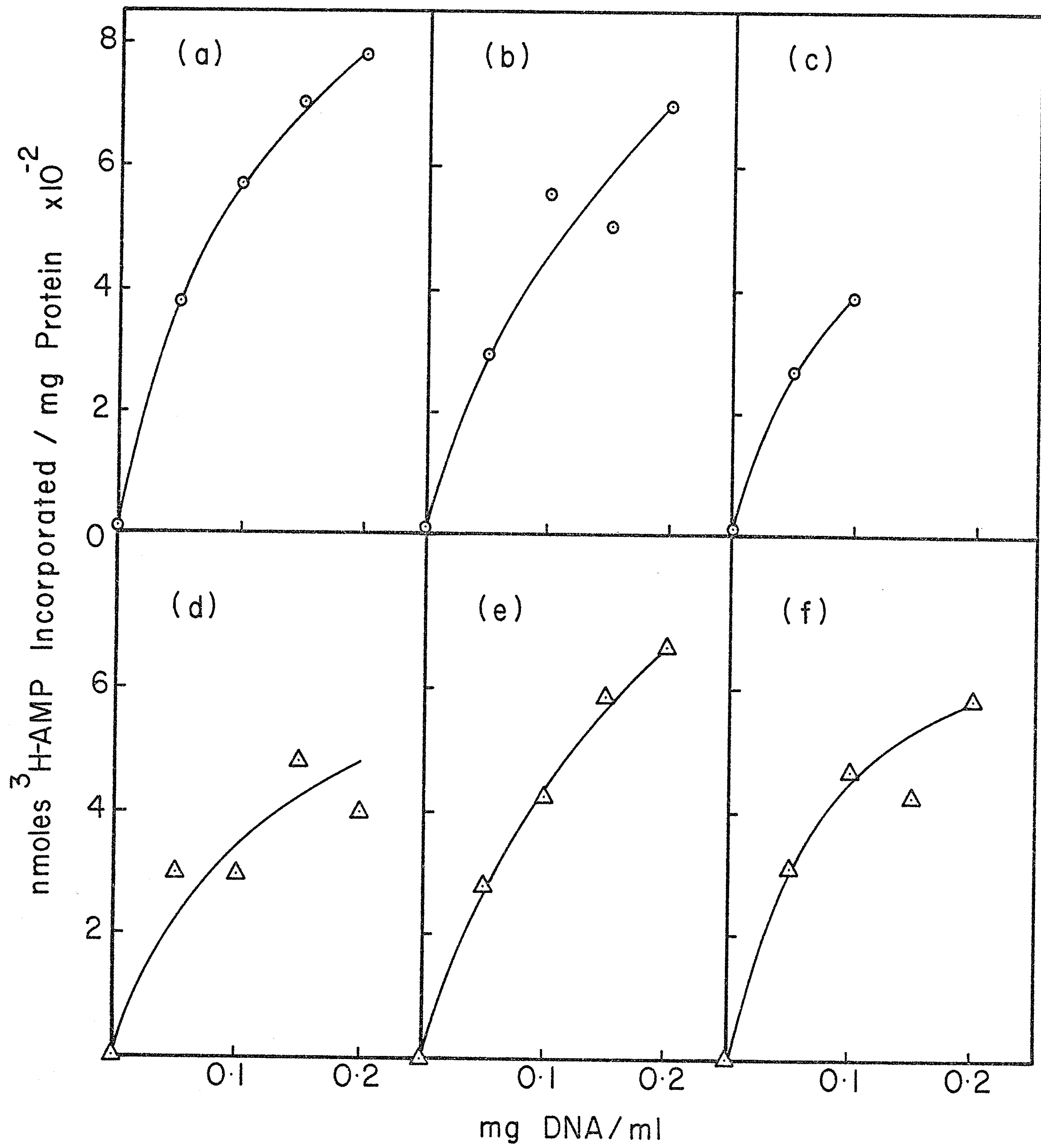
(a) The complete system contained: 50 mM TES-NaOH, pH 8.0, 2 mM $MnSO_4$, 150 μg/ml of native CT DNA and 0.5 mM ATP (specific radioactivity 10 mCi/mmole).

(b) The nucleases were added to tubes containing the complete system after an initial incubation of 50 minutes and incubation was continued at 30°C for 65 minutes.

FIGURE 13. Effect of different concentration of heterotrophic or photosynthetic *Rps. sphaeroides* native DNA or native calf thymus DNA on enzyme Fraction VI of heterotrophic or photosynthetic *Rps. sphaeroides*. Enzyme activity was measured in the standard homopolymer polymerase reaction mixture with ATP as the labeled nucleotide. The templates were: native calf thymus DNA (a and d), native heterotrophic *Rps. sphaeroides* DNA (b and e) or native photosynthetic *Rps. sphaeroides* DNA (c and f).

(⊙——⊙) Heterotrophic enzyme.

(△—△) Photosynthetic enzyme.


(a)
(b)
(c)
(d)
(e)
(f)
8
6
4
2
0
6
4
2
0·1
0·2
0·1
0·2
0·1
0·2
nmoles ^{3}H-AMP Incorporated / mg Protein x10^{-2}
mg DNA/ml

DISCUSSION

DISCUSSION

Purification of the Enzyme

As described in the Historical, a number of purification methods for bacterial RNA polymerase have been developed. All of these methods are based on six specific properties of the enzyme (Burgess, 1976):

(i) high molecular weight

(ii) reversible salt-dependent aggregation

(iii) charge

(iv) solubility

(v) DNA-binding

(vi) specific binding to substrates and inhibitors

In the present study, the Rps. sphaeroides enzymes were partially purified by methods based on only 3 of the above properties; high molecular weight, charge and solubility. The methods employed were not very effective as indicated by the per cent yield and implied by the fold of purification although this in itself is not a good indicator of protein purity (Tables II and III). The purification procedure was also not equally effective in purifying the H and P enzymes. Some explanation for this difference in effectiveness are discussed below.

From Tables II and III, it is clear that the homogenization procedure ("Materials and Methods") was not equally effective in extracting RNA polymerase from the H and the P cells. While the crude extract from the H cells contained less protein than that from the P cells (1200 mg vs 4400 mg), it contained more total enzyme activity (13300 units vs 8760 units). Thus the specific activity of the H cell crude extract was significantly higher (by a factor of five) than the P cells crude extract. This same large difference in specific activity was also evident after the high-speed centrifugation step (cf. Tables II and III, line 2). The reasons for these observations are

not clear; however, at least one explanation can be offerred.

When _Rps. sphaeroides_ is transferred from heterotrophic (dark-aerobic) to photosynthetic (light-anaerobic) conditions, a major cellular reorganization occurs. The major change is the synthesis, and the incorporation into the cytoplasmic membrane, of proteins and other components that mediate the light-dependent reactions. Concomitant with the photosynthetic pigment and protein synthesis is the formation of the chromatophores (Oelze and Drews, 1972; Schachman _et al_, 1952). In _R. rubrum_ it is known that the number of chromatophores per bacterium is inversely proportional to the light intensity to which the cells are exposed during growth (Holt and Marr, 1965). This may also be true in _Rps. sphaeroides_ where it has been shown that the pigment content of the bacteria increases in inverse proportion to the light intensity to which the cells are subjected (Cohen-Bazire _et al_, 1957; Lessie, 1965 b).

In the present study the P cells were grown in 10 or 15 liter carboys ("Materials and Methods"). By the time the cells had reached the late logarithmic phase of growth, considerable self-shading would have occurred (Sistrom, 1962 a). Thus the intracellular content of chromatophores would have been relatively high. In contrast, the heterotrophic cells, although visibly pigmented due to the decrease in oxygen tension caused by increasing cell density, likely would have contained less photosynthetic membrane material. As mentioned in "Materials and Methods", the extraction of the P enzyme was carried out after the extraction of the H enzyme. In an attempt to increase the efficiency of extract, about twice as many glass beads were used to extract the P enzyme. It is likely that this greater quantity of glass beads was more effective in rupturing the P cells since the total protein in Fraction I of the P cells was almost three times higher than the total protein in Fraction I of the H cells (4400 mg vs 1600 mg); the weight of cells used for the extraction was approximately

the same in both cases. It would be expected all other things being equal, that three times as much enzyme activity would be present. In fact, the enzyme activity in the P cell crude extract was actually about 30% lower than in the H cell crude extract (8800 units vs 13300 units). Since the major structural difference between the H and P cells was in their membrane organization, it could be proposed that the RNA polymerase activity was somehow associated with the photosynthetic membrane material and was sedimented with the other cell debris during the first (low speed) centrifugation. There are, of course, other trivial explanations such as: less polymerase synthesized in the P cells, greater loss of polymerase by adsorption onto the glass beads, etc. These explanations, however, do not explain the further loss of polymerase in the P cell extract at the next purification step. In the present explanation, it is assumed that the greater quantity of glass beads added to the P cells ruptured more cells and produced small and large membrane fragments. The large fragments would have been sedimented along with the other cell debris as mentioned above. The small fragments, with some associated polymerase, would not have been sedimented until the high speed centrifugation step. In comparing lines 1 and 2 of Table III (the P cell extraction) it can be seen that there is a 16% loss of polymerase activity. In contrast, there was no loss of polymerase activity in the H cell extraction during the same purification step (cf. lines 1, 2 in Table II), although the total protein decreased by 60%. This supports the suggestion that in the P cells, the RNA polymerase activity is associated with or occluded within the chromatophore fragments. Further supporting this suggestion were the repeated observations, in preliminary experiments, that the high speed centrifugation pellet from the P cells showed considerable RNA polymerase activity. This activity eluted from a DEAE cellulose column at 0.3 M KCl. In contrast, the pellet obtained from H cells showed

negligible activity.

The considerable drop in enzyme activity for both H and P preparations in going from the first to the second ammonium sulfate fractionation steps was likely due to, as mentioned in the "Results", too high a concentration of enzyme which may have led to aggregation and partial inactivation or the inhibitory effects of ammonium sulfate or both.

It is not known whether the change in elution conditions in the DEAE-cellulose chromatography step of the P enzyme ("Materials and Methods") did in fact improve the purification since part of the aliquot from the H cell Fraction V (pooled DEAE-cellulose) was lost and protein analysis could not be done. The large drop in total activity of the H enzyme is going from the DEAE-cellulose to the Sephadex G-200 chromatography (2290 to 617 units) was due to accidental physical loss of the enzyme during concentration of the pooled Sephadex fraction with ammonium sulfate.

The present purification scheme likely could have been improved by exploiting the other unique properties of bacterial polymerases listed in the beginning of this Discussion. The use of a DNA-cellulose column or a column to which rifampicin was covalently bound might have increased the final specific activity. In addition, the use of Polymin P (Historical) would perhaps have facilitated the separation of the polymerase from nucleic acids. The A_{280}/A_{260} ratios of Fraction VI protein (Table V) reveal that a significant quantity of nucleic acids was probably still associated with the polymerases.

Criteria of Purity

Traditional criteria of protein purity include: electrophoresis in polyacrylamide gels, measurement of the A_{280}/A_{260} ratio and sedimentation in the analytical ultracentrifuge. In this study, the H and P polymerases were sub-

jected to electrophoresis in SDS-polyacrylamide gels and their A_{280}/A_{260} ratios were measured. As mentioned above, the A_{280}/A_{260} ratios showed the presence of low but significant levels of nucleic acids in the Fraction VI H and P protein. The results of the electrophoresis will be discussed below. A more exacting criterion of protein purity, applicable to isolation of RNA polymerase, is to stain and destain polyacrylamide gels containing various precise amounts of enzyme and then to scan the stained gels to obtain densitometric tracings (Burgess, 1976). The amount of polymerase present in a preparation can then be estimated by comparing the area under the $\beta + \beta'$ peak to a standard curve obtained by using known quantitites of subunits. Similarly, the amount of impurities can be estimated by determining the amount of absorbance not due to the polymerase subunits (Burgess, 1976).

Properties of the Enzyme

Both the H and the P *Rps. sphaeroides* RNA polymerase activities demonstrated similar requirements (Tables VI and VII); however, the H enzyme showed a higher residual activity than the P enzyme in the absence of DNA or Mn^{++} and its product was almost three times more resistant to RNase T_1 than that of the P enzyme (19% vs 7%). The greater residual activity of the H enzyme in the absence of DNA or Mn^{++} may have been due to the presence of a contaminating enzyme activity such as polynucleotide phosphorylase. In the complete system, although the H enzyme incorporated only about 40% as much [^{3}H]-UMP as the P enzyme (23.8 nmoles vs 56.5 nmoles), the percentage of product that resists degradation by RNase T_1 was expected to be similar in both cases, assuming these products were identical in size and composition. Since the percentage differed almost by a factor of three (see above), this assumption of similarity in size and composition of products is probably incorrect. The results may imply that, from the same

template, qualitatively different products were synthesized. However, other considerations, such as differences in enzyme purity, could certainly complicate this interpretation.

The H enzyme of Rps. sphaeroides incorporated more [^{3}H]-UMP using H-DNA than P-DNA, whereas the P enzyme utilized both templates with equal efficiency (Fig. 9). Both enzymes were able to use double-stranded calf thymus DNA even more efficiently than either bacterial DNA template (cf. Figs. 8 and 9). These results suggest that some kind of template specificity may differentiate the two enzyme activities. However, more careful experiments such as studies of enzyme binding to specific DNA sites, ionic strength effects on template conformation and rates of initiation and elongation under various conditions must be carried out before any firm conclusions can be drawn.

Although there were also differences in the effects of KCl and rifampicin concentration on the two enzymes, no conclusion about any mechanistic differences between the two enzymes can be drawn.

The observation that both the H and P enzyme activities were excluded from Sephadex G-200 gels and eluted in the void volume (Figs. 4 and 5) has raised an interesting point: since the exclusion limit of the G-200 gels is about 800 000, these results would suggest that the molecular weight of these enzymes is higher than 800 000 in contrast to other bacterial polymerases which have a molecular weight range of 400 000 to 500 000 for the holoenzyme ("Historical"). This apparently high molecular weight value is also supported by the results of the polyacrylamide gel electrophoresis experiments (Fig. 6) in which the presence of many protein bands in both the H and P Fraction VI enzyme was observed. However, it is believed that some of these bands are due to the presence of contaminating proteins. In addition, the possibility that the apparently very high molecular weights were due to aggre-

gation of the enzyme under the elution conditions cannot be ruled out at the present stage. If present, this aggregation would be quite distinct from that of the *E. coli* RNA polymerase which aggregates under low ionic strength and dissociates at high ionic strength (Burgess, 1969 a). In the present study, the *Rps. sphaeroides* polymerases were eluted from the Sephadex column under conditions of relatively high ionic strength (0.4 M KCl).

In summary, the H and P enzymes are basically identical in the properties which are common to other bacterial polymerases such as: requirements, kinetics, presence of β and/or β', σ and α subunits, sensitivity to rifampicin, etc. Moreover, *Rps. sphaeroides* enzymes also demonstrated considerable homopolymer polymerase activities.

Properties of the Product

From the data in Table X, it is possible to calculate the Pu/Pyr (A + G/U + C) and dissymmetry (A + U/G + C) ratios in the RNA product of the H and P enzymes. For the H enzyme, the Pu/Pyr ratio is 0.58 while that of the P enzyme is 0.51, and the dissymmetry ratios of the products synthesized by the H and the P enzyme are 0.43 and 0.41 respectively. These values are significantly different from those of the calf thymus DNA template which, in native form, has Pu/Pyr and dissymmetry ratios of 1.0 and 1.35 respectively (Mahler and Cordes 1971) suggesting that one of the DNA template strands has been transcribed preferentially. Because, if both strands were transcribed, the Pu/Pyr and dissymmetry ratios of the product should be similar to those of the template. An alternative explanation is that certain short segments of both strands are transcribed. Either explanation would imply that the *Rps. sphaeroides* enzymes are specific in selecting binding and initiation sites. This apparent high selectivity is

in contrast to the early results observed in *E. coli* (Chamberlin and Berg, 1962) and in *M. luteus* (Furth *et al*, 1962) RNA polymerase systems. In both studies, the base ratios of the RNA product were very similar to those of the DNA template.

Homopolymer Polymerases

The homopolymer polymerase activities of the H and P enzymes were also of interest, not only because of their potency but also because of their ubiquitous presence in purified RNA polymerases of other bacteria (Chamberlin and Berg, 1962; Fox and Weiss, 1964; Stevens and Henry, 1964). Although the biological function if any, of this homopolymer polymerizing activity is still obscure, any obvious differences in activity between the H and P enzymes might provide a clue to the means by which transcriptional control is exerted. For this purpose the poly A polymerase activities of both the H and P enzymes unfortunately responded in a very similar manner to changes in various components (Table XI) and to different templates (Fig. 12).

Similar to the poly A polymerase activities associated with the DNA polymerase of *E. coli* (Chamberlin and Berg, 1962; 1964), *Azotobacter vinelandii*(Krakow and Ochoa, 1963) and *M. Luteus* (Fox and Weiss, 1964), the H and P poly A polymerase activities were absolutely dependent on the presence of a DNA template and a divalent cation (lines 3 and 6, Table XI). Unlike the *E. coli* polymerase, the *Rps. sphaeroides* enzymes were able to use double-stranded DNA almost as well as single-stranded DNA. Similar to the *E. coli* enzyme but unlike the *A. vinelandii* enzyme, the *Rps. sphaeroides* enzymes incorporated more ATP in the absence of three nucleotides than in their presence. The inhibition of poly A synthesis by single nucleotides was similar in magnitude to the "low efficiency inhibition" of the DNA-directed poly A synthesis catalyzed by *E. coli* polymerase (Chamberlin and

Berg, 1964). It is not possible to deduce from these similarities that the *Rps. sphaeroides* enzyme catalyze the synthesis of poly A by the same mechanism as the *E. coli* enzyme. The only significant difference between the *Rps. sphaeroides* enzymes was that the H enzyme incorporated less [^{3}H]-AMP in the complete system (single-NTP reaction) than the P enzyme (Table XI). A similar result was obtained when the RNA polymerase activity was measured (4-NTP reactin, cf. line 1 of Tables VI and VII). No attempt was made to measure template independent synthesis of [poly (A):poly (U)] as described by Smith *et al*, (1967).

In sporulating bacteria, it is known that vegetative RNA polymerase cannot transcribe sporulation genes unless it is modified, presumably by proteases (Millet *et al*, 1972, Leighton *et al*, 1972). Furthermore, RNA polymerase isolated from *Bacillus subtilis* cells in early stages of sporulation cannot transcribe DNA from its infecting phage ϕe *in vitro* (Losick and Sonenshein, 1969). Many other examples of enzyme modification by alteration of subunits (by phosphorylation or adenylation) or by association of regulatory proteins with polymerase are known (Losick and Pero, 1976). No such kind of obvious changes in *Rps. sphaeroides* grown under radically different conditions (heterotrophic vs photosynthetic) has been observed in this study; however, the possibility of such changes occurring still remains until more sophisticated techniques are employed to prove otherwise.

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