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PURIFICATION AND CHARACTERIZATION OF MULTIPLE FORMS OF DNA-DEPENDENT RNA POLYMERASE FROM THE BRINE SHRIMP, *ARTEMIA SALINA*

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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ABSTRACT

PURIFICATION AND CHARACTERIZATION OF MULTIPLE FORMS OF DNA-DEPENDENT RNA POLYMERASE FROM THE BRINE SHRIMP, ARTEMIA SALINA

by

REBECCA P. ELLISON

Advisor: Professor Carolyn Burdick

Three classes of DNA-dependent RNA polymerase have been purified several thousand fold from the brine shrimp, Artemia salina. Methods include sonication in 0.4 M ammonium sulfate, high speed centrifugation, ammonium sulfate fractionation and precipitation of nucleic acids with the synthetic polycation, Polymin-P.

In a large scale purification procedure, based on chromatography on DEAE-cellulose and DNA-cellulose, 25% of the original enzyme activity was recovered while reducing the total protein from 33.7 g (crude homogenate fraction) to 13.8 mg. Specific activity of 47 units/mg was achieved [one unit of enzyme activity is the amount required for the incorporation of one nanomole of UMP into acid-precipitable material in 10 min at 37°C]. Hydrated Artemia cysts (1,231 g) had a yield of 325 units of polymerase activity prior to separation of the enzyme classes.

Chromatographic separation of the enzyme classes was achieved by chromatography on DEAE-cellulose followed by rechromatography on DEAE-Sephadex. Artemia RNA polymerase I activity eluted from DEAE-Sephadex at 0.09 M ammonium sulfate. Polymerase I was not extensively characterized

in the present study.

Chromatographic heterogeneity of Artemia RNA polymerase II was discovered. Polymerase IIA elutes from DEAE-Sephadex at 0.16 M ammonium sulfate and polymerase IIB elutes at 0.23 M ammonium sulfate. The catalytic requirements of these two chromatographic forms of the polymerase were identical. CT DNA_{den} was the preferred template by a factor of 3.5 over CT DNA_{nat} and by a factor of 7.1 over $d(A\text{-}T)_n$. Polymerase IIA and polymerase IIB had ammonium sulfate optima between 0.075 M and 0.100 M and a Mn^{++}/Mg^{++} activity ratio of 4. Maximum activity occurred at 2 - 3 mM Mn^{++} or 6 mM Mg^{++}. Both enzyme IIA and enzyme IIB were highly sensitive to the inhibitor, alpha-amanitin, and were totally inhibited by 0.04 ug/ml. Fifty percent inhibition occurred at 0.019 to 0.028 ug/ml alpha-amanitin.

The two chromatographic forms of polymerase II appeared to differ in their stability; within 52 hours of the homogenization of the hydrated Artemia cysts, polymerase IIA was found to have a specific activity 380 times lower than that of polymerase IIB.

Artemia polymerase III eluted from DEAE-cellulose between 0.11 M and 0.19 M ammonium sulfate; upon rechromatography on DEAE-Sephadex, elution occurred at 0.32 M ammonium sulfate. The polymerase III activity of a crude Artemia cyst homogenate co-eluted with polymerase IIB on DEAE-Sephadex at 0.23 M ammonium sulfate. The existence of several chromatographic forms of Artemia polymerase III seems likely.

The Artemia polymerase III eluting at 0.32 M ammonium sulfate from DEAE-Sephadex preferred the template, $d(A\text{-}T)_n$, 2.3 fold over CT DNA_{nat} and 1.1 fold over CT DNA_{den}. Enzyme III had a Mn^{++}/Mg^{++} activity ratio of 3.2 with CT DNA_{den} as template and 12.0 with $d(A\text{-}T)_n$ as template. The

optimum Mn^{++} concentration was approximately 6 mM. Enzyme III was insensitive to 120 ug/ml alpha-amanitin.

DEDICATION

This work is lovingly dedicated to the memory of my aunt,

Mary Powel Phillips

1914 - 1976

ACKNOWLEDGMENTS

I am pleased to thank the many people who have contributed in some way to the completion of this project although not all are mentioned here. The City University Graduate Center administration, Miss F. Bloch and Dean N. Rees in particular, and Executive Officer Dr. L. Moriber and Department Chairman Dr. R. Mawe have been consistently efficient and occasionally most generous.

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INTRODUCTION

Background

The development of a complex organism from a fertilized egg is an elaborate procedure involving an intricate combination of cell division and cell differentiation. Individual cells take on particular characteristics very early in development, possibly as the result of the partition of cytoplasmic determinants present in the ooctye. In protostomes, two identifiably different cells are created by the first cleavage. As cells undergo successive divisions, they become increasingly specialized and ultimately reach the level of differentiation which is typical of their condition in the mature organism.

This specialization occurs in two stages, the first of which is cell determination. A determined cell is committed to the future expression of a particular phenotype. The determined state is transmitted vertically without expression to progeny cells. Phenotypic expression of the specialized trait is withheld until the determined cell receives a signal to differentiate. The imaginal discs of Drosophila are examples of determined cells which show no evidence of differentiation yet maintain their determined state despite serial transplantation under conditions where the stimulus to differentiate is absent [Kauffmann 1973].

In general, cell division produces daughter cells with a level of determination equal to or more specific than the parental cell. When development of the organism has been completed the individual cells

maintain their identity and perform their specialized (differentiated) functions until the death of the organism.

How the concerted events of development are programmed into the unfertilized egg is a most intriguing and difficult question. The developmental program not only determines which cells undertake certain specialized functions, it also determines the temporal sequence in which they do so. Regulation theoretically could occur at the level of the chromosome (irreversible condensation or actual loss), the gene (transcriptional control), RNA (post-transcriptional mRNA processing and modification or translational control) or protein (post-translational protein modification).

Models for Differentiation

Several models have been proposed to explain the differentiation process. One of the earliest theories held that the cells of a developing system experienced a differential loss of genetic material. The resulting differential loss of capability would result in a narrowing of functional possibilities. Actual loss of chromosomes was found in the somatic cells of Ascaris [Hogue 1910].

Although a narrowing of functional possibilities is essentially what occurs in a differentiating system, actual loss of chromatin is now known to be the exception rather than the rule [Davidson 1968]. An example of DNA which has been irreversibly modified is the Barr body or randomly inactivated X chromosome in cells of human females [Hood 1975]. The general rule is that the genetic componant of each cell is the quantitative and qualitative equal of that of the zygote.

Dramatic evidence for the absence of irreversible change during differentiation is the demonstration that nuclei from cells with

differentiated functions are able to direct development of entire animals upon transplantation into enucleated oocytes [Gurdon 1964; Briggs and King 1960].

A second explanation for the appearance of differentiated structures or functions is the selective reiteration or amplification of genes. A few examples of this have been discovered. Genes coding for the histones of developing sea urchins [Kedes 1971] and genes coding for rRNA [Brown and Sugimoto 1973; Brown and Dawid 1968; Wallace and Birnstiel 1966; Lewin 1974] and tRNA [Clarkson 1973] in Xenopus laevis somatic cells have been found in greater than 2C amounts. The ribosomal gene complement in Xenopus oocytes is multiplied approximately 1000 fold in the oocyte [Hood 1974]. Instances of such amplification are rare and restricted to situations where increased production is critical. In mice for example, 70% of the DNA is present as single copies [Britten and Kohne 1968]. Hybridization experiments show that most transcribed DNA is present as unique sequences. Reiteration and differential gene amplification are therefore unusual situations and cannot explain the totality of development.

Control on another level, that of differential gene expression, best explains what is known about the appearance of specialized cell products during development and their continued presence in particular cell types.

Differential gene expression is the result of limited and controlled gene activity. The transcriptionally active areas of the chromatin specify what proteins are synthesized. A comparison of the sequence homology of RNAs isolated from various differentiated tissues shows that different populations of mRNAs are present in different develop-

mental stages or different tissues of the same organism [Davidson and Britten 1973].

Davidson and Britten have developed a model for gene control in eukaryotes which was designed to account for the temporal control of expression of different sets of genes and the ability of a single effector to trigger production of different proteins or sets of proteins in different target cells. The model describes a cascade of positive regulatory events occuring at the level of transcription.

Cells take on their differentiated characteristics as the direct result of the production of a particular set of mRNAs coding for proteins which are either structural or functional. The array of proteins produced determines the cell's identity. Cells may share functions and this creates a range of overlapping identities.

In eukaryotic cells, the genetic material is embedded in a very complex structure. DNA is packaged in the chromosomes along with approximately equal amounts of protein and a small amount of RNA. Both histones and nonhistone proteins are included in this complex. The histones and the DNA are packaged in repeating units called nucleosomes or nu bodies which give the chromatin a beaded appearance.

Access to eukaryotic DNA is made difficult by the structural complexity of the chromosome. Production of an mRNA transcript coding for a particular protein requires a highly controlled transcripitional event. DNA site identification, initiation of transcription and termination of transcription must be precise. The complete array of determinants of transcriptional precision is unknown at the present time, however a certain amount of the precision is thought to reside in the highly complex enzyme, RNA polymerase.

Eukaryotic RNA Polymerases

Background

The eukaryotic polymerases are all very large molecules with molecular weights in excess of 500,000 daltons. They are composed of multiple subunits. SDS gel electrophoresis shows a distinct subunit composition for each polymerase class [Sklar, Schwartz and Roeder 1975]. Smaller molecular weight subunits are shared between polymerases, however the larger subunits are clearly different and unique to each class.

The heterogeneity which exists within each class is the result of size or charge differences in certain subunits. In at least one case, heterogeneity is due to the total lack of one subunit. Heterogeneity is observed after column chromatography or upon polyacrylamide gel electrophoresis under denaturing conditions.

Questions of molecular structure, catalytic properties, chromatographic behavior and intracellular localization help to identify members of each polymerase class. These aspects of identification will be considered in depth for each class of polymerase. Enzyme yield and inhibitor sensitivities as well as possible control mechanisms will be explored.

Enzyme activity levels are known to be higher in some tissues than others and the levels of enzyme classes are known to fluctuate independently under different conditions of physiological state or hormonal influence. For example, levels of polymerase I and polymerase II are independently regulated during cell division in yeast [Sebastian, Takano and Halvorson 1974].

Diversity

RNA polymerase was first discovered by Weiss and Gladstone [1959] in rat liver [see also Weiss 1960 and Weiss 1976]. The activity of this enzyme was later shown to be DNA-directed by Hurwitz, Bresler and Diringer [1960].

Although it was discovered first, the DNA-dependent RNA polymerase activity of eukaryotic cells is not as well understood as the RNA polymerase of prokaryotic organisms. Experiments investigating the transcriptional process in eukaryotic systems are hindered by the structural and biochemical complexity of the starting material. The presence in vivo of intracellular influences (hormones, vitamins and cell surface effectors) as well as the absence of fine structure genetics complicate the elucidation of transcriptional controls in higher organisms.

The study of yeast polymerases and transcriptional controls may be the best means of circumventing some of the complexities inherant in the study of transcription in higher eukaryotes. The yeast RNA polymerases are remarkably similar to those of multi-cellular eukaryotes, however the problems imposed by hormonal influences and differentiated organ systems are absent. Perhaps the most important difference that yeast offers is the possibility for a genetic approach to transcriptional control. Mutants affecting RNA polymerases have been found [Thonart, Bechet, Hilger and Burny 1976].

The discovery of multiple RNA polymerases in eukaryotic organisms provided both a major advance in the understanding of transcription and at the same time, a quantum jump in complexity. The very presence of multiple polymerases presented the possibility that they had

separate functions and could be separately controlled. There was the possibility that separate structures could be related to these separate functions. The diversity of the multiple polymerases also had the effect of precluding the design of an *in vitro* assay system favorable to all polymerase classes. A similar problem confronted *in vivo* studies.

Diversity of RNA polymerases was originally revealed by several lines of investigation. In 1967, Stirpe and Fiume showed that the toadstool toxin, alpha-amanitin, partially abolished the RNA polymerase activity of eukaryotes both *in vivo* and *in vitro*. This indicated that two forms of polymerase might exist which had different sensitivities to the toxin.

Additional indirect evidence for multiple polymerase forms came from the observation that nuclei under different conditions of incubation produced RNA products that were either DNA-like or rRNA-like [Roeder and Rutter 1970a; Roeder and Rutter 1970b].

Autoradiography under the conditions of incubation which favored synthesis of the rRNA-like (GC-rich) product (low ionic strength, Mg^{++}) resulted in incorporation of the labeled precursor over the area of the nucleolus. When the assay conditions were shifted to favor production of DNA-like RNA (higher ionic strength, Mn^{++}), incorporation of labeled precursor seemed to occur in the area of the nucleoplasm. The nucleoplasmic activity was totally inhibited by low levels of alpha-amanitin [Pogo et al. 1967].

Direct eivdence of the existence of multiple polymerases in eukaryotic cells came from the work of Roeder & Rutter [1969]. DEAE-Sephadex chromatography of the polymerase activity solubilized from sea urchin

nuclei yielded three activity peaks when the column was developed by a linear salt gradient. The order of elution of these three RNA polymerase activities from DEAE-Sephadex forms the basis of the nomenclature developed by Roeder [Roeder and Rutter 1969] (table 2). The multiple RNA polymerases demonstrated in this paper were named polymerase I, II and III. They were all extracted from isolated nuclei.

Fractionation of the nucleus [Roeder and Rutter 1970b] showed that the rRNA synthetic activity was localized in the nucleolus. Lindell et al. [1970] showed that the inhibitor, alpha-amanitin, abolished polymerase II activity without affecting polymerase I activity (see also Seifart and Sekeris [1969]). The differential sensitivity of polymerases to alpha-amanitin forms the basis of the naming system developed by Chambon [1975] in which polymerases I, II and III are called A, B and C respectively.

In the 1970 Cold Spring Harbor Symposium, Blatti, Ingles, Lindell, Morris, Weaver, Weinberg and Rutter summarized the known information about the multiple RNA polymerases. Their conclusions were that there are at least three nuclear transcriptive systems, each controlled by a specific polymerase. Polymerase I they agreed was limited to the nucleolus and was responsible for the nucleolar functions, namely the production of the 45s precursor rRNA. Polymerase II was felt to synthesize the many classes of mRNA and hnRNA. The suggestion was made that tRNA might be synthesized by polymerase III. In summary, they felt that the multiplicity of the RNA polymerases did not correspond with the complexity required for the regulation of cellular function, but since it was known that the polymerases were composed of subunits, the theory was put forth that regulatory subunits could provide appropriate selectivity, especially in combination with alteration of the chromosomal template. Studies of the

functional integration of the polymerases within the chromosome were suggested.

Investigations carried out subsequent to 1970 have served to substantiate every aspect of the summation by Blatti et al. [1970]. In a certain sense, it seems that little progress has been made since that time toward the understanding of eukaryotic transcription and transcriptional control. Many of the questions unanswered then remain unanswered now.

Yet a consideration of the totality of recent RNA polymerase research shows that progress has been made. The achievements are evident in the degree to which information previously gathered has been incorporated into a solid framework. Individual experiments for the most part have seemed repetitive; yet they gain strength from the extensive set of rather standardized experiments performed on polymerases isolated from organisms from throughout the phylogenetic range. These isolated experiments tend to support each other and the net effect is considerably greater than that of the sum of the individual contributions.

Selected literature references for the three polymerase classes

Several excellant reviews of the eukaryotic RNA polymerase literature are available [Chambon 1975; Roeder 1976]. The tripartite transcriptive system has been found in every organism in which it has been sought. The following articles are particularly recommended for their emphasis on the indicated polymerase class. The references are presented in the order of increasing biological complexity of the source organism. It is evident that despite the similarity of their polymerases, the sources are evolutionarily divergent.

Polymerase 1 has been examined in yeast [Sebastian, Takano and Halvorson 1974 ; Huet, Buhler, Sentenac and Fromageot 1975; Schultz and Hall 1976; Valenzuela, Bell, Weinberg and Rutter 1976; Valenzuela, Weinberg, Bell and Rutter 1976; Buhler, Iborra, Sentenac and Fromageot 1976], Xenopus laevis [Schwartz and Roeder 1974; Roeder 1974a], mouse plasmacytoma [Schwartz, Sklar, Jaehning, Weinmann and Roeder 1974; Sklar Schwartz and Roeder 1975], rat liver [Coupar and Chesterton 1975; Matsui, Onishi and Muramatsu 1976a; Matsui, Onishi and Muramatsu 1976b] and calf thymus [Chambon et al.1970; Kedinger, Gissinger and Chambon 1974].

Detailed information on polymerase II from a variety of sources is available in the following reports: yeast [Adman, Schultz and Hall 1972; Hildebrandt, Sebastian and Halvorson 1973; Sebastian, Bhargava and Halvorson 1973; Buhler, Iborra, Sentenac and Fromageot 1974; Buhler, Iborra, Sentenac and Fromageot 1976; Sentenac, Dezelee, Iborra, Buhler, Huet, Wyers, Ruet and Fromageot 1976; Dezelee, Wyers, Sentenac and Fromageot 1976; Valenzuela, Bell, Weinberg and Rutter 1976; Valenzuela, Hager, Weinberg and Rutter 1976], Acanthamoeba castellanii [Detke and Paule 1975], Dictostelium discoideum [Pong and Loomis 1973], Drosophila melanogaster [Phillips and Forrest 1973; Adouette, Clement and Hirshbein 1974; Greenleaf and Bautz 1975 and Greenleaf, Kramer and Bautz 1976], sea urchin [Roeder and Rutter 1969; Roeder and Rutter 1970], Xenopus laevis [Roeder 1974a; Roeder 1974b], chicken [Krebs and Chambon 1976], mouse myeloma [Schwartz, Sklar, Jaehning, Weinmann and Roeder 1974; Sklar, Schwartz and Roeder 1975], rat liver [Mandel and Chambon 1971; Roeder and Rutter 1969; Weaver, Blatti and Rutter 1971; Kedinger, Gissinger and Chambon 1974], calf thymus [Kedinger, Nuret and Chambon 1971; Chambon et al. 1970; Kedinger et al. 1972; Kedinger, Gissinger and Chambon 1974], human

KB cells [Weinmann, Raskas and Roeder 1974; Jaehning, Weinmann, Brendler, Raskas and Roeder 1976], human lymphocytes [Jaehning, Stewart and Roeder 1975], HeLa cells [Hossenlopp, Wells and Chambon 1975], and mammalian cells with amanitin-resistant polymerase II [Ingles, Beatty, Guialis, Pearson, Crear, Lobban, Siminovitch, Somers and Buchwald 1976].

In-depth information on polymerase III is available in the following articles: yeast [Valenzuela, Hager, Weinberg and Rutter 1976; Valenzuela, Bell, Weinberg and Rutter 1976], Bombyx mori [Sklar et al. 1976], Artemia salina [Renart and Sebastian 1976], Xenopus laevis [Weinmann and Roeder 1974; Roeder 1974a; Roeder 1974b; Sklar and Roeder 1976; Long, Dina and Crippa 1976; Parker and Roeder 1977], mouse plasmacytoma [Schwartz et al. 1974; Sklar, Schwartz and Roeder 1975], rat liver [Seifart, Benecke and Juhasz 1972], human KB cells [Weinmann, Raskas and Roeder 1974; Jaehning et al. 1976 and Weinmann et al. 1976], HeLa cells [Seifart and Benecke 1975; Hossenlopp, Wells and Chambon 1975] and human lymphocytes [Jaehning et al. 1975]. An especially useful paper, [Sklar, Yamamoto and Roeder 1976], compares the subunit structure of several polymerase III enzymes from different tissues (Xenopus laevis, mouse plasmacytoma [MOPC 315], Bombyx mori).

The sheer volume of mutually supporting data has greatly increased the degree of confidence with which the subject can be viewed. Previous confusion and apparant contradictions have been resolved and something approaching unified concepts have been worked out. New data can now be set into this structure and new experiments can be designed for the more unusual systems (transformed cells, dividing cells, virus-infected cells, highly specialized cells, hormonally stimulated cells) with greater assurance that one understands the baseline or "normal" conditions.

The time for a major advance in understanding transcriptional mechanisms and control seems much nearer.

The tripartite transcriptive system since 1970

Central to questions of transcriptional control, polymerase function and inhibitor sensitivity is the structural complexity of the polymerase molecules. Subunit composition was a relatively unexplored area in 1970 because of the difficulty in obtaining sufficient quantities of the purified enzymes and because of the related technical problems of finding marker proteins of sufficiently large molecular weight and of designing methods for the separation on a single gel of proteins ranging in size from 200,000 daltons to less than 10,000 daltons.

Major achievements in the area of enzyme structure include the complete elucidation of the subunit composition of the three enzyme classes from yeast [Hager et al. 1976; Sentenac et al. 1976] and mouse plasmacytoma [Sklar, Schwartz and Roeder 1975], This plus other lines of evidence including the demonstration of immunological relatedness [Ingles 1973; Hildebrandt, Sebastian and Halvorson 1973; Kedinger, Gissinger and Chambon 1974] and fingerprint analysis of tryptic digests [Buhler, Iborra, Sentenac and Fromageot 1976] indicate that although the three classes are essentially unique, there is a sharing of certain minor subunits. Since the polymerases share certain functions (DNA binding, RNA chain elongation) this discovery provides a beginning for the assessment of structure-function relationships. The next insights into the control of transcription should come from this direction.

Experiments in polymerase reconstitution have already been successfully performed in procaryotic systems (Anacystis nidulans [Herzfeld and Kiper 1976], E. coli [Ishihama and Ito 1972], QB phage [Brown and Blumen-

thal 1976]).

It is now known that polymerase II of virus-imfected cells synthesizes mRNA from viral templates as well as cellular templates [Weinmann, Raskas and Roeder 1975]. Likewise, it is known that polymerase III synthesizes small RNA's from viral templates in addition to the 5s and tRNA of the cell [Weinmann, Raskas and Roeder 1974; Weinmann, Raskas and Roeder 1975].

The previous knowledge of amanitin sensitivities has been revised considerably since 1970. It is known that polymerase III is not always fully resistant to high levels of alpha-amanitin. In vertebrate systems, polymerase III can be completely inhibited by high concentrations (approximately 1 mg/ml). However, in yeast and insects, this enzyme is fully resistant. Likewise, polymerase I is not always fully resistant, since, in yeast, a sensitivity to high levels has been found. Vertebrate and insect polymerase I on the other hand are considered to be insensitive to any concentration. (For references see section on alpha-amanitin sensitivity.)

Cytoplasmic localization of some of the polymerase III activity in certain organisms was discovered since the time of the 1970 Cold Spring Harbor Symposium; other localizations outlined at that time have found further support in recent work.

Progress is also evident in the area of the relationship between the physiological or developmental state of an organism and the level of RNA polymerase activity. Mutants containing amanitin-sensitive polymerase II are available in several cell lines [Ingles et al. 1976]. Studies have been carried out on the effects of hormones on certain target cells and their polymerase levels [Jaehning et al. 1975; Cox

1976].

Evidence has been found for post-translational RNA polymerase modifications with possible regulatory consequences. Phosphorylation of yeast polymerases [Bell, Valenzuela and Rutter 1976] and adenosine diphosphate ribosylation of quail polymerases [Muhler and Zahn 1976] have been demonstrated.

Subsequent sections of this introduction will present the recent progress in certain areas of transcription research beginning with the question of cytoplasmic localization. Amanitin sensitivities, chromatographic behavior, catalytic properties and subunit structure and polymerase relatedness will also be considered.

Cytoplasmic localization

RNA polymerase activity has been found in the cytoplasm of eukaryotic cells, however there is still debate over whether it is normally present there, possibly as the result of being synthesized there, or whether its presence is an artifact of preparation or due to nuclear leakage.

The first description of a cytoplasmic RNA polymerase was by Seifart, Benecke and Juhasz [1972] (see also [Seifart and Benecke 1975]). Titration of this enzymatic activity against increasing amanitin concentrations showed that it was amanitin-sensitive but that the amount of inhibitor required was 2 - 3 orders of magnitude greater than that necessary to inhibit polymerase II (50% inhibition occurred at 30 ug/ml). The enzyme was seen to be clearly different from enzymes I and II on the basis of chromatography on phosphocellulose (which separated it from polymerase I) and DEAE-cellulose (which separated it from polymerase II)

as well as on the basis of intermediate amanitin sensitivity. It had been isolated from rat liver cytosol and was called polymerase C.

Subsequent studies have shown that the polymerase C of Seifart, Benecke and Juhasz is the equivalent of polymerase III first described in sea urchin nuclei [Roeder and Rutter 1969]. In mouse plasmacytoma cells, there are two forms of polymerase III, polymerase IIIA and polymerase IIIB [Schwartz et al. 1974b]. Polymerase IIIB activity and some of the polymerase IIIA activity was found in the cytoplasm. The remaining polymerase IIIA was localized in the nucleus. HeLa cell nuclei have been found to contain both polymerase IIIA and IIIB activity [Weil and Blatti 1975]. When whole cell homogenates of HeLa cells were examined by DEAE-Sephadex chromatography [Hossenlopp, Wells and Chambon 1975] four polymerase III enzymes were separated.

Alpha-amanitin sensitivity of eukaryotic RNA polymerases

The elongation inhibiting toadstool toxin, alpha-amanitin, has been of central importance to the study of transcription since the discovery that the effect of the toxin on RNA synthesis was specific to the polymerase, and, furthermore, that the eukaryotic RNA polymerase activities were differentially affected [Seifart and Sekeris 1969a; Kedinger et al. 1970; Lindell et al. 1970].

Early work had indicated that polymerase I and polymerase III were resistant to the toxin while polymerase II was extremely sensitive; in 1972 Seifart and Benecke showed that rat liver polymerase C (III) was intermediately sensitive and could be completely inhibited by high concentrations of the toxin. It is now clear that while polymerase I is fully resistant, in vertebrate systems, polymerase III is 50% inhibited

by 10 - 40 ug/ml alpha-amanitin [Austoker et al. 1974; Seifart and Benecke 1975; Beebee and Butterworth 1975; Weinmann and Roeder 1974; Weinmann, Raskas and Roeder 1974; Wilhelm, Dina and Crippa 1974; Roeder 1974; Sklar 1975; Schwartz 1974].

One of the primary distinguishing features of polymerase II is sensitivity to low levels of the inhibitor, alpha-amanitin. With the exception of the less-sensitive polymerase II from yeast, the concentration required for 50% inhibition of enzymatic activity ranges from 0.004 to 0.025 ug/ml (tables 2, 11, 12 and figure 30) for vertebrate class II polymerases. The pattern of amanitin sensitivity in vertebrates is consistent and the polymerase activities can be distinguished on the basis of their amanitin sensitivity.

In the silkworm, Bombyx mori, the class II polymerases have a sensitivity equivalent to the polymerase II enzymes from higher eukaryotes [Sklar and Roeder 1975; Sklar, Yamamoto and Roeder 1976] while polymerase I and polymerase III from this organism are fully resistant. Drosophila class II enzymes are also as sensitive as the vertebrate polymerase II enzymes (see figure 30) [Phillips and Forrest 1973; Greenleaf and Bautz 1975; Greenleaf, Kramer and Bautz 1976].

In yeast, the three polymerase classes respond to the toxin differently from the cognate enzymes from vertebrate organisms and the terminology of Chambon (table 2) cannot be meaningfully applied [Schultz and Hall 1976; Valenzuela et al. 1976; Hager et al. 1976]. Polymerase I (identified by structural, chromatographic and catalytic considerations) is sensitive to high concentrations while polymerase III is insensitive. Polymerase II from yeast is somewhat less sensitive than the vertebrate polymerase II (figure 30) [Greenleaf and Bautz 1975].

Choice of nomenclature for Artemia salina polymerases

Considerable heterogeneity has been found within the three major classes of eukaryotic RNA polymerases and the two naming systems continue to grow in complexity (table 2). As mentioned, the system of Chambon is based on the sensitivity of polymerases to the inhibitor, alpha-amanitin [Chambon 1975]. Although the sensitivity of a polymerase to amanitin is simple to test for and although the results are consistent and useful in vertebrate systems, there are inconsistencies when polymerases of lower organisms are examined (table 12). The present study on brine shrimp polymerases uses the terminology of Roeder since it has been found to be equally valid for higher and lower eukaryotes. Insects and yeast present exceptions to the Chambon system since they contain polymerases which are identifiable as cognates of the polymerases of higher organisms by all criteria except that of amanitin resistance (table 12).

The only known exception to the system of Roeder is the case of Physarum [Gornicki, Vuturo, West and Weaver 1974] in which the elution order of polymerases I and II are reversed.

Summarization of polymerase functions, localizations and inhibitor sensitivities

In summary, polymerase I is localized in the nucleolus, catalyzes the transcription of the rDNA genes and is totally resistant to alpha-amanitin (with the exception of yeast polymerase I). Polymerase II is localized in the nucleoplasm, is extremely sensitive to alpha-amanitin and catalyzes the synthesis of DNA-like RNA (hnRNA and mRNA) on viral or cellular templates. Polymerase III is localized in both the nucleoplasm and the cytoplasm, is sensitive to high levels of alpha-amanitin

(exceptions are yeast and insect polymerase III) and produces transcripts of small size (tRNA and 5s RNA) whether using a viral or a cellular template. Chromatographic and electrophoretic heterogeneity exists in each class. Heterogeneity has no known effect on enzymatic activity [Roeder 1974a].

Chromatographic properties of eukaryotic RNA polymerases

Although alpha-amanitin sensitivity affords the simplest and (for animal polymerases) the most straightforward preliminary method of identification, ultimately the most reliable method is the complete elaboration of subunit composition. Subunit composition offers the most promise in the area of understanding polymerase function and transcriptional control.

Investigation of subunit composition presupposes a source of pure enzyme. It is thus dependent upon polymerase purification techniques and the separation of the polymerase classes from one another. Chromatography provides the best means for this since eukaryotic RNA polymerases bind to anion and cation exchange resins as well as to DNA-cellulose. In some cases, preliminary identification of polymerase classes can be made after chromatography since each polymerase has its characteristic elution position from a particular chromatographic material. The combination of anion and cation exchange chromatography results in a high degree of purification as does DNA-cellulose affinity chromatography.

DEAE-Sephadex is perhaps the most widely used and useful material since separation of the three polymerase classes is often possible in one chromatographic step [Roeder and Rutter 1969; Roeder 1976].

When linear gradients of ammonium sulfate are used to develop the column, the three polymerase classes elute from DEAE-Sephadex in the

general order, I, II and III. Elution positions vary somewhat from tissue to tissue, and, due to heterogeneity, more than three activity peaks may appear. Polymerase III is particularly unpredictable in this regard.

Class II polymerases characteristically elute from DEAE-Sephadex at ammonium sulfate concentrations between 0.15 M and 0.25 M [Roeder 1976; Chambon 1975]. The class II polymerases are more acidic in nature than the class I polymerases which can be more readily removed by the salt gradient (0.05 - 0.15 M ammonium sulfate is the range for elution [Roeder 1976]). The elution positions of polymerase I and polymerase II are essentially the same on DEAE-cellulose and DEAE-Sephadex (table 12).

This is in contrast to the behavior of polymerase III which elutes from DEAE-cellulose at an ionic strength lower (approximately 0.1 M ammonium sulfate) than that which would be expected on the basis of its elution from DEAE-Sephadex (0.2 - 0.3 M ammonium sulfate). Polymerase I and III therefore co-elute from DEAE-cellulose at approximately 0.1 M ammonium sulfate. Polymerase II elutes at approximately 0.2 M ammonium sulfate as it does from DEAE-Sephadex.

Since polymerase I and polymerase III are fully resistant to the concentrations of amanitin which are sufficient to abolish the activity of polymerase II, many authors using DEAE-cellulose disputed the existence of polymerase III. This confusion was resolved in 1973 [Sergeant and Krsmanovic]. It is now widely accepted that the amanitin-resistant polymerase activity eluting at low ionic strengths from DEAE-cellulose which was characterized as polymerase I in older reports should be viewed as possibly representing a composite of the characteristics of

polymerase 1 and polymerase III.

It is important to note that the first published work on the polymerases of Artemia salina [Birndorf, D'Alessio and Bagshaw 1975] is such a paper.

The inconsistant behavior of polymerase III has proved to be a useful tool, both for the purpose of polymerase identification and for the purpose of separating polymerases in those instances where polymerase 1 and polymerase III co-elute on DEAE-cellulose and can be separated on DEAE-Sephadex or, conversely, where polymerases II and III co-elute on DEAE-Sephadex and can be separated on DEAE-cellulose. A combination of these two chromatographic materials was used to advantage in the present study (figures 22, 23 and 24).

General catalytic properties of eukaryotic RNA polymerases

The three polymerase classes differ in their requirements for various assay mixture components. The divalent cation requirement, ionic strength optimum and template preference, when taken together, describe the general catalytic requirements of an enzyme class. The "characteristic" catalytic requirements are subject to variation under certain reaction conditions [Roeder 1976]. However they are sufficiently characteristic that under defined conditions, they allow the preliminary identification of an enzyme class. Taken together with chromatographic behavior and amanitin sensitivity, the preferred templates, metal ion cofactors and ionic strengths give a firm identification of an enzyme class.

The tendency of a catalytic requirement to be dependent on other reaction conditions led to a very confusing early picture of eukaryotic

RNA polymerases. Different investigators using slightly different conditions and different enzyme sources, achieved results which were often in some disagreement. As conditions became standardized and more animal systems were examined, it was realized that the discrepancies were more the result of the conditions of assay and extraction than differences between the cognate enzymes from the various organisms. For example, the use of a synthetic or denatured DNA as template can drastically alter the activity profile of a chromatographic separation of enzyme activities. Schwartz et al. [1974], using mouse plasmacytoma cell homogenate, obtained three amanitin-resistant activity peaks from a DEAE-Sephadex column using native calf thymus DNA as template. Upon reassay with $d(A\text{-}T)_n$ and the same amount of inhibitor, the activity of the first peak was unchanged, but the size of the second and third resistant peaks doubled in height. The explanation is that the mouse plasmacytoma polymerase I had no preference for $d(A\text{-}T)_n$ over CT DNA_{nat} while polymerases IIIA and IIIB were stimulated. It is worth noting that the two class III polymerases were similarly affected.

An example of the interrelatedness of variables is available from the mouse plasmacytoma system [Schwartz et al. 1974]. When the effect of ammonium sulfate on the activity of the four plasmacytoma polymerases is measured with native calf thymus and then with $d(A\text{-}T)_n$, the activity profile of polymerase I is unchanged; however the activity profile of polymerase II is shifted to a lower ionic strength with $d(A\text{-}T)_n$. When measured with $d(A\text{-}T)_n$, polymerases IIIA and IIIB have a single clear optimum at 0.05 M ammonium sulfate; measurement in the presence of CT DNA_{nat} produces a biphasic curve with optima at 0.05 M and 0.175 M and activity over a broader range of ionic strengths.

These experiments demonstrate that, although the polymerase classes differ in their properties, the members of a particular class (as seen with IIIA and IIIB) behave similarly. The cognate enzyme classes from widely differing species are also similar in their general properties [Roeder and Rutter 1960; Roeder 1976; Chambon 1975].

The ionic strength (ammonium sulfate or potassium chloride) activation profiles for polymerase I are generally narrower in range and have a lower optimum than those of polymerase II. As seen, polymerase III salt activation profiles are highly dependent on the template employed. With native DNA they are generally biphasic and, on occasion, the activation profiles plateau with increasing ionic strength.

When eukaryotic enzyme classes are compared with respect to metal ion cofactor requirements, the most obvious observation is that the activity of all three classes is reduced to zero in the absence of either Mg^{++} or Mn^{++}. Both Mg^{++} and Mn^{++} have a stimulatory effect on polymerase activity which is characteristic for the three enzyme classes. Polymerase I and polymerase III do not have a distinct preference for one metal ion cofactor over the other when polymerase activity is measured at the optimum cofactor concentration. Polymerase II is stimulated up to 10 fold by the presence of the optimum concentration of Mn^{++} over Mg^{++} [Sebastian, Bhargava and Halvorson 1973]. All three enzymes show a sharper activity profile with Mn^{++} than with Mg^{++}; generally the optimum Mg^{++} concentration is several times higher than the optimum Mn^{++} concentration.

The effect of a particular template on the enzymatic activity (net RNA synthesis) of an RNA polymerase is best presented as the ratio of the activities achieved with two different templates. Since eukaryotic

polymerases initiate non-specifically at single-stranded nicks and gaps in the DNA [Roeder 1976], there is little noticeable difference when native templates from different sources are compared. A comparison of denatured and native templates shows a different effect on polymerase II compared to polymerases I and III. Although in some cases polymerase I preferentially transcribes native DNA [Renart and Sebastian 1976], polymerase I and polymerase III often have an activity ratio of one when native and denatured DNAs from the same source are compared [Roeder 1976]. Polymerase II however has always been found to prefer denatured templates. As mentioned [Schwartz et al. 1974b], polymerase III is stimulated by the synthetic template $d(A\text{-}T)_n$. In some reports this stimulation results in up to 10 times the activity found with native DNA [Roeder 1976]. This effect is somewhat dependent on the size of the templates involved, especially on the size of the synthetic template [Roeder, personal communication].

Molecular structure of the eukaryotic RNA polymerases

Valenzuela et al. [1976c] have suggested, on the basis of the complete charactarization of the tripartite transcriptive system in mouse plasmacytoma and yeast, that structural analysis and functional studies be taken as the basis for the distinction and nomenclature of all eukaryotic polymerases. These authors feel that past criteria such as alpha-amanitin inhibition (Chambon terminology), chromatographic elution behavior (Roeder terminology), metal ion requirements and salt optima are inadequate because prominent exceptions exist. They state "these criteria should be considered only presumptive evidence until the structure and functional role of the enzymes are defined".

The question of whether certain putative subunits are true subunits (having functional significance for the enzyme) or whether they represent proteins remaining bound to the polymerase through the purification procedure is difficult to answer. A search for "factors" associating with RNA polymerase from procaryotic cells utilized anti-polymerase antibodies which had been covalently bound to sepharose to trap polymerase molecules and those molecules associated with them [Losick and Pero 1976].

A similar approach to finding 'factors' is the precipitation of polymerases from crude preparations with polymerase-specific antibody, followed by examination of the precipitate for associated factors [Losick and Pero 1976].

In the case of eukaryotic RNA polymerases, enzymes are considered pure when they run as single bands on non-denaturing gels. When this material is eluted and electrophoresed on SDS gels, the resulting polypeptides are assumed to be polymerase subunits. The combined molecular weights of the subunits should be in agreement with the molecular weight of the native enzyme as determined by sucrose gradient sedimentation.

Chromatographic and electrophoretic heterogeneity has been found for all three polymerase classes. Examination of the subunit composition of each chromatographic form has led to apparant explanations for differences in chromatographic behavior. The only eukaryotic tissues for which the subunit structure is completely known for all three enzyme classes are yeast [Valenzuela, Hager, Weinberg and Rutter 1976] and mouse plasmacytoma [Sklar, Schwartz and Roeder 1974]. Work on the other animal cell polymerases shows surprising correspondence between subunit molecular weight, size distributions and subunit number as well as the subunit differences which account for the chromatographic heterogeneity.

Because it has greater inherent stability than either polymerase I or polymerase III and because it is less sensitive to varying assay conditions, RNA polymerase II is generally the first of the three polymerase classes to be described for a particular tissue. This was the case in early experiments involving tissues now known to contain three polymerase classes [Chambon, Ramuz and Doly 1965]. Although early studies also characterized polymerase I, the results of this work must now be viewed with caution since it is likely that those preparations of polymerase I purified on DEAE-cellulose are actually mixtures of polymerase I and polymerase III (table 12).

The subunit composition of the eukaryotic RNA polymerases was slow to emerge because of two technical problems: the first was the lack of molecular weight markers in the 200,000 dalton size range and the second was the extreme range in size of the subunits themselves. The introduction of polyacrylamide gels with a linearly decreasing porosity has resulted in more and more subunits being reported for enzymes which had been examined under the less sophisticated conditions of discontinuous gradient acrylamide gels (usually 5% acrylamide layered above 10% acrylamide).

For example, in 1971, Weaver, Blatti and Rutter reported that the polymerases IIA and IIB from calf thymus and rat liver each contained four subunits. An earlier report [Blatti et al. 1970] described three subunits while Kedinger, Nuret and Chambon [1971] reported only two subunits. Although polymerases IIA and IIB are now known to contain up to ten polypeptides, Rutter's group did show that the chromatographic forms of polymerase II differed in one major subunit. They also showed that there was a high degree of correspondence between calf thymus and

rat liver polymerase II substructures. Recent work elucidating the subunit composition of polymerase II will be presented below.

The subunit composition of the two chromatographic forms of polymerase I has been determined in several animal species. In mouse plasmacytoma cells, polymerase IA contains 6 polypeptides [Schwartz and Roeder 1974]. These authors found polymerase IB to be identical except that it was lacking the third largest subunit. A very similar situation has been found in calf thymus [Gissinger and Chambon 1975] and rat liver [Muramatsu et al. 1974]. In both calf thymus and rat liver, the corresponding IB form is identical to the IA form except that the third largest subunit is missing. The sedimentation coefficient of calf thymus AI (IA) is 14.5s [Kedinger, Gissinger and Chambon 1974].

Valenzuela et al. [1976a] found eleven putative subunits in the polymerase I of yeast while Buhler, Iborra, Sentenac and Fromageot [1976] reported 14 subunits. Sajdel-Sulkowska and Halvorson [1975] described two forms of yeast polymerase I which differed in the size of the largest subunit. Yeast polymerase I thus seems to be more complex than the animal polymerase counterpart.

An exception to the generalization that the heterogeneous forms of polymerases are identical in catalytic properties is found in yeast polymerase I. Two forms have been found to differ in response to template preference and amanitin sensitivity [Huet et al. 1975].

The 1974 report of Kedinger, Gissinger and Chambon for calf thymus and rat liver polymerase II gave the subunit molecular weights (in thousands of daltons) and molar ratios as 214 (x1), 140 (x1), 34 (x1-2), 25 (x2) and 16.5 (x3-4). For polymerase BII (IIB) they found the largest subunit to be 180 (x1) and the other smaller subunits as reported for

polymerase BI (IIA). They also report that the difference between polymerase IIB1 and IIB2 lies in a charge difference in the largest subunit. Rat liver was found to contain a third polymerase II, polymerase BO (IIO). Again, the difference was found to lie in the largest subunit which, in this case, was larger than 180,000 daltons. Polymerases BI (IIA) and BII (IIB) had sedimentation coefficients of 15.5s.

Sklar, Schwartz and Roeder [1975] use 5-15% linear acrylamide gradients to separate seven subunits of polymerase II from a mouse plasmacytoma cell line (MOPC 315). They find that polymerase IIA, polymerase IIB and polymerase IIO differ only in the size of the largest subunit.

The polymerase II isolated by Greenleaf and Bautz [1975] from Drosophila contained ten subunits. Antiserum against Drosophila polymerase II inhibited the activity of polymerase II from Drosophila, yeast and calf thymus.

Yeast polymerase II was described as containing nine subunits by Valenzuela, Hager, Weinberg and Rutter [1976] while Buhler, Iborra, Sentanac and Fromageot [1976] and Greenleaf and Bautz [1975] gave values for ten yeast polymerase II subunits. These workers also showed that antibodies against polymerase B (II) gave inhibition of B (II) enzymes from Drosophila, calf thymus and yeast. These antibodies had no effect on E. coli polymerase.

Other workers, taking care to avoid proteolysis, have found that yeast contains a polymerase IIA and a polymerase IIB with native molecular weights of 465,000 daltons and 435,000 daltons respectively [Dezelee, Wyers, Sentenac and Fromageot 1976]. The only observed difference between the two enzymes was in the molecular weight of the heaviest subunit. The two enzymes are otherwise identical and contain, in addition, seven other

subunits. They found that two additional proteins of molecular weight 32,000 and 16,500 daltons were dissociated from the enzyme upon polyacrylamide gel electrophoresis or DEAE-Sephadex column chromatography. They indicate that the largest subunit can be specifically cleaved by a yeast protease and that this has no effect on the enzyme activity with either single-stranded or double-stranded templates.

The subunit structures of polymerase III isolated from mammalian (mouse plasmacytoma), amphibian (Xenopus laevis) and insect (Bombyx mori) sources are compared by Sklar, Schwartz and Roeder [1975]. Ten subunits are found in mouse, and nine in the other two tissue types. One subunit appears to be shared between polymerases I and III; one subunit is common to II and III and two subunits are shared between all three enzyme classes. Further evidence for the sharing of subunits will be presented which will show that this is probably not circumstantial. Comparison of these three polymerase III's from disparate sources is made more valid by the determinations having been performed in a single laboratory with a single set of standards.

It is worth noting that the third largest subunit is missing from the Bombyx polymerase III. This may correspond to the observation that this enzyme from Bombyx is resistant to high concentrations of alpha-amanitin while the cognate enzymes from mouse and Xenopus are sensitive. No causality has been established however, and this might be pure coincidence. In mouse plasmacytoma, the two chromatographic forms of polymerase III are essentially identical in subunit composition, however polymerase IIIB has a 33,000 dalton subunit in place of the 32,000 dalton subunit found in polymerase IIIA. The single chromatographic species from Xenopus is the only other animal polymerase III with a known subunit

structure.

Valenzuela et al. [1976c] have shown polymerase III from yeast to be composed of 12 putative subunits. The higher molecular weight subunits are clearly distinct from those of polymerase I and II as are some of the smaller subunits, however four of the smaller subunits seem identical to those of polymerases I and II of yeast.

Sharing of subunits among eukaryotic RNA polymerases

Several lines of evidence point to the sharing of subunits between various combinations of the eukaryotic polymerases. In 1973, Hildebrandt, Sebastian and Halvorson showed immunological cross-reactivity between yeast polymerases I and II. These authors felt that the immunological relatedness might reflect a similar catalytic function and that the polymerase differences were required for promoter recognition and other control functions. An alternative explanation was that these complex molecules had a shared evolutionary history, evolving to have different properties while retaining common antigenic determinants. (See also [Ingles 1973]).

Buhler et al. [1976] gave further evidence (subunit molecular weight, subunit isoelectric points and fingerprint analysis of ^{35}S-labelled tryptic peptides) for shared subunits in yeast.

A third line of evidence pointing to shared subunits (at least in yeast) came from the work of Thonart et al. [1976] in which four yeast mutants were isolated with drastically reduced RNA synthesis capabilities. Genetic analysis showed that the mutations fell into three complementation groups and showed a recessive phenotype. The synthesis of all three classes of polymerase was seen to be thermosensitive in various mutants. The conclusion of the authors was that there were at least three genes in yeast,

each of which was indispensable for the in vivo (and in vitro) activity of the three polymerases. This was thought to favor the hypothesis of shared subunits in the three polymerases.

Valenzuela, Bell, Weinberg and Rutter [1976] support this contention with two-dimensional polyacrylamide gel electrophoresis subunit mapping techniques.

Purpose of the present project

Artemia salina are protostomes belonging to the Class Crustacea and, as such, they are phylogenetically distinct from the deuterostome mammalian tissues in which RNA polymerases have been previously characterized. Brine shrimp are also phylogenetically separate from the unicellular yeast. Characterization of the chromatographic and catalytic properties and inhibitor sensitivities of the Artemia polymerases was therefore performed as a study in comparative enzymology.

Brine shrimp afford a unique opportunity to examine eukaryotic RNA polymerases at different developmental stages since the shrimp are available commercially as dehydrated cryptobiotic gastrulae (cysts) which reinitiate development upon hydration. The present study describes the RNA polymerases found in the dehydrated gastrulae; this information provides the basis for later comparison of Artemia polymerases isolated from later developmental stages.

Development of purification methods was necessary for the individual characterization of the enzymatic properties of the polymerases. Large scale purification methods were devised to examine the feasibility of obtaining large quantities of Artemia polymerases for future studies on the subunit composition of these polymerases.

METHODS

Preparation of Artemia salina Cysts

Washing

Dehydrated shrimp cysts were washed with 3.5% NaCl to dilute out the naturally occuring contaminating salts. When the dehydrated cysts were stirred into 3.5% NaCl and allowed to settle by gravity, the shrimp cysts and contaminating sand sank, permitting removal of floating debris.

Hydration

Commercially available dehydrated cysts of Artemia salina could be completely hydrated by soaking for three hours in 3.5% NaCl at room temperature. For most experiments, however, hydrated shrimp cysts were obtained by washing dehydrated cysts with 4 to 5 changes of the 3.5% NaCl and hydrating overnight at 4°C in several volumes of the 3.5% NaCl solution in a large flat dish. The liquid had a depth of no more than 1.5 cm.

Incubation

Incubation and hatching of shrimp cysts was carried out in 3.5% NaCl in the presence of antibiotics. Streptomycin and penicillin G were present in concentrations of 0.1 mg/ml and 100 units/ml respectively. The most satisfactory method of aeration proved to be the

incubation of shrimp in 250 or 500 ml Erlenmeyer flasks attached to a rotary or shaking water bath. The optimal temperature for hatching was 27 to 28°C. The maximum tolerable temperature was 32°C. Air bubbled directly into the shrimp cyst suspension proved unsatisfactory despite the introduction of an air pressure regulator in the line and maintenance of a very slow stream of air. Bubbling caused breakage and clumping of the developing embryos when employed over long periods of time (overnight or longer). Typically 3 g (dry weight) washed shrimp cysts were incubated in a volume of 100 ml in a 500 ml Erlenmeyer flask with gentle swirling at 27°C.

Hatching

Hatching of Artemia cysts begins after 8-9 hours incubation at 27°C and is manifested by the appearance of pre-nauplius larvae. Although hatching will continue to occur over a period of several days incubation, a synchronized population of pre-nauplius larvae can be obtained by the selective separation of hatched embryos from unhatched embryos at chosen incubation times. The embryos which hatch within a particular incubation time can be obtained by first removing hatched embryos from a population of incubating brine shrimp, allowing others to hatch and then removing the recently hatched synchronized individuals.

Appearance of swimming nauplii first occurs after 20 hr at 28°C and then, as is the case with hatching, the number of swimming individuals increases throughout several days incubation. Large numbers of shrimp, synchronized with respect to their emergence as swimming nauplii, can be obtained by separating them from other developmental stages at an appropriate time.

Buffer Preparation

All buffers were made with distilled water. Buffer A was used during shrimp cyst disruption and for all stages of RNA polymerase purification with the exception of phosphocellulose chromatography, DNA-cellulose chromatography, gel electrophoresis and glycerol gradient centrifugation. The composition of buffer A was 0.05 M Tris·HCl (pH 7.8), 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 10^{-5} M phenylmethylsulfonyl fluoride (PMSF) and 25% (v/v) glycerol. The glycerol concentration of buffer A was changed to 5% (v/v) for shrimp cyst homogenization and 50% (v/v) for freezing and storage of purified polymerase.

Phosphocellulose chromatography was performed using buffer A without Mg^{++}.

Buffer B was used for DNA-cellulose column chromatography. The composition of buffer B was 0.05 M Tris·HCl (pH 7.8), 1.0 mM EDTA, 0.5 mM DTT, 10^{-5} M PMSF, 0.15 M NaCl and 25% (v/v) glycerol.

Polyacrylamide gel electrophoresis tray buffer was as described in Methods under "gel electrophoresis".

Buffer for glycerol gradient centrifugation contained, in addition to glycerol (15 to 30% v/v), 0.05 M Tris·HCl pH 7.9 , 0.1 M ammonium sulfate, 2 mM $MnSO_4$, 0.1 mM EDTA and 0.1 mM DTT (see also "glycerol gradient formation" in Methods). This buffer was generally made up as a 2x stock (with Mn^{++} withheld until time of use) so that the glycerol concentration could be varied easily as required. After addition of $MnSO_4$, the final volume was adjusted with distilled water on the day of use.

Ion Exchange Resin Preparation

DEAE -Sephadex

DEAE-Sephadex A 25-120 was prepared for use by hydrating for several hours at room temperature in distilled water, then soaking in 0.5 M NaOH at room temperature for 30 min. The resin was put into a Buchner funnel and exhaustively rinsed with distilled water before being made 1 M with HCl. After 30 min at room temperature, the resin was again exhaustively rinsed with distilled water. This was followed by rinsing with 50 mM Tris·HCl buffer at pH 7.8 until the pH had stabilized at this level. The pH-equilibrated DEAE-Sephadex was loaded into a chromatography column, allowed to settle, and equilibrated by running several column bed volumes of buffer A containing 0.05 M ammonium sulfate through it (the equilibration process usually ran overnight). Washed resins could be refrigerated for long periods of time without bacterial contamination because of the presence of PMSF in the buffers.

DEAE-cellulose

DEAE-cellulose was prepared for use in the same manner as DEAE-Sephadex. Both DEAE-Sephadex and DEAE-cellulose were regenerated after use by this washing procedure.

DNA-cellulose

DNA-cellulose was prepared in the laboratory as follows: high molecular weight DNA was dissolved in a buffer containing 0.01 M Tris·HCl at pH 7.4 and 10^{-3} M EDTA at a final concentration of approximately 2 mg/ml (the DNA had been previously denatured by heating to 90°C for

15 min with quick recooling). Enough cellulose (Whatman CF11) was added to form a thick pulp. The mixture was allowed to stand overnight and then lyophilized (or allowed to dry with occasional stirring). After lyophilization, 10 volumes of the Tris-EDTA buffer were added and the mixture left in the cold for 12-24 hours. The DNA-cellulose was collected by centrifugation and washed two times with Tris-EDTA buffer. It was equilibrated with buffer B for direct use or frozen for storage. Prior to application of the enzyme preparation, the column was washed with buffer B until the A_{260} reached a minimum level.

Phosphocellulose

Phosphocellulose was prepared by a method adapted from Gissinger and Chambon [1972]. Dry phosphocellulose (Whatman P11) was stirred into 0.5 N KOH. The volume used was 3 l 0.5 N KOH for each 100 g dry weight phosphocellulose. After 30 min the phosphocellulose was rinsed with distilled water until the pH reached approximately 9. Three liters of 0.5 N HCl were added with gentle stirring. After 30 min the supernatant was aspirated off and two liters of 0.5 N HCl were added and allowed to stand for 20 min. The phosphocellulose was rinsed again with distilled water and when the pH was approximately 4, it was washed with 1M Tris buffer until the pH was stable at 7.9. After washing with buffer A with no Mg^{++}, it was stored at 4°C or loaded into a column. The phosphocellulose was regenerated after use with 1 M ammonium sulfate.

Glycerol Gradient Centrifugation

Glycerol gradient formation

A Beckman motorized gradient maker was used and the settings

required to fill the 13.2 ml SW41 rotor tubes to the appropriate level were -4 to 72. Using the Beckman nomograph, it was determined that the formation of a 15-30% glycerol gradient (v/v) required 4% glycerol buffer in the low-concentration syringe and 35% glycerol buffer in the high-concentration syringe. The buffer used was previously described.

Glycerol gradient centrifugation

An SW41 rotor was used for the glycerol gradient centrifugation in a Beckman ultracentrifuge. The run lasted 20 hr and 54 min at 30,000 rpm at 3° C. Various equivalent time/speed ratios were used in different experiments. These were calculated to be 11.2 hr at 41,000 rpm, 11.8 hr at 40,000 rpm and 15.4 hr at 35,000 rpm. The SW41 tubes hold 13.2 ml each; a sample volume of up to 0.5 ml can be run.

Glycerol gradient fractionation

An ISCO (Instrumentation Specialties Co.) Model 183 Density Gradient Fractionator was used for fractionation of the glycerol gradients. Tubes were punctured from below and mineral oil was forced in from the top at a constant rate. Fractions of 25 drops each were collected (approximately 0.55 ml). Fractionation of the glycerol gradients and the subsequent assay of the collected fractions for RNA polymerase activity were carried out in a cold room at 4° C.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis under non-denaturing conditions was done according to the method of Kedinger, Gissinger and Chambon [1974] adapted from Krakow [1971] and according to the method of Maizel [1969]. Since the method of Kedinger, Gissinger and Chambon was used

in most experiments, it is outlined below.

Stock solution A contained 36.3 g Tris·OH, 4 ml HCl, 0.23 ml TEMED and distilled water to 100 ml (resulting in pH 8.9); stock solution C contained 25 g acrylamide, 0.67 g methylene bisacrylamide and distilled water to 10 ml. All stock solutions were stored in the dark at 4°C and prepared every two weeks except stock solution G which contained 14 mg ammonium persulfate in 10 ml distilled water and which was made fresh every day. For 10 ml of separating gel, in addition to the amount of stock solution C necessary for the desired acrylamide concentration, 1.25 ml stock solution A, 5.0 ml stock solution G, 0.1 mM DTT and water sufficient to bring the volume to 10 ml were combined and, after careful mixing, put into glass tubes. In most cases, 5% acrylamide concentrations were used. The electrophoresis tubes had inner dimensions of 5 x 90 mm and were filled with approximately 1.2 ml each of separating gel. Stacking gels were not used. Before hardening of the gel mixture, it was carefully overlaid with 0.2 ml of 8 fold diluted stock solution A containing 0.1 mM DTT.

The upper and lower tray buffers contained 25 mM Tris·OH, 33 mM glycine, 1 mM ammonium sulfate and 0.1 mM DTT, pH 8.9. The gels were pre-electrophoresed at 2 mA/tube for at least one hour prior to sample loading. All samples were dialyzed against the tray buffer prior to loading and at the time of loading they were each mixed with 5 ul of 0.15% bromphenol blue in water and a sufficient amount of a saturated sucrose solution to prevent mixing with tray buffer after loading.

Electrophoresis was carried out in the cold room at 1.5 mA/gel until the tracking dye reached the bottom of the gel or as described in figure legends. Upon removal of the gels from the gel tubes, a

dilute solution of BSA was injected with a syringe needle into the tracking dye band. In those gels in which the tracking dye had left the gel, BSA was injected a short distance into the bottom of the gel so that, after staining, the correct orientation of the gel would be known.

Gels were stained in Coomassie blue (0.25% in 12.5% TCA or 0.04% in a solution which was five parts absolute methanol; five parts distilled water; one part acetic acid) or acid fast green (0.5% in 7% acetic acid). The staining period was overnight or longer at room temperature. Destaining was accomplished electrophoretically with a Canalco gel destainer or by several changes of the 5:5:1 MeOH:H_2O:HAc mixture (or 7% acetic acid, depending on the composition of the staining solution.) After destaining, gels were scanned at 600 nm in the gel scanning attachment of a Gilford spectrophotometer and the absorbance profile traced onto chart paper.

The relative amounts of protein in selected absorbance bands were determined by cutting out the appropriate absorbance peaks generated on paper by the gel scanning procedure and comparing the weights of the cut out area of paper. The weight of the cut out absorbance peak was taken to be directly proportional to the area of the absorbance peak; the ratio of either absorbance peak area or absorbance peak weight was taken to be the same as the ratio of the amount of protein present in any two gel bands compared in this manner, provided that the manner of staining and scanning were identical.

Miscellaneous Measurements

Conductivity measurements

Conductivity measurements were carried out directly with a Radiometer Type CDM 2d conductivity meter equipped with a 0.62 cm flow-through probe

or with a Yellow Springs Instrument Co. Model YSI 3400. For column fractions and other samples where volumes were small, conductivity measurements were made on 1/200 dilutions (25 ul in 5 ml distilled water). Standard curves were prepared with buffers of known ionic strength (either diluted or undiluted and at 0°C or at room temperature, 21°C).

Protein concentrations

Protein concentrations were determined by the method of Lowry [1951]. The proteins were first precipitated by addition of a small sample to 1 ml of cold 15% TCA and centrifugation for several minutes at top speed in a clinical desk top centrifuge or in a Beckman J-21 at 3,000 rpm. Alternatively, protein concentrations were determined by the method of Kalckar [E. Fronk, personal communication] which uses the absorbance at 280 nm in the formula $(A_{280} \times 1.45) - (A_{260} \times 0.74) = mg/ml$.

DNA concentrations

DNA concentrations of the highly purified templates were arrived at by assuming that an absorbance at 260 nm of 20 was equivalent to 1 mg/ml whether the template was Artemia, calf thymus or salmon sperm DNA or the synthetic template, poly d(A-T). Indication of the proportion of nucleic acids in a polymerase preparation was arrived at from the A_{280}/A_{260} ratio.

Nucleoside triphosphate concentrations

The determination of nucleoside triphosphate concentrations was carried out spectrophotometrically using the following absorption information: ATP $A_m = 15.4 \times 10^3$ at 259 nm and pH 7, CTP $A_m = 9.1 \times 10^3$ at 271 nm and pH 7, GTP $A_m = 13.7 \times 10^3$ at 252 nm and pH 7 and UTP $A_m = 10.0 \times 10^3$ at 262 nm and pH 7.

The labeled UTP was prepared at the desired specific activity by adding 90 ul ^{3}H·UTP at 1 mCi/ml to 10 ul UTP at 0.1 M to obtain 0.01 M ^{3}H·UTP with a specific activity of approximately 60 cpm/pmol. The actual determination and subsequent adjustment of specific activity was done by measuring the A_{262} of a dilution of the 0.01 M isotope and then determining the radioactivity of aliquots of the dilution which had been spotted and dried on GF/C filters and counted in a toluene-based scintillant. The values obtained for several aliquots were averaged.

Preparation of Templates

Poly [d(A-T)]

Poly [d(A-T)] ($d(A-T)_n$) was purchased from General Biochemicals (with a mw of 1-5 x 10^6 daltons) or it was synthesized in the laboratory as follows: 50 ul Tris·HCl pH 7.4 (1M), 10 ul $MgCl_2$ (0.5 M), 80 ul dATP (0.01 M), 80 ul d(TTP) (0.01 M), 20 ul $d(A-T)_n$ (5 O.D.$_{260}$/ml) and 100 ul PCMS (10^{-3} M) were combined and brought to 1 ml with distilled water. This reaction mixture was incubated for 90 min at 37°C. Aliquots of 20 ul were taken at intervals and added to a dilute solution of ethidium bromide in order to determine the progress of the polymerization reaction and to ascertain the point at which the rate of polymerization ceased to be linear. For this purpose, the emission at 590 nm of the product polymer-ethidium bromide complex was measured in an Aminco-Bowman Spectrophotofluorimeter (American Instrument Co.).

The polymerization reaction was terminated with the addition of 0.6 ml EDTA (0.2 M) per 30 ml reaction mixture. The reaction mix was made 0.5 M with KCl and heated at 60°C for 5 min. Silicic acid was added at 10 ug/ml and the mixture was centrifuged at 10,000 rpm for 1 min.

The supernatant was filtered through a millipore filter and two volumes of 95% ethanol were added on ice. After centrifugation at 10,000 rpm for 5 min, the pellet was resuspended and dialyzed against 0.01 M Tris·HCl (pH 7.8), 0.1 M KCl and 1 mM EDTA.

Calf thymus DNA and salmon sperm DNA

Calf thymus DNA and salmon sperm DNA purchased from Sigma were further purified according to Kedinger et al. [1974]. In this procedure, the DNA was solubilized in buffer containing 10 mM Tris·HCl (pH 7.5), 10 mM NaCl and 0.1% SDS and then twice phenol extracted. The second phenol extraction was followed by ethanol precipitation and dialysis against a buffer containing 10 mM Tris·HCl (pH 7.5), 10 mM NaCl and 0.1 mM EDTA. The purified DNA was stored at 4°C at a concentration of approximately 1.5 mg/ml ($O.D._{260}$ = 30).

Artemia DNA

Artemia DNA was prepared from Artemia salina cysts by a modification of the method of Marmur [1961]. Sixty grams (wet weight) of hydrated shrimp cysts were washed several times with distilled water and ground in a porcelain mortar, followed by dilution with saline-EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0) to a final concentration of 1.2 g/5 ml. Eleven ml 25% sodium lauryl sulfate was added per 100 ml shrimp homogenate, followed by incubation at 60°C for 10 min. After incubation, sodium perchlorate was added to a final concentration of 1 M.

An equal volume of a mixture of chloroform-isoamyl alcohol (24:1) was added, followed by shaking for 30 min. The mixture was centrifuged for 10 min at 9,500 rpm in a Sorvall GSA rotor. The upper layer was removed and the DNA was ethanol precipitated from it.

The DNA spool was resuspended in 0.1 x SSC (saline-sodium citrate, 0.15 M NaCl, 0.015 M sodium citrate). The volume at this point was 200 ml and the concentration of DNA was 2 mg/ml (as determined by absorbance at 260 nm).

The crude DNA preparation was treated with 50 ug/ml pancreatic RNAse at 37°C and the chloroform-isoamyl alcohol procedure was then repeated two times. An equal volume of 90% phenol was added and the mixture was shaken for 10 min. The phenol was removed by centrifugation and the DNA-containing upper layer was shaken gently with 1/3 volume of ether. The ether was removed by aspiration and the DNA was alcohol precipitated. The DNA spool was resuspended in 0.1 x SSC and dialyzed against a buffer containing 10 mM Tris·HCl at pH 7.5, 10 mM NaCl, and 0.1 mM EDTA. The purified DNA was stored in this buffer at 4°C or, for longer periods, at 0°C as an ethanol precipitate.

All denatured DNA templates were obtained by heating the native form at 90°C for 10 min followed by quick cooling.

Assay of Enzyme Preparations for Nuclease Activity

Assay of enzyme preparations for nuclease activity was done by incubation of the polymerase sample with a known amount of $^{3}H \cdot d(A\text{-}T)_n$ and measuring the loss of acid precipitable counts after incubation at 37°C. An indirect method of measuring nuclease as well as protease activity was to measure the incorporation of $^{3}H \cdot UMP$ into an acid-precipitable RNA product over time. Linear incorporation over 15 min indicated that there was little breakdown of template, product or polymerase enzyme under the standard conditions of assay.

Standard Assay for RNA Polymerase Activity

The standard assay for RNA polymerase activity contained, in a final volume of 125 ul, 7 umoles Tris·HCl (56 mM), 0.7 umoles NaF (5.6 mM), 0.5 umoles PEP (4 mM), 0.075 umole each ATP, CTP and GTP (0.6 mM), 0.0125 umole ^{3}H·UTP (0.1 mM, approximately 60 cpm/pmole), 1 umole KCl (8 mM), 2.5 ug pyruvate kinase (20 ug/ml), 0.2 umole mercaptoethanol (1.6 mM), calf thymus DNA or d(A-T)$_n$ and either Mn^{++} or Mg^{++} at 4 mM. The polymerization reaction was halted by the addition of 0.2 M sodium pyrophosphate at pH 6 with KH_2PO_4.

Ammonium sulfate was added in the amounts indicated or, in the assay of column fractions, was present in amounts determined by the concentration of the ammonium sulfate gradient for that particular column fraction. The volume of enzyme was usually 30 or 50 ul in a final volume of 125 ul.

The RNA product of the reaction was precipitated by the addition of several ml cold 5% TCA. The precipitated product was collected after 10 min at 0°C by filtration through acid-washed, ATP-soaked GF/C filters. The precipitate was washed with 10-15 ml cold 5% TCA after which the filters were dried and counted for radioactivity in scintillation grade toluene containing New England Nuclear omnifluor. Full tritium windows of Nuclear Chicago, Beckman and Intertechnique scintillation counters were used for different experiments. All data was normalized for one scintillation counter by using tritium standards.

GF/C filters (glass fiber filters, high retention, Whatman) were found to non-specifically bind ^{3}H·UTP, resulting in a high and variable background. To prevent this, the filters were hydrated in distilled

water, then boiled gently in 0.1 M HCl for approximately one hour. The temperature was raised very slowly to prevent disruption and disintegration of the filters. After washing with several changes of distilled water, the filters were brought to pH 7.9 with 1 M Tris·HCl buffer. They were stored at 4°C in a beaker containing a solution of 0.01 M ATP brought to pH 7 with pyrophosphate.

Protection of enzyme activity was enhanced by the addition of 0.5 mg/ml BSA to column fractions and RNA polymerase preparations subjected to freezing and storage. (BSA elutes from DEAE-Sephadex at lower ammonium sulfate concentrations and because of its small size, is easily distinguished from RNA polymerase in gel electrophoresis and glycerol gradient absorbance profiles.)

Purification of RNA Polymerase

A summary of the purification procedure is presented in figure 1. A detailed description of each procedure is given below.

Homogenization

Homogenization of *Artemia* cysts was carried out by various methods. Grinding in a porcelain mortar followed by homogenization with a motor-driven Potter-Elvehjem (glass and teflon) homogenizer proved to be the best. Freeze-thaw methods did not disrupt the cysts nor did a Waring blender. A colloidal mill was successful in disrupting the cysts but it caused much aeration and was not used in the experiments described.

All procedures were carried out on ice or in a 4°C cold room. After hydration (usually overnight at 4°C as described) the *Artemia* cysts were washed several times with distilled water and collected on a large disk of Whatman No. 1 filter paper. The resulting cake of shrimp cysts

(containing contaminating sand in small amounts) was weighed (wet weight).

The shrimp were then placed in a very large porcelain mortar (held at 4°C with ice) and suspended in a small volume of buffer A (5% glycerol). The buffer volume was that sufficient to make a paste. Grinding with a pestle was carried out for up to 10 min. The appearance of a bright orange color indicated shrimp cyst breakage. The shrimp past was diluted with additional buffer A (5% glycerol) until the consistancy was appropriate for homogenization in the Potter-Elvehjem. For 100 g wet weight shrimp, 150 ml buffer A was required. Homogenization in the teflon-glass homogenizer was not used in the chromatographic and catalytic characterization experiments in the interest of saving time and avoiding aeration and heating. Grinding alone and grinding followed by homogenization were both followed by filtration through several layers of cheesecloth (previously prepared by boiling in EDTA at 0.1 mM).

Sonication

Sonication of the shrimp homogenate in 0.32 M ammonium sulfate resulted in breakage of the nuclei, fragmentation of the chromatin and release of the polymerase molecules and other nuclear proteins from the chromatin. The volume of the homogenate was doubled by the addition of buffer A (5% glycerol) to lower the protein concentration and then the ammonium sulfate concentration was brought to 0.32 M by the gradual addition of 0.08 ml of 4 M ammonium sulfate per ml of homogenate. (The 4 M ammonium sulfate had been adjusted to a pH of 7.8 by the addition of ammonium hydroxide). After gentle stirring, the homogenate became extremely viscous and was subjected to approximately 60 seconds of sonication. This was delivered as 10 seconds of sonication followed by a minute of slow stirring to prevent heating and aeration. The large probe

of a Heat Systems-Ultrasonics, Inc. Sonifier Cell Disrupter, Model W185, or a Branson sonicator was used at full strength. Large samples were sonicated as smaller aliquots. The temperature was controlled by a salt ice bath and stirring with a thermometer provided constant sample monitoring. Sonication was judged complete when the homogenate flowed easily from the constricted end of a Pasteur pipette.

High speed centrifugation

In order to remove debris, unbroken cysts, cyst shells and the large amounts of fat present, the homogenate was centrifuged for 75 min at 35,000 rpm in a Beckman 35 rotor or 30,000 rpm in a Beckman 30 rotor. The surface layer of lipid was removed with a cold spatula and the supernatant decanted, filtered through glass wool, and brought to 55% saturation with ammonium sulfate (the ammonium sulfate was slowly added in pulverized form with constant stirring). After 20 to 30 min the proteins were pelleted by centrifugation in the 35 rotor (or the 30 rotor) at 25,000 rpm for 20 min. The pellet was resuspended in buffer A (30% glycerol).

Polymin-P titration

In order to remove contaminating nucleic acids, polymin-P titration was carried out on 1 ml aliquots of the resuspended ammonium sulfate precipitate of the high speed supernatant by adding increasing amounts (0 through 60 ul) of a 2.5% polymin solution. After mixing and allowing to stand on ice for 15-20 min, the shrimp-polymin mixture was spun at 10,000 rpm in a Sorval J-21 centrifuge (JA-20 rotor) for 10 min. The supernatant was assayed for RNA polymerase activity and an aliquot was brought to 1 ml with a solution which was 1 M NaCl and 7.5 mM Tris·HCl, pH 7.8. The high salt was necessary to prevent light scattering due to

aggregation of the polymin. Absorbance was measured at 260 nm and at 280 nm in a Beckman Acta III spectrophotometer and the A_{280}/A_{260} ratio was plotted against polymerase activity.

The remaining high speed supernatant was reacted with polymin at the ratio of polymin to shrimp extract which gave the highest A_{280}/A_{260} ratio without loss of enzymatic activity. In order to remove traces of polymin after the removal by centrifugation of the nucleic acid-polymin complex, the supernatant containing the polymerase activity was again made 55% ammonium sulfate and precipitated by centrifugation.

Storage of the post-polymin polymerase fraction

Following ammonium sulfate precipitation of the post-polymin supernatant, the pellet was resuspended in buffer A (50% glycerol), assayed for RNA polymerase activity and protein concentration and then frozen in liquid nitrogen (when available) or by placing in a Revco low temperature (-70°C) freezer. After freezing all samples were stored in a Revco at -70°C. Enzymes stored in liquid nitrogen are reported to maintain their activity longer than enzymes stored at higher temperatures, however facilities for this have never been available. Increasing the glycerol content of the buffers to 50% has been seen to improve enzyme stability (data not shown).

RESULTS

Development of an Assay System for Artemia salina RNA Polymerase

Prior to this project, the RNA polymerase activity of Artemia salina had never been examined. Although assay systems existed for sea urchin [Roeder and Rutter 1969], rat liver [Seifart, Benecke and Juhasz 1972] and calf thymus [Kedinger et al. 1972] RNA polymerases, it was not known if these would be adequate for measurement of the RNA polymerase of the brine shrimp cyst. Major concerns were the possibility of high nuclease, protease and phosphatase levels. These enzymes were assayed for either directly or indirectly as will be described. The possibilities of insufficient template or excess enzyme in the assay mix were considered and the correct amounts of these components established.

Figure 2 shows the incorporation of substrate (^{3}H·UMP) into an acid-insoluble product to be essentially linear over a period of 15 min when either shrimp cyst homogenate or highly purified shrimp cyst RNA polymerase is incubated in the standard reaction mixture at 37°C. This indicates that neither the template, the product, nor the enzyme is adversely affected by elements of the assay procedure. A decrease in the rate of incorporation with time would have indicated that product was no longer being formed, or that it was being broken down at a rate exactly equal to its formation. A decrease in the amount of acid-precipitable ^{3}H·UMP with incubation would have indicated nucleolytic cleavage of the RNA product.

The reaction mixture used to obtain the data in figure 2 was the same as that used in all subsequent assays for polymerase activity, with the exception of characterization studies, in which the concentration of metal ion cofactor, ionic strength and concentration of alpha-amanitin were varied as indicated.

The standard reaction mix contained, in addition to template, enzyme and nucleoside triphosphate substrates, a nucleoside triphosphate regenerating system in the form of phosphoenol pyruvate and pyruvate kinase. Sodium fluoride was included as an inhibitor of phosphatases. The metal ion cofactor was either manganese or magnesium and ionic strength was maintained by the presence of KCl and the addition of ammonium sulfate. The pH was held constant at 7.8 by Tris·HCl. (See methods section for the actual molar concentrations of the reaction mixture componants and appendix 1 for the schedule of additions and final concentrations of the test substance stock solutions.)

Brine shrimp homogenate was assayed for endogenous nuclease activity as described in Methods and the results are presented in table 3. The nuclease activity is seen to be low and time-dependent. Radioactive template (^{3}H·d(A-T)$_n$) was incubated with shrimp RNA polymerase preparations at different stages of purification in a complete reaction mix at 37°C. Nuclease activity in the form of template degradation is indicated by the release of acid-soluble radioactivity.

Reduction of acid-precipitable material is not extensive, even after 20 min incubation at 37°C. When the nuclease activity of shrimp crude homogenates was measured, the activity was only slightly enhanced in the presence of Mg^{++} relative to the activity with Mn^{++}.

The results presented in part B of table 3 cannot be compared in an

absolute sense with the data from part A of table 3 since protein determinations were not carried out on those samples. The average amount of template remaining after 20 min at 37°C in assays containing homogenate, first supernatant and second supernatant in the presence of Mn^{++} average 91 ± 1% (table 3, part B). After 10 min at 37°C (table 3, part A) the homogenate and first supernatant average 97 ± 0.5% template remaining. The effects of nucleases on the total amount of template available under the conditions of RNA polymerase assay were considered negligible on the basis of these results.

In order to determine the range of template concentrations adequate to obtain optimum activity of a highly purified preparation of shrimp RNA polymerase, a radioactive template was again employed (see Methods section for the preparation of $^{3}H{\cdot}d(A\text{-}T)_n$). Shrimp cyst RNA polymerase activity purified by DEAE-cellulose column chromatography followed by chromatography on DNA-cellulose (figure 3) was added in amounts from 0 to 9 ug protein to a series of standard polymerase assay mixes lacking only the labeled nucleotide substrate and containing a constant amount of the labeled template (figure 4). After 5 min incubation at 37°C, the mixture was poured onto a millipore filter. Only that portion of the template bound to protein was held by the filter. The amount of $^{3}H{\cdot}d(A\text{-}T)_n$ held by the filter increased linearly until a plateau was reached at which 71% of the template was bound. Departure from linearity began at the point where the ratio of template (micrograms) to protein (micrograms) was 0.31.

Since the effect of increasing the protein concentration in these reactions was initially to generate a straight line, the indication is that one polymerase molecule is sufficient to bind one $d(A\text{-}T)_n$ molecule.

The point at which linearity is lost indicates the transition from a one to one ratio to a situation where more than one polymerase molecule is bound to the template molecule. Polymerase molecules can actively transcribe while sharing a particular template, however, any polymerase assay containing $d(A\text{-}T)_n$ as the template in a 0.3 ratio to the amount of protein could safely be considered to contain a non-limiting or excess amount of template.

The effect of increasing the amount of shrimp protein in the standard RNA polymerase assay mixture was examined. Figure 5 relates ^{3}H·UMP incorporation (RNA synthesis) to protein concentration. Shrimp cyst RNA polymerase extracts at several stages of purification were employed. Although the incorporation remained linear when 550 ug crude homogenate protein was added in a final assay volume of 125 ul, some componant of the standard reaction mix was found to be rate limiting when the amount of protein in the post-DNA-cellulose stage enzyme was present in amounts greater than 5 ug protein per 125 ul assay.

Observation of Multiple RNA Polymerase Activities in Artemia salina

Initial assays for RNA polymerase activity in homogenates of hydrated shrimp cysts showed that the RNA polymerase activity present was partially resistant to the toadstool toxin, alpha-amanitin. This toxin is known to inhibit elongation of RNA chains catalyzed by class II eukaryotic RNA polymerases. The finding of inhibition by alpha-amanitin of polymerase activity in brine shrimp homogenates was the first indirect evidence of the existence of more than one class of RNA polymerase in Artemia salina.

Direct evidence of the presence of several forms of RNA polymerase

in Artemia came when DEAE-Sephadex column chromatography (figure 6 and figure 7) resulted in the complete separation of two major enzyme activity peaks. The first peak to be eluted from the DEAE-Sephadex by the linear gradient of ammonium sulfate was totally resistant to 8 ug/ml amanitin (fiugre 7). The second enzyme activity peak had partial amanitin resistance. This made it possible that Artemia had three distinguishable and potentially separable RNA polymerase forms.

Following the nomenclature developed by Roeder and Rutter [1969] for eukaryotic RNA polymerases, the enzyme activity eluting first from DEAE-Sephadex will be called enzyme I while amanitin-sensitive polymerase activity eluting at the higher ionic strength will be called polymerase II. In sea urchins, where this naming system was first applied, a third polymerase eluted after polymerase II and was named polymerase III [Roeder and Rutter 1969]. Since that time, tissues have been found in which polymerase III elutes at an ionic strength similar or identical to polymerase II. The amanitin-resistant activity co-eluting with polymerase II of brine shrimp will be tentatively called polymerase III.

Prior to chromatography on DEAE-Sephadex (figure 6) the RNA polymerase activity extracted from incubated (24 hrs at 28°C) Artemia was measured using three templates, each in the presence and absence of alpha-amanitin.

Template	Template Amount	Polymerase Activity	% Resistance to 8 ug/ml Alpha-amanitin
$d(A\text{-}T)_n$	5 ug	128 pmol/mg/10'	77
CT DNA_{den}	30 ug	95 pmol/mg/10'	68
CT DNA_{nat}	30 ug	30 pmol/mg/10'	75

Originally (prior to freezing) the extract had incorporated

170 pmol/mg protein/10 min at 37°C with 5 ug $d(A\text{-}T)_n$ in a final volume of 125 ul. After thawing and prior to chromatography, the activity was as given above. The choice of template greatly affected the total activity of the crude extract. Choice of template also had an effect on the degree of amanitin resistance displayed. Clearly $d(A\text{-}T)_n$ is the favored template of at least one of the enzymes. The elevated amanitin resistance with that template allows a preliminary assignment of $d(A\text{-}T)_n$ as the favored template of the amanitin-resistant form(s). There are too many variables for further interpretation of this data, however, the existence of several enzyme forms which differ in their template preferences is strongly suggested.

It should be noted that incorporation of ^{3}H·UMP by Artemia extract became dependent upon the addition of DNA to the reaction mixture during the pre-chromatography purification procedure. Most endogenous DNA was removed by the high speed centrifugation. Remaining nucleic acid fragments were separated from the enzyme when the latter was precipitated from the high speed supernatant with ammonium sulfate. The A_{280}/A_{260} ratios presented in figure 17 show that nucleic acids were significantly reduced by treatment of the shrimp extract with polymin-P. Incorporation of ^{3}H·UMP became dependent on endogenously added DNA at the time of ammonium sulfate precipitation. DNA-dependence of the reaction is strong evidence that the reaction product is RNA. Further evidence of this is the dependence of the reaction on the presence in the reaction mix of other nucleoside triphosphates (ATP with the template $d(A\text{-}T)_n$ and ATP, GTP and CTP with native and denatured DNAs).

In an attempt to elucidate the enzyme composition of the second DEAE-Sephadex activity peak, aliquots from polymerase-containing fractions

were subjected to polyacrylamide gel electrophoresis. The hypothesis was that two polymerase molecules of differing molecular weight or electrophoretic properties might be co-eluting in the activity peak from the column and that these might correspond to an amanitin-sensitive enzyme and an amanitin-resistant enzyme. Demonstration of enzymatic activity in an actual gel slice is impossible with such low enzymatic activity, however protein bands might be separated by electrophoresis which could be tentatively identified as polymerase molecules. The basis for this tentative identification would be the characteristic slow migration of polymerase molecules in gels of low acrylamide concentration and correspondance of the column fraction polymerase activity with the amount of protein in the resulting gel band. As figure 8 shows, electrophoresis of polymerase-containing fractions from a DEAE-Sephadex column did result in single protein bands. Although the bands could be RNA polymerase on the basis of their slow migration, the amount of protein in the stained band was not directly proportional to the amount of enzymatic activity in the fraction which was electrophoresed.

Thus it appeared doubtful that the proteins which had been seen by the electrophoresis procedure were polymerases although they were very similar in electrophoretic behavior. The results of this work are shown since later experiments provide a possible explanation for the data in figure 8. This subject is treated in greater depth in the Discussion section.

Further experiments aimed at understanding the nature and the number of enzymes eluting as the second DEAE-Sephadex activity peak capitalized on the observation that the amanitin-resistant activity was more labile than the amanitin-sensitive activity. At the time of elution, this RNA

polymerase activity from the DEAE-Sephadex column of figures 7 and 8 showed the usual partial resistance to amanitin (25% resistance to 4 ug/ml amanitin with CT DNA_{den} as template). After several rounds of freezing and thawing in a Revco at -70°C, the amanitin-resistant activity was completely abolished. The remaining polymerase activity was re-assayed in the presence of increasing amounts of amanitin. The results are presented in figure 9. Total inhibition was achieved at 0.4 ug/ml amanitin. The high degree of linearity and the total abolition of enzymatic activity indicates that a single species of enzyme is active.

Disappearance of the polymerase activity resistant to 4 ug/ml amanitin indicates that the amanitin-resistant polymerase is differentially labile. This is supported by figure 7 in which loss of the amanitin-resistant activity occurs under conditions which leave the amanitin-sensitive polymerase at the original level of activity.

Further strong evidence of multiple polymerases in Artemia is found in figure 10 which shows the the effect of ammonium sulfate concentration on the RNA polymerase activity of fraction 17 of the DNA-cellulose column whose activity profile is given in figure 3. This polymerase preparation had been batch-eluted from DEAE-cellulose prior to DNA-cellulose chromatography. Because the DNA-cellulose column was developed with a step gradient, the activity peak contains a highly purified mixture of amanitin-resistant and amanitin-sensitive polymerases.

Fraction 17 of the DNA-cellulose column (figure 3) was 57% resistant to 8 ug/ml alpha-amanitin when assayed with $d(A\text{-}T)_n$ as template. The effect of ionic strength (ammonium sulfate) was examined under three sets of conditions and the results are given in figure 10. An aliquot of the enzyme mixture was frozen and thawed to remove its amanitin-resistant

activity while the remainder of the sample was assayed in the absence and presence of alpha-amanitin. The untreated enzyme assayed without amanitin gave a biphasic activity profile with maxima at 0.025 and 0.075 M ammonium sulfate. The enzyme assayed in the presence of amanitin had an activity optimum at 0.075 M and the frozen and thawed aliquot containing only amanitin sensitive-material had an activity optimum at 0.025 M. The untreated enzyme mixture clearly contains at least two enzyme activities which differ in their ionic strength optima as well as in their sensitivity to amanitin and freezing and thawing.

For comparison, assays of the amanitin-sensitive RNA polymerase activity of the second activity peak of a DEAE-Sephadex column eluted with a linear salt gradient (and not previously exposed to DNA-cellulose) was examined. After freezing and thawing no amanitin-resistant activity was present and the enzyme was assayed with different templates at various ammonium sulfate concentrations and metal ion cofactors (figure 11 and figure 12). Figures 10, 32, 33 and 35 show other instances in which Artemia RNA polymerase II was found to have an ionic strength optimum near 0.075 M ammonium sulfate with CT DNA_{den} as template. Thus Artemia contain a polymerase II activity which is similar to that of other species [Roeder 1976] and separable from other Artemia polymerase activities by several methods.

The Artemia RNA polymerase II used in the activity assays of figure 11 and figure 12 was examined by glycerol gradient centrifugation as described in the legend to figure 13. The enzyme was layered onto a preformed glycerol gradient (15 - 30% v/v) and centrifuged at 35,000 rpm for 15.4 hr in a SW41 rotor at 3°C. The gradients were fractionated and assayed for RNA polymerase activity. Selected fractions were run on 5% polyacrylamide

gels under non-denaturing conditions in order to determine whether fractions containing enzymatic activity contained protein(s) which migrated in a manner suggestive of an RNA polymerase. The results are shown in figure 13. Although the enzymatic activity was very low, those gels corresponding to the peak fractions were the ones found to contain single absorbance bands. Other gels contained no bands whatsoever. The gels were scanned in a spectrophotometer at 600 nm and the relative sizes of the absorbance peaks of different gels were determined as described in the figure legend. The actual absorbance profiles of two of these gels are shown in figure 44. In figure 44, the Artemia glycerol gradient fractions were run on two gels and compared to a gel containing a sample of E. coli RNA polymerase (Sigma) which had been purified by centrifugation on a similar glycerol gradient (fraction 14, Appendix 2).

Because phosphocellulose chromatography of E. coli RNA polymerase causes the σ subunit of that enzyme to become dissociated from the core polymerase, Artemia polymerase behavior on DEAE-Sephadex was checked after chromatography on DNA-cellulose. Figure 14 shows that DNA-cellulose chromatography made no changes affecting polymerase II and polymerase III behavior on DEAE-Sephadex. The elution position of polymerase II treated in this manner is virtually identical to the elution position of Artemia polymerase II from a crude homogenate (figures 6 and 7). Polymerase III activity is similarly unchanged with respect to elution position; it co-elutes with polymerase II. It was essential to ascertain this since DNA-cellulose was a very effective purification step.

Large Scale Purification

Introduction

Further investigation of the multiple Artemia RNA polymerase classes and their similarity to each other and to the cognate polymerases isolated from other eukaryotic tissues required separation followed by catalytic characterization. A number of problems were encountered.

The activity of enzyme I as well as that of the amanitin-resistant activity in the DEAE-Sephadex activity peak belonging to enzyme II was low relative to the amanitin-sensitive activity; these amanitin-resistant activities were also the most labile. Larger amounts of these enzymes would therefore be needed for full catalytic characterization and identification. Due to the extreme and unequal lability of the enzymes as they were eluted from the column, re-assay of the column fractions at a later time resulted in significantly less product being formed although identical incubation conditions were employed (figure 7, part B). As previously shown, this differential lability of the co-eluting enzymes permitted their separate characterization prior to the development of chromatographic separation techniques.

Attempts to obtain large amounts of separated polymerase classes from DEAE-Sephadex columns of shrimp homogenate failed since increasing the amount of protein resulted in an overloading of the column material as well as overlap of the enzyme activities. Increasing the amount of column material as well as the amount of protein had the effect of prolonging the loading and running time of the column and, because of the longer time at 4°C, reducing the yield (in terms of activity) to approximately that of the smaller columns. The problem of diminishing returns

was made more acute by the low protein concentration of the collected fractions. Eukaryotic RNA polymerases are unstable under these conditions so BSA (bovine serum albumin) was sometimes added to column fractions to bring the protein concentration to approximately 0.5 mg/ml. DEAE-Sephadex column fractions, although they contained detectable enzymatic activity, generally had insufficient polymerase protein for extensive characterization by gel electrophoresis.

Due to the complexity of the purification procedure, large amounts of hydrated shrimp cysts could not be processed to the stage of polymerase chromatography in a single day. Bulk purification required that shrimp enzyme be purified up to the stage of column chromatography and then frozen. Enzyme preparations were then pooled for chromatographic separation and catalytic characterization studies.

Large scale purification not involving chromatographic separation of RNA polymerase activities

On three occasions, Artemia cysts (total wet weight = 1,231 g) were homogenized and the RNA polymerase activity partially purified by the pre-chromatography procedures outlined in the flow-diagram, figure 1. The procedures used were as described in Methods and include sonication, nigh speed centrifugation and precipitation with polymin-P. The recovery of enzymatic activity at the various stages of purification are given in table 5 for these three separate experiments (1, 2 and 3). The F/S (frozen/stored) fractions of these three experiments were pooled for further purification by chromatography. Table 5 also gives the results of six additional Artemia cyst homogenizations (experiments 4 - 9) in which the RNA polymerases from a total of 2,242 g hydrated shrimp cysts were purified to the F/S stage according to the procedures of figure 1.

The procedure of polymin titration to remove nucleic acids from the high speed supernatant of the shrimp cyst extract has already been described in Methods. Data from the polymin titration of the high speed supernatants of experiments 2 and 3 are presented in figure 17 A and figure 17 B respectively as well as in table 4. It can be seen from the results of these and other polymin titrations (data from experiments 9 and 6 in figure 17 C and figure 17 D and from experiments 4 - 9 in table 4) that RNA polymerase activity in the Artemia preparations tended to be at an optimum when the A_{280}/A_{260} ratio was 0.77. This ratio corresponds to a nucleic acid content of 7.5% relative to the protein concentration. Prior to treatment with polymin-P, the shrimp high speed supernatant had, on the average, an A_{280}/A_{260} ratio of 0.71 or 10% nucleic acid relative to protein. The effect of this reduction in nucleic acids is to double the enzymatic activity of the polymerases. Figure 17 shows data from four representative experiments while table 4 gives the summary of eight experiments.

For experiments 1 - 3 the increase in specific activity (polymerase activity units/mg protein) of the shrimp extracts and the degree of recovery of the original homogenate enzymatic activity is detailed in table 6. The specific activity increased over a thousand fold from the crude homogenate to the DNA-cellulose peak fraction. The total amount of protein in the combined homogenates of experiments 1 - 3 was 33,700 mg. This was reduced to 13.8 mg after the DNA-cellulose step; yet 24% of the polymerase activity originally present in the homogenate was recovered. In terms of protein alone, this represents a greater than 2,400 fold purification.

The DEAE-cellulose procedure is outlined in figure 15 and described in detail below. No column activity profile can be shown since the

enzymatic activity was pooled without assaying the individual fractions for polymerase activity.

The pooled F/S fractions of experiments 1 - 3 contained 5,001 mg of protein. The thawed sample was stirred with DEAE-cellulose (previously equilibrated with buffer A at 0.05 M ammonium sulfate) at a ratio of 6 mg dry weight DEAE-cellulose/mg protein. The mixture was kept in the cold room and stirred occasionally for 45 min at which time the slurry was poured into a large plastic Buchner funnel. The cellulose was washed with 500 ml buffer A at 0.07 M ammonium sulfate and transferred to a glass chromatography column 4 cm in diameter. All polymerase activity was removed by elution with buffer A at 0.5 M ammonium sulfate. Fractions of 100 drops were collected and those 16 tubes containing the UV absorbance were pooled and made 51% saturated with ammonium sulfate. The proteins were precipitated by centrifugation at 25,000 rpm in a Beckman 35 rotor. The pellet was resuspended in 150 ml buffer B in preparation for loading the sample onto a DNA-cellulose column.

Figure 3 shows the polymerase activity and protein concentration of the collected fractions of this DNA-cellulose column of the pooled F/S fractions of experiments 1 - 3. The legend to this figure describes the procedures in detail.

The DNA-cellulose RNA polymerase activity peak contained 24% of the original activity of the crude homogenate while the peak fraction of the column contained only 0.013% of the homogenate protein. The specific activity of the peak fraction is 1,100 times that of the crude homogenate.

Large scale purification and separation of RNA polymerase classes

As previously mentioned, the F/S fractions of six additional shrimp

cyst homogenizations (experiments 4 - 9) were pooled and subjected to chromatography on DEAE-cellulose followed by chromatography on DNA-cellulose (figure 1, figure 16). The enzymatic activity and protein concentration of the pre-chromatography purification steps is shown in table 7 for experiments 5, 6, 7 and 8. Table 5 gives a summary of the recovery of activity units from homogenization to the thawing of the stored F/S fractions in experiments 4 - 9.

The chromatographic treatment of this second bulk preparation of shrimp polymerase activity differed from that described in figure 15 in that the overall purpose was to separate enzyme activities on the basis of their elution position from DEAE-cellulose as well as to provide a purification step. DEAE-cellulose chromatography was followed by DNA-cellulose chromatography; assay of enzymatic activity with and without alpha-amanitin was used to indicate the degree of separation achieved.

Figure 16 is a flow diagram of this large scale purification scheme. The pooled F/S fractions from experiments 4 - 9 contained 1,892 units of RNA polymerase activity in 15,147 mg protein. This material was mixed with DEAE-cellulose in buffer A at 0.045 M ammonium sulfate as described in the legend to figure 18. After pre-loading with shrimp extract, the DEAE-cellulose was collected in a Buchner funnel. The flow-through volume was reserved for assay and recovery of polymerase activity unable to bind DEAE-cellulose under these conditions.

The DEAE-cellulose was then washed with buffer A (0.08 M ammonium sulfate) and the slurry was transferred from the Buchner funnel to a large chromatographic column as described in the legend to figure 18. Polymerase activity was eluted stepwise by buffer A (0.5 M ammonium sulfate). The enzymatic activity and protein concentration of the three

preparations, the flow-through volume, the 0.08 M ammonium sulfate wash and the 0.5 M ammonium sulfate-eluted enzyme were measured. The RNA polymerase assays indicated that all three fractions contained significant enzymatic activity.

Sample	Total Protein	Total Activity	% Resistance
flow-through	n.d.	726 units	72
0.08 M wash	980 mg	516 units	45
eluted activity	400 mg	413 units	30

The DEAE-cellulose procedure described above is called DEAE-cellulose 1 in figure 16 and table 8. Table 8 presents a summary of the yield at each step of the purification procedure, in terms of protein, enzymatic activity, alpha-amanitin resistance and degree of purification.

The DEAE-cellulose 1 activity peak (fractions 27 - 32 of figure 18) fractions were pooled and re-chromatographed on DNA-cellulose (DNA-cellulose 1; figure 19). The DEAE-cellulose 1 flow-through fraction was mixed with additional DEAE-cellulose at a lower ionic strength and step-eluted with buffer A (0.5 M ammonium sulfate). The procedure is described in the legend to figure 20 and the results are presented in figure 20 (the activity profile of DEAE-cellulose 2) and table 8 (item 4, DEAE-cellulose 2).

The 0.08 M ammonium sulfate wash of DEAE-cellulose 1 was ammonium sulfate precipitated, resuspended in buffer B with no NaCl and applied to a DNA-cellulose column (table 8, item 3, DNA-cellulose 2, column profile not presented).

Comparing the results of the two bulk purification procedures as summarized in table 6 and table 8, it is seen that the pooled experiments

1 - 3 (1,231 g wet weight shrimp cysts) had a final yield of 325 units of enzymatic activity in 13.8 mg protein. This is a specific activity of 23.6 units/mg (peak fraction = 4.3 mg, 189 units, 44.0 units/mg).

The results of pooled experiments 4 - 9 are similar to those of experiments 1 - 3 in terms of yield and degree of purification (specific activity) achieved. The 2,241 g wet weight shrimp yielded 248 units of enzymatic activity in 7.0 mg at 35.4 units/mg (peak fraction = 0.45 mg, 21.3 units, 47.3 units/mg) after DNA-cellulose chromatography of the portion of the original F/S enzymatic activity which remained bound to DEAE-cellulose after the 0.08 M wash (figure 19). The lower alpha-amanitin resistance of this enzyme activity indicates that it is enriched for enzyme II while the flow-through of the DEAE-cellulose 1 had an amanitin resistance (72%) indicating an enrichment for amanitin-resistant polymerase activity.

Further substantiation of the partial separation of polymerase classes is seen in the relatively high elution position (0.13 M - 0.25 M ammonium sulfate) of the enzyme remaining bound to the original DEAE-cellulose column after the 0.08 M wash. The break-through volume of this first DEAE-cellulose column not only did not bind to the first DEAE-cellulose column when loaded at a relatively low ionic strength, it also had a low elution position (0.05 M - 0.19 M) from the subsequent DEAE-cellulose column to which it was bound at a very low ionic strength (less than 0.04 M ammonium sulfate).

Chromatographic Characterization

In-depth characterization of the enzymes, RNA polymerase II and RNA polymerase III from hydrated cysts of Artemia salina was made possible by

the extensive information gained in the early experiments of this project which dealt with chromatographic behavior and catalytic properties of the shrimp polymerases. This information permitted the design of a chromatographic scheme which resulted in apparently complete separation of enzyme activities. Characterization of the catalytic requirements could be done with greater assurance that the sample did not contain a mixture of enzymes. Because of the lability of certain enzyme forms, speed of experimentation had become an important variable. Only 70 hours elapsed between the homogenization of the hydrated Artemia cysts and the running and assay of the DEAE-cellulose column (figure 22), the two DEAE-Sephadex columns (figures 23 and 24) and all of the catalysis characterization in the set of experiments presented. The phosphocellulose column of polymerase II was run several days later using the pooled fractions 12 - 24 from DEAE-Sephadex (figure 24) which had been frozen and stored at -70°C.

The rationale behind the experimental design for separation of RNA polymerases by a combination of DEAE-Sephadex and DEAE-cellulose column chromatography is presented in the Discussion section. A flow diagram of the chromatographic characterization scheme is presented as figure 21. The results from experiments on the catalytic requirements and preferences of the separated classes of Artemia polymerases are given following the presentation of the results of the various chromatographic procedures.

As indicated in figure 21, 112 g (wet weight) of hydrated Artemia cysts were ground in a porcelain mortar. After filtering through cheesecloth, this homogenate was sonicated in 0.4 M ammonium sulfate and centrifuged at 25,000 rpm in a Beckman 30 rotor. The supernatant from this high speed centrifugation was brought to 55% saturation with ammonium sulfate. The ammonium sulfate-precipitated pellet contained 882 mg protein

and had a nucleic acid content of 7.5% relative to the protein concentration as judged from the relative absorbance at 280 nm and 260 nm.

DEAE-cellulose chromatography

The material described above was loaded onto DEAE-cellulose as described in the legend to figure 22. The time-consuming step of running the sample onto a pre-equilibrated column of DEAE-cellulose was eliminated by mixing DEAE-cellulose (pre-equilibrated with buffer A at 0.05 M ammonium sulfate) directly with the high speed supernatant fraction, and, after allowing time for adsorption of the enzyme, gently removing the loaded DEAE-cellulose by centrifugation. The DEAE-cellulose was washed four times by centrifuging it from several volumes of buffer A (0.05 M ammonium sulfate). After washing, the slurry was transferred to a chromatographic column and the DEAE-cellulose was allowed to settle while the column was running. A linear gradient of buffer A containing ammonium sulfate (0.05 M to 0.40 M) was used to elute the polymerases. Fractions were collected and assayed for RNA polymerase activity in the presence and absence of alpha-amanitin (figure 22).

Polymerase activity eluted from the DEAE-cellulose over a wide range of ammonium sulfate concentrations. Amanitin-resistant activity was confined to those fractions eluting at lower ionic strengths. Examination of the A_{280} and A_{260} absorbance of the column fractions shows that the nucleic acids which had bound the DEAE-cellulose eluted at an ionic strength higher than the major polymerase activity peak.

DEAE-Sephadex chromatography

Fractions 36 to 55 of the DEAE-cellulose column were pooled and rechromatographed on DEAE-Sephadex as described in the legend to figure 24.

Figure 24 shows the activity profile in the presence and absence of alpha-amanitin. The single activity peak (fractions 12 - 24) of this DEAE-Sephadex column was pooled for rechromatography on phosphocellulose as described and shown in figure 25 and the accompanying figure legend.

Fractions 21 through 35 from the DEAE-cellulose column were pooled and rechromatographed on DEAE-Sephadex as described in the legend to figure 23. It can be seen that the polymerase activity is distributed into five peaks, only two of which show significant resistance to alpha-amanitin at 4 ug/ml. The amount of polymerase activity resistant to 4 ug/ml is shown graphically by figure 26 in which the degree of resistance is presented for each fraction as the percentage of the total activity of each fraction which is resistant to the inhibitor at this concentration.

Fractions 12, 17, 25 and 34 of the DEAE-Sephadex column of figure 23 were chosen for the study of catalytic preferences since each was the most active fraction of what appeared to be four separately eluting RNA polymerase activity peaks. Catalytic properties were examined with respect to the effect of metal ion cofactor (Mn^{++} or Mg^{++}) concentration, amanitin concentration and ionic strength (ammonium sulfate) as well as the effect of template structure and base composition on the polymerization reaction.

Catalytic Characterization

Preliminary data characterizing the Artemia polymerases with respect to catalytic requirements were presented with the purification data as figures 9, 10, 11 and 12. The results which follow were obtained from study of the catalytic properties of the four chromatographically separated Artemia RNA polymerases, fractions 12, 17, 25 and 34 of the DEAE-Sephadex column of figure 23.

Template preference

Three templates were employed in preliminary assays of the four fractions chosen for characterization since comparison of activity with different templates is critical to RNA polymerase identification. The effect of different templates is seen in table 9. The templates employed were CT DNA_{den} (denatured calf thymus DNA), CT DNA_{nat} (native calf thymus DNA) and the synthetic heteropolymer, $d(A\text{-}T)_n$. Data are presented in the first column as [^{3}H]·UMP incorporated. The units are pmol/50 ul enzyme/ 10 min incubation in the standard reaction mix at 37°C. The final volume of the assays was 125 ul. The calf thymus DNA (native or denatured) was present as 3 ug/125 ul assay and the concentration of $d(A\text{-}T)_n$ was 5 ug/ 125 ul assay. Since the radioactive label was in the substrate nucleotide, UTP, it has to be assumed that the product RNA formed from the $d(A\text{-}T)_n$ template contained approximately twice as much label as an equivalent amount of product formed using calf thymus DNA as template. This is due to the relatively greater amount of AMP in the $d(A\text{-}T)_n$ template which determines placement of UMP in the product.

In table 9, the template effectiveness is also shown as the ratio of the activity generated by the single-stranded and synthetic templates to the activity of native DNA and again as the ratio of activity with CT DNA_{den} to activity with $d(A\text{-}T)_n$. A striking observation based on the activity ratios of table 9 is that fractions 12 and 34 behave similarly and that fractions 17 and 25 likewise behave similarly. Fractions 17 and 25 were most active with CT DNA_{den}, but were 5 - 6 times more active with CT DNA_{nat} than with $d(A\text{-}T)_n$. Fractions 12 and 34 were most active with $d(A\text{-}T)_n$. However, since there was such a stimulation of activity with

$d(A\text{-}T)_n$ in the cases of fractions 12 and 34, further catalytic characterization of these fractions was performed in the presence (in separate assays) of both $d(A\text{-}T)_n$ and CT DNA_{den}. CT DNA_{den} was used exclusively as the template for the catalytic characterization of fractions 17 and 25. All assays were performed with the standard reaction mix containing 2 mM Mn^{++} and 0.025 M ammonium sulfate unless otherwise indicated. Templates were present as indicated and incubation was for 20 min at 37°C.

The effect of template choice on RNA synthesis was explored in greater depth with Artemia polymerase II from the DEAE-Sephadex column of figure 24. Fractions 12 - 24 of this column had been pooled for chromatography on phosphocellulose (figure 25). An aliquot of this polymerase II preparation was reserved for determination of enzymatic activity in the presence of equal amounts of various templates in native, denatured and synthetic forms. Artemia DNA was prepared as described in Methods; commercially obtained calf thymus and salmon sperm DNA was further purified as described in Methods. Aliquots of the three DNA preparations were denatured by heating at 90°C for 10 min followed by rapid cooling. The results of the assays are presented in table 10. The template effectiveness of each of the seven nucleotide polymers was indicated by the amount of substrate incorporated into acid-soluble material. The second column of table 10 compares the amount of product formed with each template to that formed with native Artemia DNA. The third column of figures gives the activity ratio obtained by comparing the incorporation of substrate in the presence of the denatured forms of each DNA type with the activity achieved with $d(A\text{-}T)_n$.

As indicated by table 10, polymerase II from Artemia does not have any preference for the homologous Artemia DNA over calf thymus and salmon

sperm DNA. In agreement with the results of table 9, Artemia polymerase II consistently prefers the denatured form of natural DNA's over the native form or the synthetic template.

Alpha-amanitin resistance

Determination of the effect of the elongation inhibitor, alpha-amanitin, is central to the identification of eukaryotic RNA polymerases. The effect of inhibitor concentration on the polymerase activity of fractions 17 and 25 is given in figures 27 and 28. Fifty percent inhibition of fraction 17 was achieved with 0.028 ug/ml alpha-amanitin. For fraction 25, fifty percent inhibition was achieved at 0.019 ug/ml amanitin. Polymerase acitvity of each fraction was totally abolished by 0.4 ug/ml amanitin.

Fraction 12 was assayed against increasing concentrations of alpha-amanitin using both CT DNA_{den} and $d(A\text{-}T)_n$ under conditions identical to those of figure 27 and figure 28. However the activity was too low to be certain of significance. Repeat of the assays gave still lower incorporation (within 50 cpm of background). The suggestion was that this highly labile enzyme activity is totally resistant to the inhibitor, however this has not been satisfactorily demonstrated.

Fraction 34, when assayed with alpha-amanitin, showed no diminution of enzyme activity through the concentration range measured (0 to 40 ug/ml) although the counts incorporated were again low (figure 29). The points shown are the results of single determinations. The enzymatic activity is not reduced with alpha-amanitin; however there is no ready explanation for the apparant increase in enzyme activity with increase in amanitin concentration. It may be due to the combined effect of having relatively low

incorporation in single determinations coupled with a relatively high background which had been seen to fluctuate somewhat. Whatever the cause for this slight increase, the effect of the inhibitor on fraction 34 is different from the effect on fractions 17 and 25. The results are considered suggestive of complete resistance to alpha-amanitin since the polymerases inhibited by very high concentrations (class III polymerases) are all partially inhibited by 40 ug/ml (figure 30).

Ionic strength effects

The RNA polymerase activity of each of the four chosen DEAE-Sephadex fractions was measured over the range of ionic strengths provided by 0 to 0.25 M ammonium sulfate. Assay conditions were as described in the figure legends; in all cases, the concentration of Mn^{++} was 2 mM and the template concentration was 3 ug/125 ul assay (CT DNA_{den}) or 5 ug/125 ul assay $(d(A\text{-}T)_n)$.

The RNA polymerase activity of fraction 12 assayed in the presence of increasing ammonium sulfate concentration is shown in figure 31. The activity profiles obtained with the two templates differ greatly. With $d(A\text{-}T)_n$, the optimum activity occurs at 0.025 M ammonium sulfate and falls off quickly at higher ionic strengths. In the presence of CT DNA_{den}, the activity profile has a distinctly biphasic shape, with maxima occurring at 0.05 M and 0.25 M ammonium sulfate. Taking into account the differential incorporation of the label, [^{3}H]·UTP, into the RNA products of these two templates, the net increase in activity with $d(A\text{-}T)_n$ at 0.025 M ammonium sulfate is approximately 4.5 fold.

Figure 32 shows the RNA polymerase activity of fraction 17 at various ammonium sulfate concentrations. Maximum activity is obtained at an ammonium sulfate concentration of 0.075 M. The polymerase does not show a narrow requirement for ionic strength; the activity falls off slowly in

the higher concentrations measured.

Figure 33 shows the polymerase activity of fraction 25 measured at varying ionic strengths under conditions identical to those described for fraction 17. Again the catalytic preferences of fraction 25 are seen to be similar to those of fraction 17; the optimum ammonium sulfate concentration is 0.075 M and the shape of the curve is rather broad.

Figure 34 shows the result of assaying fraction 34 for polymerase activity at varying ionic strengths using both $d(A\text{-}T)_n$ and CT DNA_{den} as templates. Fraction 34 gives a pattern of response which is similar to that of fraction 12. The optimum with $d(A\text{-}T)_n$ is at 0.025 M ammonium sulfate and is quite narrow. The shape of the curve with CT DNA_{den} is generally similar to the one generated by fraction 12 under the same assay conditions.

To simplify comparison of the four fractions with respect to their behavior over a range of ionic strengths, data indicating incorporation has been converted to the percentage of maximum RNA synthesis (figure 35). For fractions 12 and 34, one hundred percent RNA synthesis has been taken as the amount of product formed at 0.025 M ammonium sulfate with $d(A\text{-}T)_n$ as template, while fractions 25 and 17 are compared with CT DNA_{den} as template.

Metal ion requirements

The effect of metal ion cofactor identity and concentration on the polymerization reaction is important to the identification of eukaryotic RNA polymerase classes. The effect of varying concentrations of Mg^{++} and Mn^{++} on the DEAE-Sephadex fractions 12, 17, 25 and 34 was measured in the standard reaction mix with ammonium sulfate at 0.025 M and either CT DNA_{den}

(fractions 17 and 25) or CT DNA_{den} (fractions 12 and 34) as template. The data was converted to percent maximum RNA synthesis for purposes of comparison (figures 36, 37, 38 and 39).

Figure 36 shows a distinct preference by fraction 12 for low Mn^{++} when $d(A\text{-}T)_n$ was the added template. The Mn^{++} optimum lies between 1 and 2 mM and the activity fell off rapidly on either side of this concentration. Enzymatic activity was essentially zero unless a metal ion cofactor was added. Although addition of Mg^{++} had a stimulatory effect, it was not as great as that of Mn^{++} nor did it occur over such a narrow concentration range. There was no obvious optimum concentration between the range of 1 and 10 mM Mg^{++}. The effect of Mg^{++} on the rate of RNA synthesis by fraction 12 in the presence of CT DNA_{den} was seen to be similar to that of Mg^{++} with $d(A\text{-}T)_n$.

Figure 37 shows the effect of metal ions on fraction 17 RNA polymerase activity. Mn^{++} had much greater stimulatory effect than Mg^{++} and the optimum concentration range was both lower and more narrow. There was very little enzymatic activity in the total absence of a metal ion cofactor (approximately 5% of the maximum); the optimum concentration for Mn^{++} was 2 mM and the activity fell to 50% at 10 mM while the optimum for Mg^{++} was 6 mM and 50% activity was reached at 20 mM.

Assay of fraction 25 for RNA polymerase activity in the presence of increasing metal ion concentration (figure 38) again indicated a similarity in the behaviors of fractions 17 and 25. The greatest stimulation was obtained with 2 to 3 mM Mn^{++} and again there was zero activity when no metal ion was present. The activity profile with Mg^{++} showed a broad curve with the optimum concentration of Mg^{++} being at 6 mM. Approximately 50% of the maximum activity remained at 20 mM Mg^{++}.

Fraction 34 was assayed against increasing Mg^{++} or Mn^{++} concentration in the presence of either CT DNA_{den} or $d(A-T)_n$. The results are presented in figure 39. The results using CT DNA_{den} as template are presented in terms of the activity obtained with that template at 2 mM Mn^{++} (taken as 100%) while the results with $d(A-T)_n$ as template were in terms of the value achieved with $d(A-T)_n$ and 2 mM Mn^{++} (taken as 100%). A comparison with figure 36 shows that the data obtained with fraction 12 was similar to that of fraction 34 with respect to the effect of Mn^{++} and Mg^{++} on reactions using $d(A-T)_n$ as template and to the effect of Mg^{++} on the CT DNA_{den} directed reactions.

In summary, comparison of figures 36 and 39 shows that the effects of metal ion cofactor on incorporation of label into RNA product are similar in all four fractions. However, careful inspection of the effects of Mn^{++} concentration reveals subtle differences. Fractions 12 and 34 measured with $d(A-T)_n$ had lower optimum concentrations (1 mM) and for these fractions, the polymerase activity fell with increasing Mn^{++} concentrations, reaching 50% at 4 mM. Fractions 17 and 25 showed maximum activity between 2 and 3 mM. These fractions approached 50% activity at a more gradual rate and reached it at 10 mM.

Polyacrylamide Gel Electrophoresis

Various Artemia RNA polymerase preparations were electrophoresed on 5% polyacrylamide gels under non-denaturing conditions. In some instances, the RNA polymerase preparations from Artemia were co-electrophoresed with other Artemia polymerase preparations or with E. coli RNA polymerase.

Polyacrylamide gels were prepared and electrophoresis carried out as described in Methods. Samples were as described in the figure legends.

Representative gels were scanned after staining in a spectrophotometer. Their absorbance profiles are presented in figures 40, 41, 42, 43 and 44. Figure 40 is the absorbance profile resulting from electrophoresis of fraction 12 from figure 23. In gel A, the sample was fraction 12 (60 ul) and in gel B, the sample was a mixture of fraction 12 (45 ul) and E. coli RNA polymerase (6.3 ug). Fraction 12 electrophoresed alone gave a single absorbance band. In gel B of figure 40, E. coli polymerase had been mixed with fraction 12 prior to electrophoresis. The two proteins migrating relatively slowly (left side of gel) are assumed to be the dimeric and monomeric forms of the E. coli polymerase. The other minor bands are contaminants of the commercial E. coli preparation.

Figure 41 shows the absorbance profiles of fraction 17 electrophoresed alone and as a mixture with E. coli polymerase. Fraction 17 appears to consist of a single major protein species migrating at a rate intermediate to that of the E. coli monomer and dimer. Again, the E. coli control is not presented.

The gels scanned in figures 42 and 43 belong to a second set of gels. Although they were prepared and run under identical conditions, inspection of this set showed that destaining was not complete. There was no obvious reason for this difficulty and various attempts to further destain the gels failed. Since the preparation of E. coli polymerase which produced low background in the gels of figures 40 and 41 also retained stain in a gradient along these gels, it seemed that the problem was not isolated to the Artemia preparation or due to proteins in the gel, but instead was an artifact of staining.

Figure 42 compares the electrophoretic behavior of pooled fractions 24 and 26 from figure 23 with E. coli RNA polymerase. The Artemia poly-

merase sample had a single protein band which was easily discernable despite the high background. This protein migrated between the dimeric and monomeric forms of E. coli RNA polymerase as clearly shown by gel C. Figure 43 shows the absorbance profile of fractions 24 and 26 (figure 23) and the pooled polymerase II activity fractions (12 - 24) of figure 24 electrophoresed singly and in combination. Electrophoresis of each preparation alone yielded a single slowly migrating protein band. Electrophoresis of the mixture of these two Artemia preparations also resulted in a single slowly migrating protein band. Comparison of the absorbance profiles shows that the proteins of the slowly migrating bands of gel A and gel B actually comigrated when mixed and run on a third gel (C). The effect on the absorbance profile was strictly additive.

Highly purified Artemia and E. coli polymerases were run on parallel gels under conditions identical to those of the gels previously presented. The enzyme samples were from the activity peaks of separate glycerol gradients (figure 13 and appendix 2). Trace A shows E. coli polymerase electrophoresed alone--it migrates as a monomer because the glycerol gradient was run under ionic conditions (0.1 M ammonium sulfate) which favor this form. In addition, the glycerol gradient fraction (fraction 14) chosen for electrophoresis was in the major activity peak of the gradient rather than the heavier minor peak thought to contain aggregated polymerases.

Traces B and C show the absorbance profiles of fractions 15 and 16/17 (pooled) of the Artemia polymerase glycerol gradient. Although the Artemia and E. coli polymerases were not run on the same gel, it seems clear that the Artemia polymerase migrates slower than the E. coli monomer under these conditions.

DISCUSSION

The original purpose of this project was twofold--the investigation of the RNA polymerase activities of the brine shrimp, Artemia salina, and the comparison of these polymerase activities with cognate polymerases described in other tissues. The Results section provides substantial information on the chromatographic and catalytic properties and inhibitor sensitivities of the Artemia polymerases. In the Discussion, the results of these experiments will be summarized and then considered in greater detail. The limitations of the experiments will be explored and directions for future research will be outlined.

Results of other researchers working with polymerases from other eukaryotic tissues will be compared to the results obtained with Artemia. Particular attention will be given to three published papers [Birndorf, D'Alessio and Bagshaw 1975; Bagshaw 1976; Renart and Sebastian 1976] which partly describe the RNA polymerases of Artemia. Only one of these papers [Birndorf, D'Alessio and Bagshaw] had been published at the time that this project was still being carried out. The other two papers were published several months after the last experiment was completed.

Artemia salina certainly contains multiple RNA polymerases. The demonstration of this is the chromatographic separation of RNA polymerase activities which differ in their catalytic properties and inhibitor sensitivities. The polymerase activity of hydrated brine shrimp cysts is quantitatively comparable to other tissues. As will be shown, the multiple RNA polymerases of brine shrimp cysts share the properties of RNA

polymerases isolated from higher eukaryotes. The catalytic and chromatographic similarities are so great that identification of the predominant brine shrimp polymerases in terms of their cognate forms can be done with confidence even though they have not been investigated with respect to intracellular localization and function (class of RNA synthesized).

It might be well to begin discussion of the experimental results with some observations on the importance of the assay system for RNA polymerase. The assays used in this work measured only the net synthesis of RNA. No determination of the length of the product RNA, the proportion of active polymerases or the amount or area of the template transcribed could be made.

The complexity of transcription as a process, coupled with the complexity of DNA which is an integral componant in the reaction makes it difficult to assay for anything other than net production of RNA [Chamberlin 1976]. Net production is the sum of several discrete transcriptional events, any one of which could be rate-limiting under different circumstances. When polymerase preparations are impure, there may be pre-transcriptional or post-transcriptional events affecting the net RNA production, including the actions of other enzymes such as nucleases, proteases, polyadenylic acid polymerases and polynucleotide phosphorylase. Measurement of net production encompasses the events of enzyme-template binding, chain initiation, chain elongation and chain termination. It is difficult to determine which step of the reaction or which componant (enzyme, template, product or substrate) is affected by a particular variable.

RNA chain lengths can be estimated in experiments using two substrate labels. [Gamma-^{32}P]-labeled nucleoside triphosphates will retain their

label only when they are placed in the 5' terminal position of the product. The ratio of incorporated [^{32}P] to internal [^{3}H] label gives the average chain length after the original specific activities of the isotopes are considered. Transcription is so complex that determination of the average chain length may be misleading unless a defined template is used. For the purpose of monitoring the polymerase purification procedures and for preliminary characterization experiments, the measurement of net RNA production was adequate. This method is also adequate for comparison of polymerases from other tissues since all researchers share these limitations. As described previously, precautions were taken (figures 2, 4, 5; table 3) to insure that the standard assay mix used in these experiments was adequate for the measurement of net RNA production.

In examining the literature, it seems that authors sometimes underestimate the complexity and sensitivity of the transcription process. Although the question is interesting, it is very difficult, for example, to make absolute statements about whether changes in RNA polymerase activity are related to physiological or developmental states of a tissue or organism. Many factors determine or influence the activity of a polymerase *in vitro* so that it is difficult to determine the true situation *in vivo*. Reported shifts are often on the order of 2 to 10 fold increases or reductions. An example is found in an *Artemia* paper [Renart and Sebastian 1976] which states that between 36 hours of development and 72 hours of development, polymerase II activity is reduced by one half. The authors feel that this is related to the concurrant reduction of RNA synthesis. Yet figure 38 of this project shows that *Artemia* RNA polymerase activity (assayed with CT DNA_{den} as in the published work) varies from 0 to 100% activity over the range of from zero to 2 mM Mn^{++}. Without

singling out the work of these authors except for purposes of illustration, it is important to stress that RNA polymerases are sensitive to many variables. Probably they are sensitive to variables as yet unknown. Caution must be used when comparing the results of different preparations and different systems. Specific activities fluctuate between otherwise identical preparations (table 7) despite care taken to duplicate procedures. An obvious but generally unreported variable is the length of time required to complete various procedures. The length of time elapsing between homogenization and assay of a particular fraction is critical; other variables such as the effect of protein concentration on enzyme stability are also often overlooked. To add another dimension of complexity, the three polymerase classes from eukaryotes are differentially stable (figure 7) and so false impressions about the relative activity or amounts of the enzyme forms are easily obtained.

Experiments presented in the first section of the Results show that the standard reaction mix is not limited by componants of the reaction mixture itself over a period of 15 to 20 min incubation (figure 2). Figures 4 and 5 show that with the appropriate amounts of template and Artemia protein, the incorporation of labelled substrate could procede in an essentially linear fashion for this period of time. From the time of the first RNA polymerase assay of a crude Artemia cyst homogenate with alpha-amanitin, it was apparent that multiple RNA polymerase activities existed in brine shrimp. DEAE-Sephadex column chromatography of either incubated Artemia embryos or hydrated Artemia cysts resulted in the separate elution of two major polymerase activities (figures 6 and 7). An amanitin-resistant activity eluted at an ionic strength typical for polymerase I in other eukaryotic systems [for reviews see Roeder 1976 and

Chambon 1975]. (A single exception is the polymerase I of Physarum [Gornicki, Vuturo, West and Weaver 1974] which elutes after the polymerase II activity.) Artemia polymerase I was not extensively characterized beyond noting its amanitin-resistance and DEAE-Sephadex elution position.

Nevertheless, Artemia polymerase I was included in the development of the purification and chromatographic techniques and certain enzymatic properties became evident as outgrowths of the effort to separate and characterize polymerases II and III. Consequently, discussion of Artemia polymerase I will occur only in the context of other endeavors.

The second DEAE-Sephadex polymerase activity peak of Artemia proved somewhat unusual. No difference was found between the elution position and the degree of amanitin-resistance of this activity when hydrated cysts and 24 incubated embryos were compared. The polymerase activity eluted at a point considered characteristic for polymerase II [see Introduction and table 12] but the observation that it was not completely inhibited by levels of alpha-amanitin sufficient to abolish known polymerase II's [see Introduction and tables 2 and 12] led to initial confusion.

This confusion was resolved by two lines of evidence which supported the theory that the second DEAE-Sephadex peak was a mixture of Artemia polymerase II and Artemia polymerase III and that these two separate enzymes elute from this material at the same ionic strength. Experiments performed on this DEAE-Sephadex activity peak under conditions in which the enzyme activities were physically mixed (the condition in which they eluted from the column) but differentially inactivated (as will be

described) are in perfect agreement with characterization experiments performed after physical separation of these activities had been accomplished by column chromatography.

Evidence for Polymerase II in Artemia salina

There seems to be no question that the amanitin-sensitive activity of the second DEAE-Sephadex activity peak is Artemia polymerase II. A strong argument can be made that the co-eluting amanitin-resistant activity is polymerase III. This latter argument relies partly on comparison with the behavior of the polymerases of other systems during chromatography and will be developed throughout this discussion.

For the purpose of discussing the properties of the Artemia polymerase II enzyme, several preparations will be considered to be enzymatically pure polymerase II. In those instances where polymerase II is assayed in the physical presence of an inactivated polymerase, this is made clear in the text of the discussion or in the legend to the figure mentioned.

Fractions 17 and 25 from the DEAE-Sephadex column of figure 23 are considered pure polymerase II on the basis of sensitivity to amanitin. The pooled fractions 12 - 24 from the DEAE-Sephadex column of figure 24 are also considered to be pure polymerase II for the same reason; however, because of chromatographic considerations, pooled fractions 12 - 24 are considered physically , as well as enzymatically, pure polymerase II.

The discovery that the amanitin-resistant polymerase activity of the second DEAE-Sephadex peak (figures 6 and 7) was relatively labile, and that it could be abolished by several rounds of freezing and thawing with little effect on the amanitin-sensitive activity, permitted separate

characterization of the amanitin-sensitive activity (figures 9 - 13). As figure 9 shows, the Artemia RNA polymerase which is relatively stable to freezing and thawing is totally inhibited by 0.4 ug/ml amanitin (with 60% activity remaining at 0.004 ug/ml). This high degree of sensitivity to amanitin is characteristic of eukaryotic RNA polymerase II [see tables 2 and 12 and Introduction]. The degree of sensitivity varies somewhat between higher and lower eukaryotes, but with the exception of the less sensitive yeast polymerase II [Greenleaf and Bautz 1975], total inhibition is achieved by 0.1 to 1.0 ug/ml amanitin.

The behavior of fractions 17 and 25 and the pooled fractions 12 - 24 (figures 23 and 24 respectively) when assayed in the presence of amanitin can be seen in figures 23 to 28 and figure 30. Fraction 17 is 50% inhibited by 0.028 ug/ml while fraction 25 is 50% inhibited by 0.019 ug/ml alpha-amanitin. Both are totally inhibited by 0.4 ug/ml amanitin. Fractions 12 - 24 (pooled) were totally inhibited by 4.0 ug/ml, the only concentration of amanitin tested.

On the basis of amanitin sensitivity, these Artemia polymerase activities are all polymerase II and there is no discernable difference between the chromatographically purified polymerase II preparation and the polymerase II assayed in the presence of inactivated amanitin-resistant polymerase III.

In addition to amanitin sensitivity, Artemia polymerase II has other attributes of the class II polymerases. Figures 12, 37 and 38 show that Artemia polymerase II assayed under various ionic strength conditions is sharply stimulated by 2 - 3 mM Mn^{++}. Figures 37 and 38 also show the effect of Mg^{++} concentration on the polymerization reaction. The broader shape of the curve with Mg^{++} and the relatively greater activity with Mn^{++}

are identical to the effect seen on sea urchin and rat liver RNA polymerase II in the first paper to demonstrate multiple RNA polymerase activities [Roeder and Rutter 1969]. Since Roeder and Rutter's 1969 description, essentially all subsequent papers have been in agreement on this point despite a diversity of sources for the polymerase II's which were tested [see selected bibliography for polymerase II given in Introduction].

The Artemia DEAE-Sephadex polymerase II which had been frozen and thawed was maximally stimulated by ionic strength conditions between 0.075 and 0.100 M ammonium sulfate (figure 10). This is the same optimum found for polymerase II in other tissues (see table 12 and the selected bibliography for polymerase II given in the Introduction]. Figures 10 and 11 show the activity curve with CT DNA_{den} as template. Figure 11B shows that use of $d(A\text{-}T)_n$ as template has the effect of lowering the optimum ionic strength for the net production of RNA by the Artemia polymerase II.

The behavior of fractions 17 and 25 with respect to metal ion cofactor concentration and identity (figures 37 and 38 and table 11) are essentially identical to the literature values for polymerase II from other organisms as summarized in table 12. Figure 12 shows that the effect of Mn^{++} concentration is similar when the enzyme tested is the alpha-amanitin-sensitive activity from the DEAE-Sephadex chromatography of a shrimp cyst homogenate. The activity curve measured for this enzyme with increasing Mg^{++} concentration (data not shown) was also identical to those of figure 37 and 38 as well as the literature values for polymerase II from other species. The stimulatory effect of a denatured DNA template (tables 9, 10 and 11) on fractions 17 and 25 further confirms their identity as class II polymerases (table 12).

Evidence for Polymerase III in Artemia salina

The existance of polymerase III in Artemia salina is best demonstrated by a comparison of the elution patterns of the polymerase activities shown in figure 22 (DEAE-cellulose chromatography) and figure 23 (DEAE-Sephadex chromatography). Perhaps the most unique property of the RNA polymerase III's described in other tissues [Hossenlopp, Wells and Chambon 1975; Roeder 1976] is the difference in the point of elution of this enzyme from DEAE-cellulose and DEAE-Sephadex. Polymerase I and polymerase II are each consistent in their behavior on the two materials and elute at a point characteristic for each.

As shown by table 12, polymerase III from other tissues elutes from DEAE-cellulose at approximately 0.1 M ammonium sulfate and from DEAE-Sephadex at 0.2 - 0.3 M ammonium sulfate. The result of this is that polymerase III and polymerase I tend to co-elute from DEAE-cellulose but upon rechromatography on DEAE-Sephadex, polymerase III tends to co-elute with polymerase II or to elute after polymerase II. In Artemia, polymerase IIB elutes from DEAE-Sephadex at 0.24 M ammonium sulfate (σ = 0.02, n = 13) while polymerase III elutes at 0.32 M ammonium sulfate (σ = 0.05, n = 12). Also on DEAE-Sephadex, Artemia polymerase IIA elutes at 0.17 M ammonium sulfate (σ = 0.01, n = 11) and polymerase I elutes at 0.11 M ammonium sulfate (σ = 0.02, n = 9).

Examination of the DEAE-cellulose activity profile shown in figure 22 shows that the alpha-amanitin-resistant polymerase activity (amanitin resistance is a property of polymerase III as well as polymerase I) elutes between 0.05 M and 0.20 M ammonium sulfate. The fractions eluting between 0.11 M and 0.19 M ammonium sulfate (fractions 21 - 35) were pooled and

rechromatographed on DEAE-Sephadex (figure 23).

In figure 23, amanitin resistance is present in fractions eluting at greater than 0.19 M ammonium sulfate. Fraction 34 (eluting at 0.32 M ammonium sulfate) was chosen for further study. The effect of alpha-amanitin on this fraction is shown by figure 29; other catalytic properties are investigated in experiments whose results are shown in figures 34, 35 and 39 and table 9. Information gathered from these experiments is included in the summary of Artemia RNA polymerase activities presented in table 11. In this table, Artemia polymerase IIA is represented by fraction 17, polymerase IIB is represented by fraction 25 and polymerase III is represented by fraction 34. The question of the identity of the polymerase activity in fraction 12 will be dealt with at a later point.

Comparison of the observed properties of Artemia polymerase III (in the form of fraction 34) (see table 11) with the general properties of nuclear RNA polymerases summarized by Roeder [Roeder 1976] (see table 12) completely substantiates the contention that Artemia salina contains polymerase III.

The question of the identity of the amanitin-resistant polymerase co-eluting with polymerase II in figures 6 and 7 cannot be fixed with certainty using the results presented. However, for several reasons, it is felt to be a chromatographically separate form of polymerase III. Because of its amanitin-resistance, this enzyme must belong to either class I or class III polymerases. Class I polymerase from Artemia has already been demonstrated to elute at approximately 0.10 M ammonium sulfate (figure 6 and figure 7). Although different chromatographic forms of the class I polymerases exist, they typically elute from DEAE-Sephadex at lower rather than higher ionic strengths.

In Artemia, a class III RNA polymerase has been shwon to elute from DEAE-Sephadex at 0.32 M ammonium sulfate. In mouse plasmacytoma, a class III polymerase (IIIB) elutes at 0.31 M ammonium sulfate while a second class III polymerase (IIIA) elutes slightly after the plasmacytoma polymerase II (at 0.24 M ammonium sulfate) [Schwartz, Sklar, Jaening, Weinmann and Roeder 1974].

In HeLa cell preparations [Hossenlopp, Wells and Chambon 1975], class III polymerases are seen eluting from DEAE-Sephadex at 0.09 M, 0.15 M, 0.26 M and 0.35 M ammonium sulfate. They have been named CI, CII, CIIIa and CIIIb. Polymerases CIIIa and CIIIb seem to correspond to the polymerases IIIA and IIIB of the mouse plasmacytoma.

In the Artemia system, careful examination of figure 22 shows no amanitin-resistance eluting at a point greater than 0.20 M, however, rechromatography of the fractions eluting between 0.11 M and 0.19 M results in a certain amount of amanitin-resistance co-eluting with polymerase II (0.23 M) (fraction 25) as well as the polymerase III activity centered around fraction 34 (0.32 M ammonium sulfate). This supports the possibility that the amanitin-resistant activity co-eluting with polymerase II in figure 6 and figure 7 is polymerase III. A possible reason for the absence of a separately eluting polymerase III activity may be the high Mn^{++} (4 mM) concentration used in the assays of the DEAE-Sephadex column fractions shown in figures 6 and 7. Although polymerase III has a relatively broad activity profile over increasing ionic strengths when CT DNA_{den} is used as the template (figure 34), table 9 shows that (at 0.025 M ammonium sulfate and 2 mM Mn^{++}), polymerase II is stimulated up to 6 fold over its activity with $d(A\text{-}T)_n$ while the activity of polymerase III is somewhat reduced (from 1.0 to 0.9) with CT DNA_{den}. Measured at 3 mM Mn^{++} (table 10)

polymerase II is 10 times more active with CT DNA_{den} than with $d(A\text{-}T)_n$. Any column whose fractions are assayed for polymerase activity with CT DNA_{den} as template will appear to contain at least 5 times more polymerase II activity. However, assay with $d(A\text{-}T)_n$ subjects both polymerase I and polymerase III to sharper optima with respect to metal ion cofactors and ionic strengths. It can be seen that no single set of assay conditions equally favors all three enzyme classes.

However, since the DEAE-Sephadex columns of figure 6, 7 and 23 were assayed with CT DNA_{den}, and the ionic strengths of the assay varied similarly (although in figures 6 and 7, 30 ul of enzyme was assayed in a final volume of 125 ul, while in figure 23, 50 ul of enzyme was assayed in a final volume of 125 ul), the Mn^{++} concentration of figure 6 and figure 7 was 4 mM while that of figure 23 was 3 mM. The material chromatographed on these DEAE-Sephadex columns differed in previous history: the sample loaded onto the column in figure 6 was prepared from 24 hour incubated Artemia embryos, while that of figure 7 and figure 23 were prepared from hydrated Artemia cysts. Figure 23 differed from both figure 6 and figure 7 in that the material loaded had previously eluted from DEAE-cellulose at low ionic strengths (0.11 to 0.19 M ammonium sulfate). The possibility exists that prior treatment on DEAE-cellulose affected the proportion of polymerase molecules co-eluting with polymerase II. It should be remembered that the mouse plasmacytoma studies [Schwartz et al. 1974] indicate that the two polymerase III species (IIIA and IIIB) have different intracellular locations.

Measurement of the RNA polymerase activity of fraction 34 in the presence of varying concentrations of alpha-amanitin (figure 29), Mn^{++} and Mg^{++} (figure 39), ammonium sulfate (figure 34) and various templates

(table 9) confirms the preliminary identification based on the differential behavior of this enzyme on DEAE-cellulose and DEAE-Sephadex. Fraction 34 is a class III polymerase as further seen by comparison of table 11 (fraction 34 summary). and table 12 (literature summary).

Fraction 12 of the DEAE-Sephadex column shown in figure 23 behaves very similarly to fraction 34 (table 11) and class III polymerases have been seen to elute from DEAE-Sephadex at the same ionic strength [Hossenlopp, Wells and Chambon 1975], however, the actual activity of this fraction was very low. There is no certainty that this fraction does not contain a mixture of polymerases I and III. Positive identification of fraction 12 is impossible without further chromatographic separation and, ultimately, the determination of its subunit composition.

Polymerase III was not found by Birndorf et al. [1975] when they described polymerase I and polymerase II from Artemia embryos. The only chromatographic step used in their purification procedure was DEAE-cellulose. Since polymerase I and III are known to co-elute from this material, the characterization of Artemia polymerase I by these authors is probably the characterization of a mixture of polymerases I and III.

Polymerase III was found by Renart and Sebastian [1976] to elute at approximately 0.38 M ammonium sulfate from DEAE-Sephadex. However, they don't include amanitin-sensitivity data for their column activity profile and so it is impossible to know whether any polymerase III is co-eluting with polymerase II.

Neither of the above-mentioned preliminary characterizations [Birndorf et al. 1975 ; Renart and Sebastian 1976] give any indication of two chromatographic forms of polymerase II in Artemia. One possible reason for this is that polymerase IIA is highly labile while polymerase

IIB is the most stable of all the shrimp polymerases. Although polymerase IIA is present in greater amounts, its activity is entirely overshadowed by the highly active polymerase IIB eluting near it. Polymerase IIA most often appears as a shoulder on the polymerase IIB activity peak. It was initially only through the gel electrophoresis of figure 8 that the existence of polymerase IIA was suspected. The successive chromatographic steps (DEAE-cellulose followed by DEAE-Sephadex, figures 22, 23 and 24) separated the majority of polymerase IIB activity from polymerase IIA and gave the first good chromatographic separation of these two forms. Thus, fraction 17 of figure 23 is polymerase IIA while fraction 25 is polymerase IIB. Polymerase IIA had been relatively enriched by the separation from the late-eluting polymerase II activity fractions from figure 23. The polymerase II activity peak of figure 24 is thus identical to fraction 25 of figure 25 in being polymerase IIB.

Physical Studies on *Artemia salina* RNA Polymerases

Physical studies on *Artemia* RNA polymerase IIA and polymerase IIB indicate that these enzymes are similar to other eukaryotic RNA polymerases in having a high molecular weight. Polyacrylamide gel electrophoresis under non-denaturing conditions and glycerol gradient centrifugation were the methods used. In each procedure, the behavior of the *Artemia* enzymes was compared directly or indirectly to that of the *E. coli* RNA polymerase (Sigma).

Figures 41 and 42 show the absorbance profiles of *Artemia* polymerase IIA and IIB after polyacrylamide gel electrophoresis on 5% acrylamide gels under non-denaturing conditions. Each preparation appears to contain a single major protein species. This protein migrates between the *E. coli*

RNA polymerase monomer and the E. coli RNA polymerase dimer, regardless of whether the sample was Artemia polymerase IIA or polymerase IIB.

The molecular weight of the σ-containing E. coli RNA polymerase holoenzyme is variously given as 422,000 daltons [Berg, Barrett and Chamberlin 1971], 440,000 daltons [Zillig, Zechel and Halbwachs 1970] and 454,000 daltons [Burgess 1969]. E. coli holoenzyme aggregates to a dimer a ionic strengths below 0.1 M [Chamberlin 1976]. The commercially obtained E. coli RNA polymerase used in these studies had been stored at 0.1 M ammonium sulfate so the presence of both forms could have been predicted. (The core enzyme is known to aggregate at up to 0.2 M ionic strength [Chamberlin 1976]). Glycerol gradient centrifugation at 0.1 M ammonium sulfate (15 - 30% glycerol) separated the E. coli holoenzyme monomers from the dimers (appendix 2); fraction 14 from appendix 2 was electrophoresed on 5% polyacrylamide gels under non-denaturing conditions and the profile is shown in figure 44A. Only the monomeric form of the enzyme is present on this gel.

Figure 44 compares the electrophoretic behavior of the E. coli holoenzyme monomer (trace A) with Artemia polymerase II (traces B and C) from the activity peak of a similar glycerol gradient (figure 13). The absorbance profiles support the contention that the native Artemia polymerase migrates more slowly than the E. coli polymerase monomer under identical conditions of electrophoresis. Conversely, these absorbance profiles support the contention that the apparent activity peak in the glycerol gradient (figure 13) of Artemia polymerase II is a true polymerase activity peak despite its extremely low polymerase activity. (A published report appearing after completion of this project reports that "use of nitrocellulose tubes results in severe loss of enzyme [Artemia RNA polymerase]

activity" [Bagshaw 1976]. This is a possible explanation for the low activity seen in figure 13 as well as the total loss of activity when glycerol gradient centrifugation of other Artemia polymerase preparations was attempted (for example fractions 13 and 14 of the phosphocellulose column of figure 25--data not shown).

The absorbance profiles shown in figure 43 compare migration rates of Artemia polymerase IIB (pooled fractions 24/26 from figure 23) in 5% acrylamide with those of polymerase IIB from pooled fractions 12 - 24 of figure 24. These enzymes comigrate when run on the same gel. Since pooled fraction 24/26 migrates behind the E. coli polymerase monomer (see figure 42), one can conclude that polymerase IIB from figure 24 would behave similarly.

In 5% gels where polymerase IIA (fraction 17) and polymerase IIB (fraction 25) were electrophoresed together, no separation could be detected. [Data not shown]

The degree of separation of the E. coli monomer and dimer shows the effect of a molecular weight difference of approximately 450,000 daltons. Discounting the possible effects of dimerization on the net charge of the E. coli polymerase, it is clear that a relatively large size difference between Artemia polymerase IIA and polymerase IIB could escape detection. Longer gels or gels of lower porosity would optimize separation due to different migration rates, however SDS gel electrophoresis is a far more reliable method for measuring molecular weights and detecting molecular weight differences [Laemmli 1970]; Weber and Osborn 1969]. Electrophoresis in the presence of SDS has the double advantage of eliminating charge effects and of producing smaller molecules through dissociation of the native polymerase into subunits. Few molecular

weight markers exist in the size range of the complete eukaryotic RNA polymerase. This is the most common cause for disagreement between the results of different investigators.

Satisfactory SDS gel electrophoresis of Artemia polymerase preparations was not accomplished for several reasons. The lack of large amounts of highly purified (and otherwise characterized) sample was a major obstacle. Since other eucaryotic RNA polymerases are known to contain up to 12 subunits, a correspondingly greater amount of starting material is required for SDS gel electrophoresis. The evidence indicates that most subunits are present in equimolar amounts. Further complication is caused by the range of subunit sizes to be expected in eucaryotic polymerases (between 200,000 and <10,000 daltons) which requires complex gels, each one containing a linearly decreasing porosity (usually 8 - 16% acrylamide).

In order to be certain of the origin of the subunits, the starting material should be protein eluted from a single band on non-denaturing gels. [Preferably this protein should show enzymatic activity although this requirement is particularly difficult to fulfill with such a labile enzyme. The binding of ^{3}H·alpha-amanitin to polymerase II is so highly specific that this is a reasonable substitute for enzymatic activity in identification of polymerase II among several protein bands in polyacrylamide gels.]

The difficulties inherant in bringing Artemia polymerase II to the point of identification of subunit number, molecular weight and molar ratios can be more fully appreciated on examination of the published attempt of Bagshaw [1976] to do precisely that. The first problem Bagshaw encountered was in the purification of polymerase II to homogeneity.

Polymerase II was extracted from nuclei isolated from Artemia embryos incubated for 24 - 72 hours (no temperature was given). The extract was purified by two cycles of DEAE-cellulose chromatography followed by discontinuous sucrose gradient centrifugation. Rather surprisingly, "all efforts to purifiy polymerase II beyond this stage led to complete (95-100%) loss of enzyme activity. These efforts included further chromatography on DEAE-cellulose, DEAE-Sephadex, phosphocellulose, DNA-sepharose, isoelectric focusing and gel electrophoresis under non-denaturing conditions." No possible explanations for this loss of activity are put forward. The activity profile of the sucrose gradient is given only in "% of maximum" and no protein determinations are done. Aliquots of each fraction of the activity peak were run on SDS gels and a correlation was sought between density of staining of a particular SDS gel protein band and the polymerase activity of the corresponding sucrose fractions used as the sample.

Although the gel traces presented by Bagshaw [1976] showed multiple protein bands, four proteins were found whose polypeptide content corresponded to the RNA polymerase activity of the sample. The molar ratios were calculated relative to one of the protein bands which was arbitrarily set at unity. In four sucrose fractions, the molar ratios vary from 0.81 to 1.50. The molecular weights of the four proteins are 170,000, 130,000, 36,000 and 14,000 daltons.

A linear (10 - 30%) sucrose gradient in the presence of apoferritin (mw = 400,000) led Bagshaw to conclude that the native enzyme had a molecular weight which was slightly less than 400,000 dlatons. Assuming equimolar amounts of the four subunits, the added value would be 360,000 daltons. The assumption was made that RNA polymerase and apoferritin

have similar densities. The author concludes that these results support each other and that the native molecule is therefore composed of those four subunits whose molecular weight totals 360,000 daltons and whose presence corresponds to enzyme activity.

However, the sudden loss of enzyme activity with the sucrose centrifugation leaves open the possibility of lost subunits. The author [Bagshaw 1976] did not attempt SDS gel electrophoresis with enzyme prepared in a different manner, nor did he try recombination of sucrose gradient fractions in an attempt to restore activity. (Since the sucrose gradient was a discontinuous one, only three fractions were involved.) He gave no values for enzymatic activity at any stage of purification, either after homogenization or before and after the sucrose gradient centrifugation. It seems remarkable that no evidence of the native enzyme appeared on gels run under non-denaturing conditions. Certainly this difficulty encountered by Bagshaw makes it impossible to compare his work with the results of the non-denaturing gel electrophoresis presented by this author. Figures 13 and 41 - 44 of this report strongly suggest a molecular weight of greater than 450,000 daltons for the native form of the Artemia polymerase II.

SUMMARY AND CONCLUDING REMARKS

A thorough characterization of the chromatographic behavior and catalytic requirements of RNA polymerases II and III from the protostome, Artemia salina has shown them to be remarkably similar to the cognate polymerases of deuterostomes in all parameters examined (tables 11 and 12).

The development of large scale purification procedures for Artemia polymerases has made it feasible to study the subunit structure of these enzymes in greater detail. Structural analysis should reveal the basis upon which the labile and chromatographically distinct polymerase IIA differs from the stable form, polymerase IIB. Although these two forms of polymerase II are indistinguishable in catalytic properties, they must differ in structure.

Further experiments examining subunit structure and the possibility of subunit modification should contribute greatly to comparitive enzymology, developmental biology and the molecular basis for amanitin resistance, as well as, ultimately, transcriptional control mechanisms. The unique phylogenetic position and developmental properties of the brine shrimp, Artemia salina, establishes a unique role for the study of Artemia RNA polymerases in precisely these areas of molecular biology.

TABLE 1.--Localization and General Functions of Animal Cell RNA Polymerases

Enzyme Class	Subcellular Localization	Cellular Gene Transcripts	Viral Gene Transcripts
I	nucleolus	18s, 28s rRNAs	none identified
II	nucleoplasm	HnRNAs, mRNAs	mRNA precursors
III	nucleoplasm	tRNAs, 5s RNA	low molecular weight RNAs

SOURCE: R. Roeder. 1976. Eukaryotic Nuclear RNA Polymerases. In "RNA Polymerase". (R. Losick and M. Chamberlin, eds.) Cold Spring Harbor Laboratory, Cold Spring Harbor.

TABLE 2.--Terminology, Localization and Amanitin Sensitivity of Animal DNA-dependent RNA Polymerases

Terminology of Chambon		Terminology of Roeder			
Class	Enzyme	Class	Enzyme	Localization	Amanitin Sensitivity
A	AI (a + b)	I	IA	Nucleolus	Insensitive to 1 mg/ml
	AII		IB	Nucleolus	"
B	BO	II	IIO	Nucleus	Sensitive to 0.001-0.01 ug/ml
	BI		IIA	Nucleus	"
	BII (a + b)		IIB	Nucleus	"
C	CI				Sensitive to 10-100 ug/ml
	CII				"
	CIIIa	III	IIIA	Nucleus	"
	CIIIb		IIIB	Cytoplasm	"

SOURCE: Adapted from P. Chambon. 1975. Eucaryotic nuclear RNA polymerases. Ann. Rev. Biochem. 44: 613-638.

TABLE 3.--Assay of *Artemia* Cyst Fractions for Nuclease Activity

A. Incubation of shrimp cyst protein with labeled template under various ionic conditions.

Tube	Time	Temp.	Ion	CPM	% Maximum	Sample
1	0'	0°C	Mn^{++}	31,659	100.0	Homogenate (290 ug)
2	10'	37°C	Mn^{++}	30,734	97.1	"
3	20'	37°C	Mn^{++}	28,484	90.0	"
4	10'	37°C	Mg^{++}	28,019	88.5	"
5	20'	37°C	Mg^{++}	28,374	89.6	"
6	0'	0°C	Mn^{++}	33,339	100.0	4,000 rpm supernatant (210 ug)
7	10'	37°C	Mn^{++}	32,170	96.5	
8	20'	37°C	Mn^{++}	28,570	85.7	"
9	10'	37°C	Mg^{++}	31,428	94.4	"
10	20'	37°C	Mg^{++}	26,268	78.8	"

B. Repeat of nuclease assay with hydrated cyst fractions

Tube	Time	Temp.	Ion	CPM	% Maximum	Sample
1	0'	0°C	Mn^{++}	43,521	100.0	Homogenate
2	20'	37°C	Mn^{++}	39,044	89.7	Homogenate
3	20'	37°C	Mn^{++}	40,019	92.0	4,000 rpm supernatant
4	20'	37°C	Mn^{++}	30,125	69.2	4,000 rpm pellet
5	20'	37°C	Mn^{++}	31,478	91.2	15,000 rpm supernatant
6	20'	37°C	Mn^{++}	25,050	57.4	15,000 rpm pellet
7	300'	0°C	Mn^{++}	571	1.3	4,000 rpm supernatant

TABLE 4.--Stimulation of Artemia RNA Polymerase Activity by Polymin-P

Exp.	Original[a] Activity	Post-Polymin Activity	Activity Ratio	Original[b] A_{280}/A_{260}	Post-Polymin A_{280}/A_{260}
2	4,453	7,132	1.6	0.71	0.95
3	8,561	16,166	1.9	0.71	0.74
4	5,144	10,334	2.0	0.71	0.73
5	4,580	15,108	3.3	0.70	0.77
6	1,323	2,205	1.7	0.76	0.95
7	5,686	6,290	1.1	0.73	0.75
8	535	864	1.6	0.70	0.81
9	1,096	1,115	1.0	0.77	0.78
			Ave. = 1.8		

[a] Original activity and post-Polymin activity are given in counts per minute above background.

[b] A_{280}/A_{260} is the ratio of absorbance at 280 nm and 260 nm of a 1/100 or 1/500 dilution of polymerase sample made into 1 M NaCl.

TABLE 5.--Recovery of RNA Polymerase Activity through Pre-chromatography Purification of Hydrated *Artemia* Cyst Enzymes

Exp. No.	Wet Wt. *Artemia* (grams)	Total Activity Units[a] Homog.	Sonic.	Post-P	F/S	F/S[b]	F/S Units/ g Shrimp	Percent Recovery
1	272	529	260	633	n.d.	n.d.		
2	458	387	402	519	368		1.38	95
3	501	432	307	3,003	592	344	1.18	137
TOTAL	1,231	1,348	970	4,155		1,377		
4	129	87	62	403	108	47	0.84	125
5	449	340	437	1,053	841	205	1.87	247
6	324	255	368	733	255	113	0.79	100
7	509	111	137	1,729	279	173	0.55	252
8	497	259	335	864	255	129	0.51	98
9	333	309	379	317	155	38	0.47	50
TOTAL	2,242	1,361	1,718	5,073	1,892	706		

[a] One unit of enzyme incorporates 1 nmol [^{3}H]-UMP into acid-insoluble material in 10' at 37°C

[b] F/S fraction assayed after freezing and storage at -70°C

Abbreviations used : homogenate, Homog.; sonicate, Sonic.; post-Polymin, Post-P; frozen/stored, F/S.

TABLE 6.--Summary of the Purification of Hydrated Artemia Cyst RNA Polymerase Activity

Fraction	Total Protein (mg)[a]	Total Activity (units)[b]	α-amanitin Resistance (percent)[c]	Specific Activity (units/mg)	Fold Purification[d]
Homogenate	33,700	1,348		0.040	
Post-Polymin	6,205	4,155		0.670	17
F/S[d]	5,001	1,377		0.275	
DEAE-cellulose	n.d.	583	35		
DNA-cellulose fractions 16-23	13.8	325		23.6	590
DNA-cellulose fraction 17	4.3	189	57	44.0	1,100

NOTE: Combined values of experiments 1-3; total weight of hydrated shrimp cysts was 1,231 g.

Activity assays were carried out in the standard reaction mix containing 30 ul enzyme, 4 mM Mn^{++} and 2.5 ug/assay $d(A\text{-}T)_n$ in a final volume of 125 ul. Incubation was for 15 min at 37°C.

[a]Protein determinations were by the method of Lowry after precipitation in 15% TCA.

[b]One unit of enzyme activity is the amount necessary to incorporate 1 nmol substrate into acid-precipitable material in 10 min at 37°C.

[c]Percent resistance to alpha-amanitin was determined by comparing the polymerase activity measured in the presence and absence of 8 ug/ml alpha-amanitin.

[d]Fold purification refers to the increase in specific activity relative to the specific activity of the homogenate.

TABLE 7.--Recovery of Enzymatic Activity and Protein throughout the Purification Scheme of Figure 1. Results of Four Typical Experiments

Fraction	Volume	Protein Conc.[a]	Total Protein	Total Activity[b]	α-amanitin Resistance[d]	Specific Activity
	(ml)	(mg/ml)	(mg)	(units)[c]	(percent)	(units/mg)
Experiment 5 - 449 g wet weight hydrated Artemia cysts						
Homog.	650					
Sonic.	705	14.0	8,515	437	62	.051
HSS	610	13.5	8,235	340	88	.041
Post-P	133	26.3	3,498	1,053		.301
F/S	102	22.9	2,336	841	37	.360
Experiment 6 - 324 g wet weight hydrated Artemia cysts						
Homog.	500					
Sonic.	540		6,246	368		.059
HSS	468					
Post-P	303	8.3	2,490	733		.249
F/S	138	13.3	1,835	255	59	.139
Experiment 7 - 509 g wet weight hydrated Artemia cysts						
Homog.	675			111	41	
Sonic.	729	14.5	10,584	137	74	.013
HSS	685					
Post-P	350	19.2	6,720	1,729		.257
F/S	138		1,545	279	88	.181
Experiment 8 - 497 g wet weight hydrated Artemia cysts						
Homog.	675	12.6	8,493	259		.030
Sonic.	680	12.7	8,675	335		.039
HSS	665					
Post-P	370	12.8	4,736	864		.182
F/S	150			255	42	

[a] Protein determination was by the method of Lowry [1951].

[b] RNA polymerase activity was measured in the standard reaction mix containing 30 ul enzyme and 0.75 ug $d(A\text{-}T)_n$ in a final volume of 125 ul.

[c] One activity unit of enzyme incorporates one nanomole of [^{3}H]·UMP in 10 min at 37°C.

[d] Alpha-amanitin resistance is given in terms of percent resistant activity remaining after incubation with 4 ug/ml alpha-amanitin.

Homog., homogenate; Sonic., sonicate; HSS, high speed supernatant; Post-P, post-Polymin; F/S, frozen stored.

TABLE 8.--Results of the Chromatographic Purification and Partial Separation of RNA Polymerase Activity of Hydrated Artemia Cysts

Fraction/Procedure	Total Protein[a] (mg)	Total Activity (units)[b]	α-amanitin Resistance[c] (percent)	Specific Activity (units/mg)	Fold Purif.
1. F/S (pooled)	15,147	1,892		0.125	4.2
2. DEAE-cellulose 1					
flow through (see 4) 0.08 M wash (see 3) fractions 27-34	400	413	30	1.03	34.4
fraction 30		39.7			
DNA-cellulose 1 (figure 19)					
fractions 32-48	7.0	248		35.4	1,180
fractions 35-40	2.7	115		42.6	1,420
fraction 36	.45	21.3		47.3	1,577
3. 0.08 M wash of DEAE-cellulose 1	980	516	45	0.53	31.7
DNA-cellulose 2 (column profile not shown)					
fractions 28-40		57	33		
4. DEAE-cellulose 2 (figure 20)					
flow through		556	72		8.7
fractions 19-26	666	170	72	0.26	
fraction 23		30.2			

Combined extracts from exp. 4 - 9; 2,241 g total wet weight Artemia cysts.

[a]Protein determination by the method of Lowry [1951].

[b]One unit of enzyme incorporates one nmol ^{3}H·UMP in 10 min at 37°C

[c]Percent activity remaining after incubation with 4 ug/ml amanitin

TABLE 9.--Effect of Template on RNA Polymerase Activity Fractions from a DEAE-Sephadex Column[a]

Fraction	Template	[^{3}H]·UMP Incorp. (pmol/50 ul/10')	Stimulation Relative to CT DNA_{nat}	Activity Ratio $\frac{CT\ DNA_{den}}{d(A\text{-}T)_n}$
12	CT DNA_{den}	1.05	1.14	0.56
	CT DNA_{nat}	0.92		
	$d(A\text{-}T)_n$	3.76	2.04	
17	CT DNA_{den}	17.06	3.52	6.16
	CT DNA_{nat}	4.84		
	$d(A\text{-}T)_n$	5.54	0.57	
25	CT DNA_{den}	46.27	3.40	4.91
	CT DNA_{nat}	13.62		
	$d(A\text{-}T)_n$	18.84	0.69	
34	CT DNA_{den}	4.44	2.10	0.91
	CT DNA_{nat}	2.11		
	$d(A\text{-}T)_n$	9.74	2.10	

NOTE: All assays were carried out in the presence of 0.025 M ammonium sulfate and 2mM Mn^{++}. The concentration of CT DNA was 3 ug/125 ul assay and $d(A\text{-}T)_n$ was 5 ug/125 ul assay.

[a]Figure 23 gives DEAE-Sephadex column activity profile

TABLE 10.--Effect of Template on Artemia salina RNA Polymerase II

Template	[^{3}H]·UMP Incorp. (pmol/50 ul/10')	Stimulation Relative to Artemia DNA_{nat}	Activity Ratio $DNA_{den}/d(A\text{-}T)_n$
$d(A\text{-}T)_n$	0.73	0.47	----
Artemia DNA_{den}	2.28	2.9	6.2
Artemia DNA_{nat}	0.78		
Calf thymus DNA_{den}	3.71	4.7	10.1
Calf thymus DNA_{nat}	1.03	1.3	
Salmon sperm DNA_{den}	2.38	3.0	6.5
Salmon sperm DNA_{nat}	1.64	2.1	

NOTE: The standard reaction mixture contained 0.6 ug of template, 3mM Mn^{++} and 1.8 ug protein in a final volume of 125 ul. Incubation was for 25 min at 37°C. Each value is the average of three determinations.

TABLE 11.--General Properties of Artemia RNA Polymerase Activities

DEAE-Sephadex Fraction[a]	12	17	25	34
Elution Position (M ammonium sulfate)				
DEAE-cellulose	0.11-0.19	0.11-0.19	0.11-0.19	0.11-0.19
DEAE-Sephadex	0.09	0.14	0.225	0.32
Catalytic Properties				
$(NH_4)_2SO_4$ optima (mM)				
with CT DNA_{den}	50 & 250	100	75-100	0-250
with $d(A\text{-}T)_n$	25			25
Mn^{++} optima (mM)				
with CT DNA_{den}	4	2-3	2-3	1-2
with $d(A\text{-}T)_n$	1			1-2
Mg^{++} optima (mM)				
with CT DNA_{den}	2	6	6	6
with $d(A\text{-}T)_n$				6
Mn^{++}/Mg^{++} Activity Ratio				
with CT DNA_{den}	2.3	4.0	3.2	3.2
with $d(A\text{-}T)_n$	4.3			12.0
CT DNA_{den}/CT DNA_{nat} Activity Ratio	1.1	3.5	3.4	2.1
$d(A\text{-}T)_n$/CT DNA_{nat}	2.0	0.57	0.69	2.3
α-amanitin Sensitivities				
ug/ml for 50% inhibition	insensitive to 4 ug/ml	.028	.019	insensitive to 40 ug/ml
ug/ml for 100% inhibition		.040	.040	

[a]Figure 23 gives DEAE-Sephadex column activity profile

TABLE 12.--General Properties of Nuclear RNA Polymerases

	Enzyme Class		
	I	II	III
Chromatographic Elution Positions (M ammonium sulfate)			
DEAE-Sephadex	~0.1	~0.2	0.2-0.3
DEAE-cellulose	~0.1	~0.2	~0.1
Phosphocellulose	~0.17	~0.11	~0.13
Catalytic Properties			
Ammonium Sulfate Optima (M)	~0.05	~0.10	0.05-0.20
Mn^{++}/Mg^{++} Activity Ratio	1-2	5-10	~2
$d(A\text{-}T)_n$/DNA Activity Ratio	1-1.5	0.5-1.0	5-15
Alpha-amanitin Sensitivities (ug/ml for 50% inhibition)			
Animal Cells	insensitive	0.01-0.05	10-25
Insects	insensitive	0.03-0.06	insensitive
Yeast	300-600	1	insensitive

SOURCE: R. Roeder. 1976. Eukaryotic Nuclear RNA Polymerases. In "RNA Polymerase". (R. Losick and M. Chamberlin, eds.) Cold Spring Harbor Laboratory, Cold Spring Harbor.

NOTE: The elution positions indicated are those for the major forms of RNA polymerases I, II and III. Minor enzyme forms may elute at slightly different positions. Catalytic properties refer to those which are apparent under defined conditions with DNA templates in excess. Alpha-amanitin insensitivity indicates no inhibition at toxin concentrations up to at least 1 mg/ml. All numerical values are approximate; some exceptions are known.

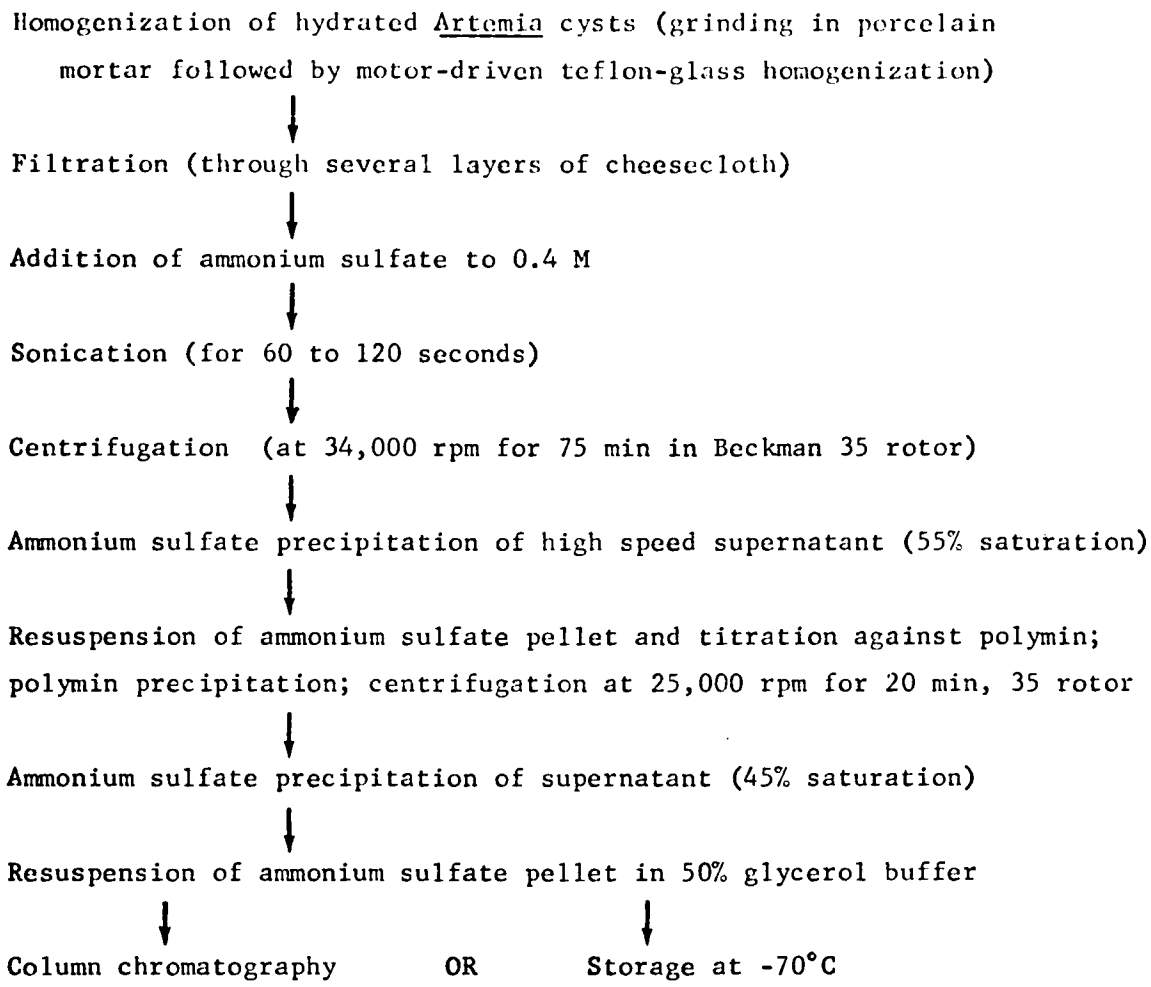


Fig. 1. Purification scheme for RNA polymerases of Artemia salina. All procedures carried out on ice or in a 4°C cold room.

Fig. 2. Incorporation of substrate by Artemia cyst RNA polymerase measured after different times of incubation. Shrimp cyst polymerase at two stages of purification was measured for activity after various incubatimes in the standard reaction mix at 37°C.

(-▲-▲-) Shrimp cyst homogenate. This homogenate contained a mixture of shrimp RNA polymerase enzymes and had 44% resistance to 8 ug/ml alpha-amanitin. Assays contained 93 ug protein, 2.5 ug $d(A\text{-}T)_n$ and 4 mM Mn^{++} in a final volume of 125 ul. Other components were as described in Methods for the standard reaction mix. The reaction was terminated with cold 5% TCA and the acid-insoluble product collected and washed on GF/C filters. The filters were dried and the radioactivity counted. Each point is the average of two determinations.

(-●-●-) Shrimp polymerase activity peak fraction from a DNA-cellulose column (column profile not shown). Each assay contained 9.5 ug protein in a final volume of 125 ul; the composition of the reaction mix and incubation procedures were identical to those used for the crude homogenate (above). Each point is the average of two determinations.

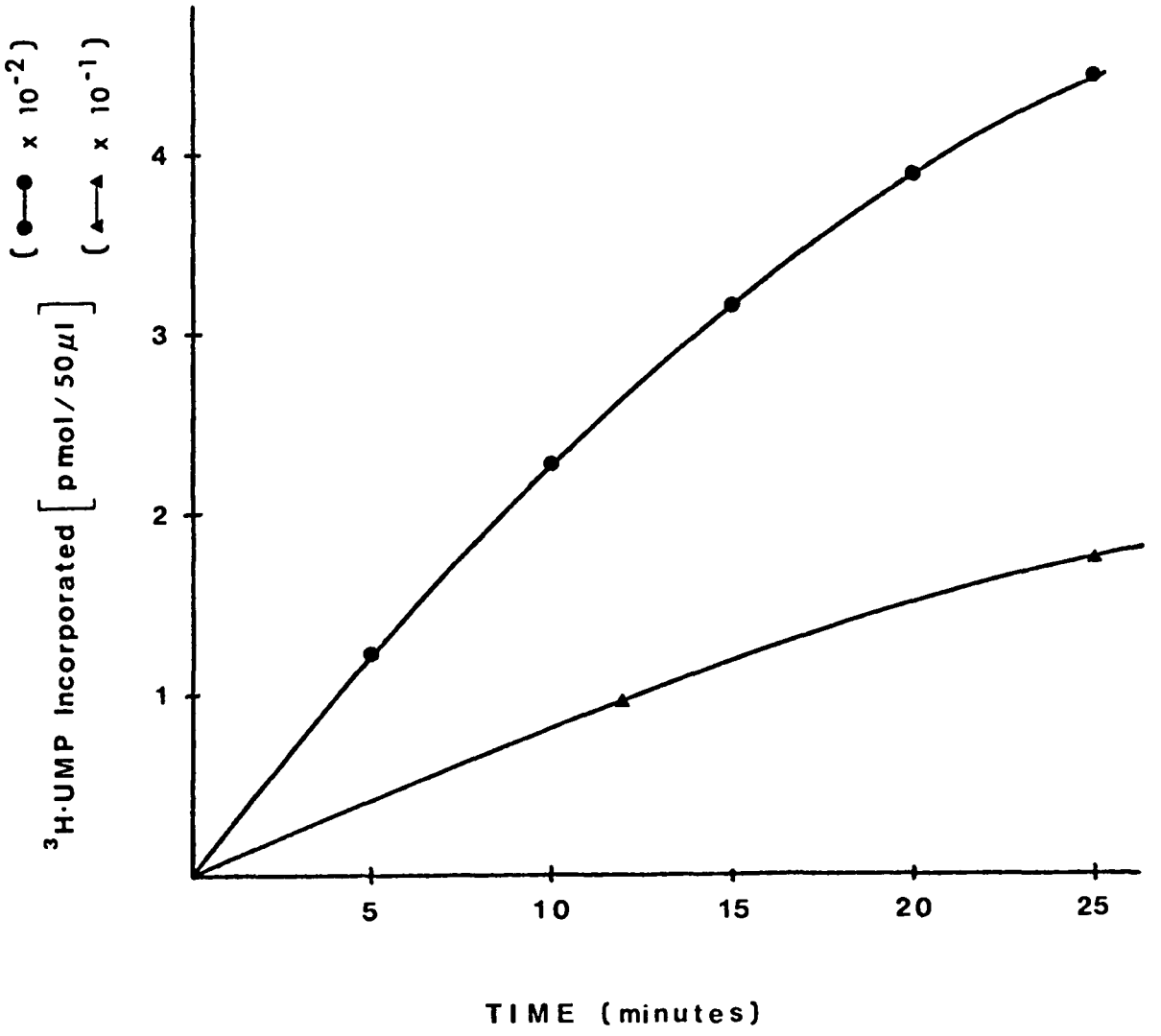

(●—● x 10^-2) (▲—▲ x 10^-1)
3H·UMP Incorporated [p mol / 50µl]
1
2
3
4
5
10
15
20
25
TIME (minutes)

Fig. 3. Rechromatography on DNA-cellulose of the DEAE-cellulose activity peak of Artemia cyst F/S fractions pooled from experiments 1 - 3. As explained in the text, the polymerase sample applied to this DNA-cellulose column was the pooled shrimp polymerase F/S fractions of experiments 1 - 3. This activity had been previously batch-eluted from a DEAE-cellulose column. The polymerase-containing fractions were collected from the DEAE-cellulose and pooled on the basis of their UV absorbance. The volume of the pooled fractions was 93 ml. This was made 50% saturated with ammonium sulfate and the proteins were precipitated by centrifugation at 25,000 rpm in a Beckman 35 rotor.

The pellet was resuspended in 150 ml buffer B. The conductivity indicated an ionic strength equivalent to 0.15 M NaCl in buffer B. The total enzymatic activity was 233 units of RNA polymerase activity which was 35% resistant to 8 ug/ml alpha-amanitin when assayed with $d(A\text{-}T)_n$ as template.

The DNA-cellulose column had been equilibrated to 0.15 M NaCl in buffer B. The dimensions of the column were 7 cm x 2.25 cm; the sample was loaded overnight. After washing with 30 ml buffer B (0.15 M NaCl), the column was batch eluted with 50 ml buffer B (0.5 M NaCl). Fractions of 50 drops/tube were collected and the UV absorbance, protein concentration and enzymatic activity were measured (table 6).

(···▲···▲··) Protein concentration determined by the Lowry method.

(-●—●-) RNA polymerase activity measured in the absence of amanitin. (Fraction 17 was found to be 57% resistant to 8 ug/ml. The standard reaction mix contained no added ammonium sulfate, 4 mM Mn^{++} and $d(A\text{-}T)_n$.)

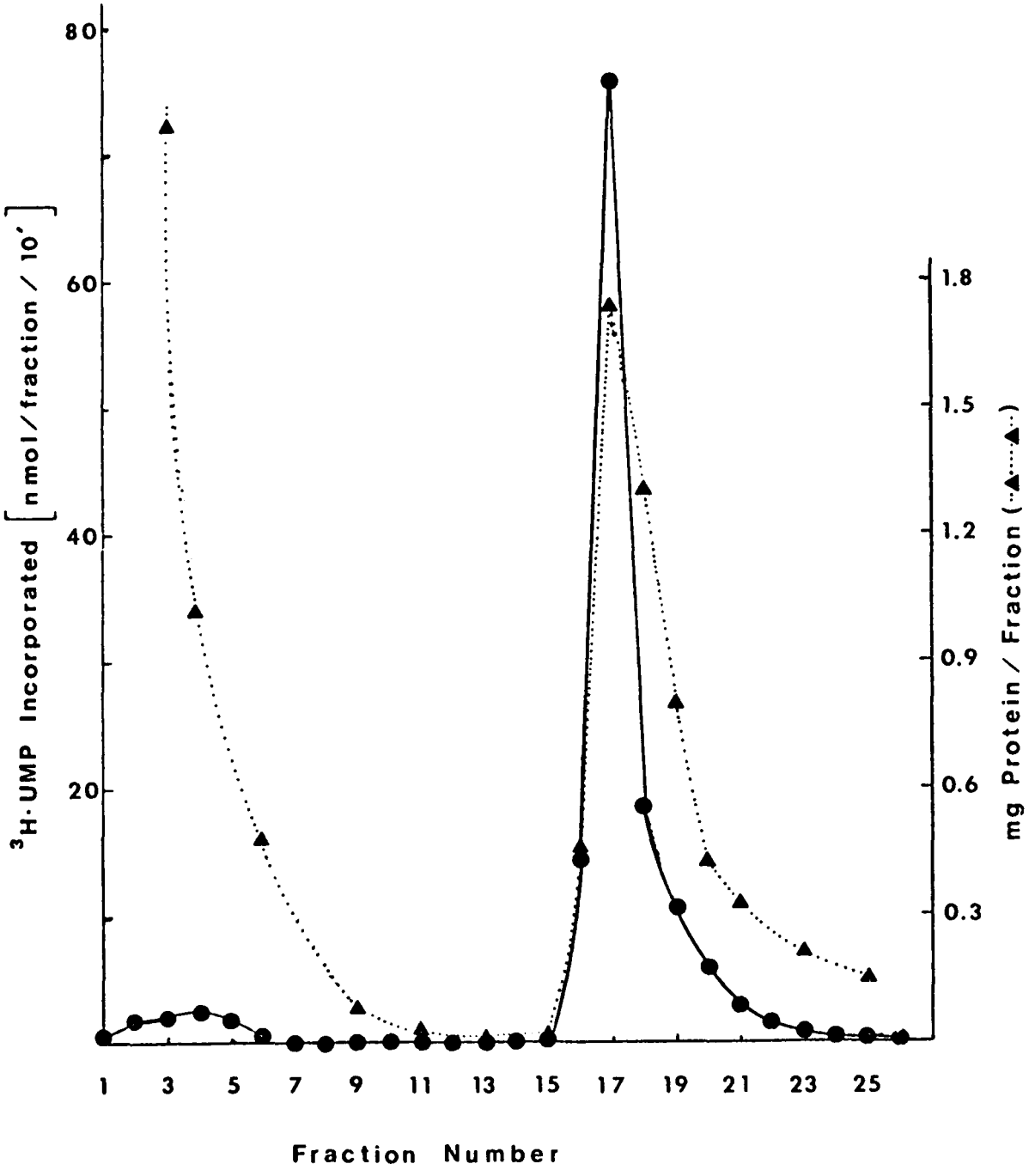

80
60
40
20
^{3}H-UMP Incorporated [n mol / fraction / 10']
1.8
1.5
1.2
0.9
0.6
0.3
mg Protein / Fraction (▲····▲)
1 3 5 7 9 11 13 15 17 19 21 23 25
Fraction Number

Fig. 4. Retention of [^{3}H]-d(A-T)$_n$ on millipore filters by Artemia RNA polymerase from a DNA-cellulose activity peak. Complete reaction mixes containing 0.75 ug [^{3}H]-d(A-T)$_n$ and cold triphosphates and 0 to 9 ug shrimp polymerase in a final volume of 125 ul was incubated for 5 min at 37° C. The 0.75 ug [^{3}H]-d(A-T)$_n$ contained 3,632 cpm. The reaction was terminated by the addition of "low salt" solution (0.02 M Tris·HCl at pH 7.6 and 0.05 M NaCl) on ice. The reaction mixtures were poured through millipore filters under vacuum. Filters had been pre-soaked and washed with 0.1 M HCl and stored in 0.01 M ATP. The loaded filters were washed with 5 ml of the low salt solution, dried and the bound radioactivity was counted.

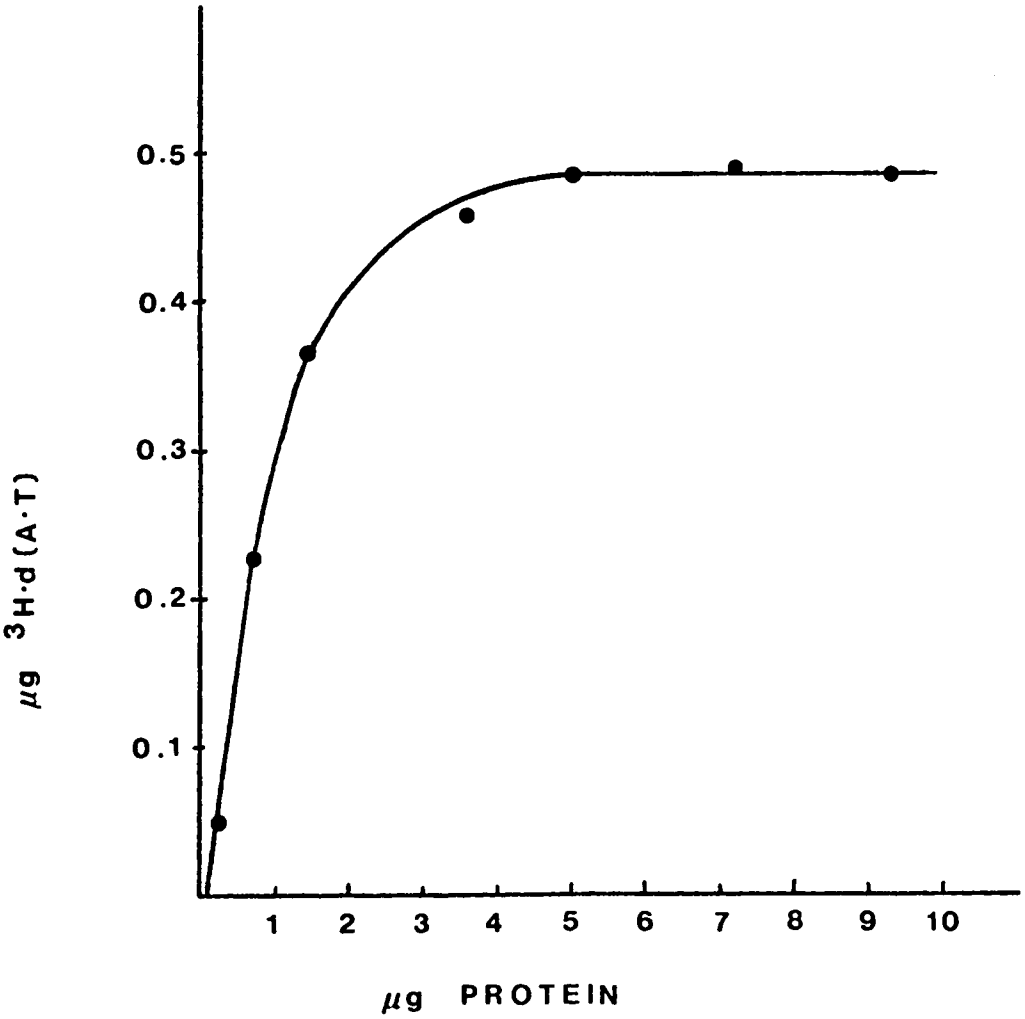

0.5
0.4
0.3
0.2
0.1
μg ³H·d(A·T)
1
2
3
4
5
6
7
8
9
10
μg PROTEIN

Fig. 5. The effect of protein concentration on RNA polymerase activity. RNA polymerases at various stages of purification from hydrated Artemia cysts were assayed for polymerase activity at increasing protein concentrations in the standard reaction mix. All incubations were for 20 min at 37°C in the presence of 4 mM Mn^{++}, 5 ug $d(A\text{-}T)_n$ and no added ammonium sulfate.

(-▲-▲-) Crude homogenate

(-■-■-) 15,000 rpm supernatant of the crude homogenate

(-●-●-) 35,000 rpm supernatant of the crude homogenate

(-□-□-) DNA-cellulose column activity peak fraction (column activity profile not shown)

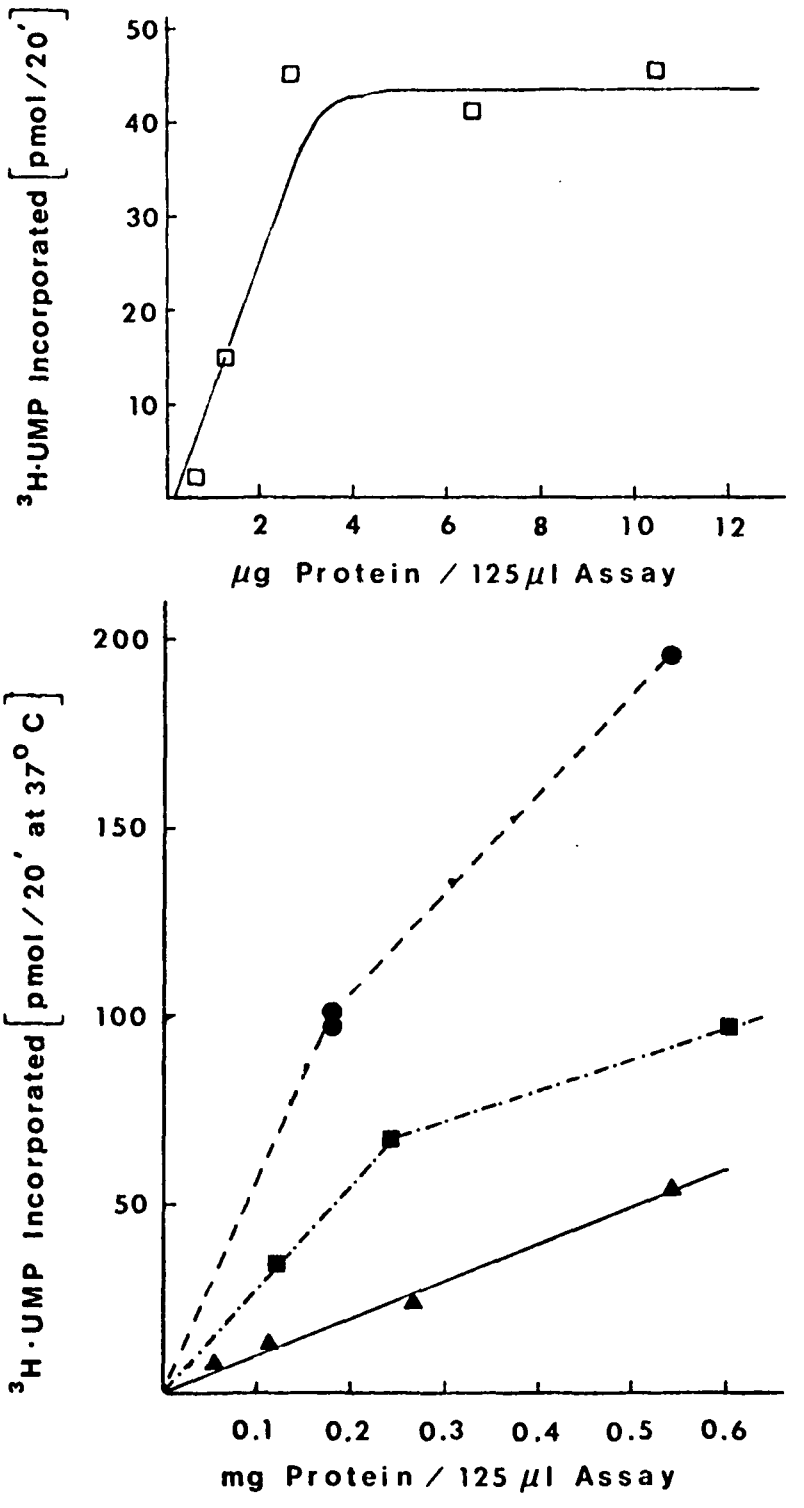

^{3}H·UMP Incorporated [pmol/20']
50
40
30
20
10
2
4
6
8
10
12
μg Protein / 125μl Assay
^{3}H·UMP Incorporated [pmol/20' at 37° C]
200
150
100
50
0.1
0.2
0.3
0.4
0.5
0.6
mg Protein / 125 μl Assay

Fig. 6. DEAE-Sephadex column chromatography of RNA polymerase activity extracted from Artemia salina after 24 hr incubation at 28°C. Artemia cysts were hydrated, washed and incubated in 3.5% NaCl at 28°C with gentle shaking as described in Methods. The shrimp polymerase extract was prepared as described by Kedinger and Chambon [1972]. This method differed from the described method in that the sonication was carried out at a higher ammonium sulfate concentration (0.225 ml saturated ammonium sulfate at pH 7.5/ml extract). The shrimp extract was frozen in liquid nitrogen and stored at -70°C for chromatography.

Shrimp protein (7.4 mg, Lowry method) was loaded onto the DEAE-Sephadex column, the column was washed with one column volume of 0.05 M ammonium sulfate buffer, followed by 20 ml 0.1 M ammonium sulfate and then the enzyme activity was eluted with a linear gradient of 25 ml each 0.1 and 0.4 M ammonium sulfate buffer A. Fractions of 0.9 ml (nos. 1-19) and 0.45 ml (nos. 20-131) were collected. Every other fraction was assayed with (-●-●-) and without (-O-O-) alpha-amanitin at 1.6 ug/ml. Conductivity was measured with a Radiometer conductivity meter with a flow-through probe and compared to standards of known ionic strength. BSA had been placed in the collecting tubes so that the final BSA concentration of the collected RNA polymerase fractions was 0.5 mg/ml. Mn^{++} concentration was 4 mM in all assays.

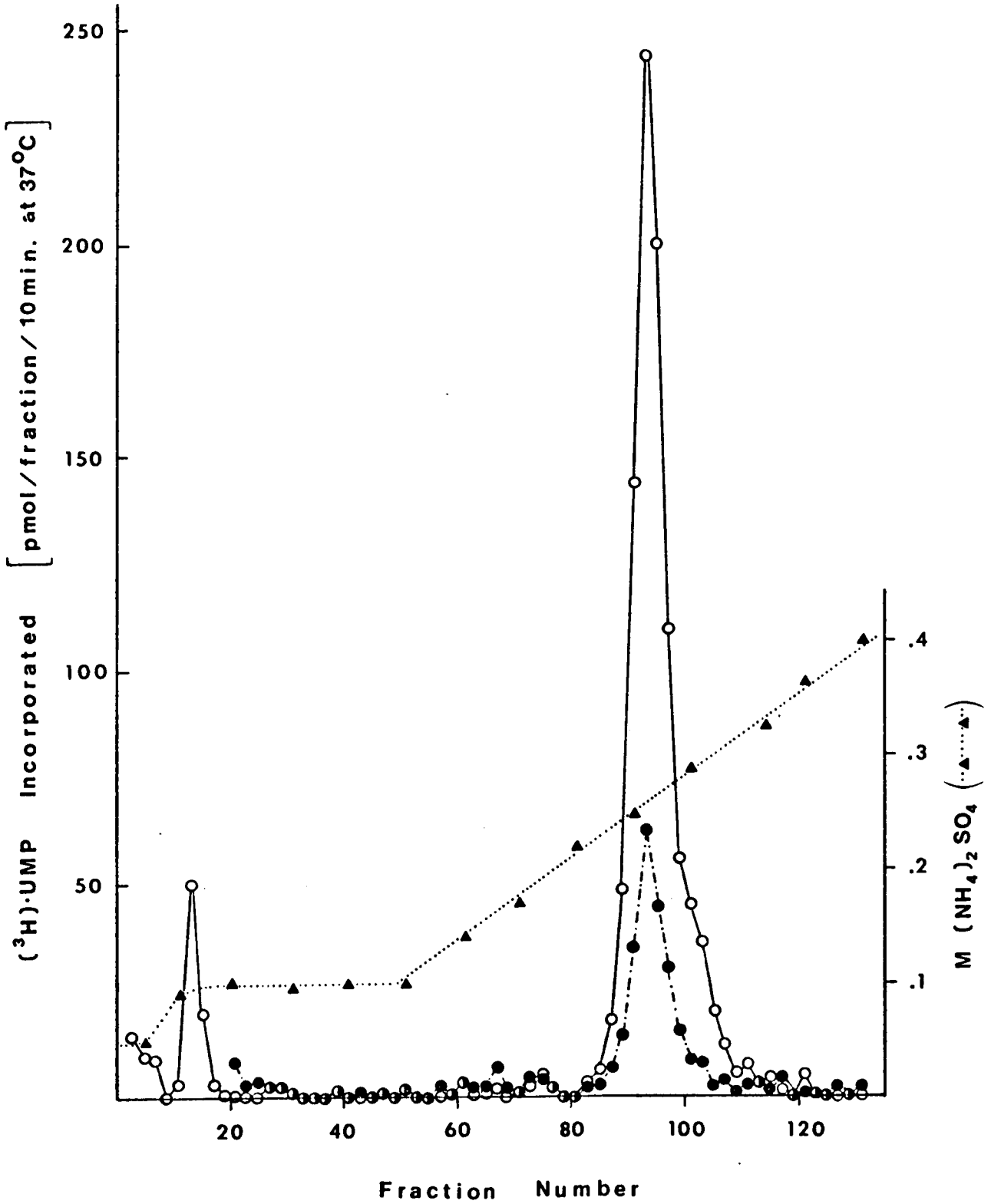
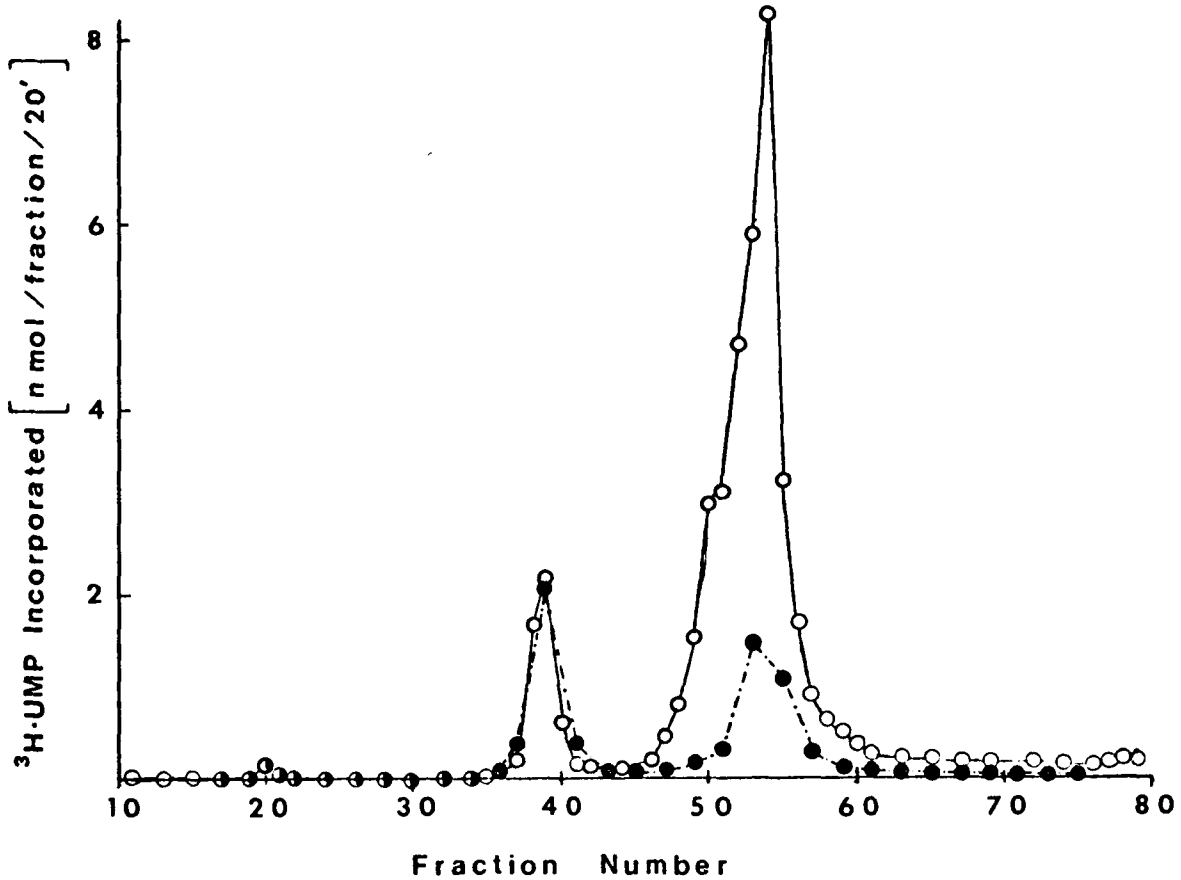

250
200
150
100
50
(3H)-UMP Incorporated [pmol/fraction/10min. at 37°C]
.4
.3
.2
.1
M (NH4)2 SO4
20
40
60
80
100
120
Fraction Number

Fig. 7. DEAE-Sephadex column chromatography of hydrated Artemia cyst homogenate and re-assay of the collected fractions after freezing in liquid N_2 and storage at -70°C. A. 55 mg protein (measured by the Lowry method) of shrimp cyst polymerase fraction F/S was loaded onto a DEAE-Sephadex column measuring 1.5 cm x 17 cm at a flow rate of 60 ml/hr. The column was washed with 40 ml buffer A containing 0.05 M ammonium sulfate and the enzyme activity was eluted with a linear gradient made from 140 ml each of 0.05 M and 0.40 M ammonium sulfate buffer A. Fractions of 3 ml each were collected and assayed as they came off the column. The standard reaction mix contained 50 ul enzyme and 1.5 ug CT DNA_{den} in a final volume of 125 ul. Incubation was for 20 min at 37°C in the presence (-●-●-) and absence (-O-O-) of 4 ug/ml alpha-amanitin. B. Re-assay of the column fractions after 18 days storage at -70°C in a Revco (the fractions had been frozen in the Revco and had no additional glycerol or BSA added to them prior to freezing and assay). The assay conditions were identical to the original assay conditions described above and did not include alpha-amanitin.


^{3}H·UMP Incorporated [n mol / fraction / 20']
8
6
4
2
10
20
30
40
50
60
70
80
Fraction Number

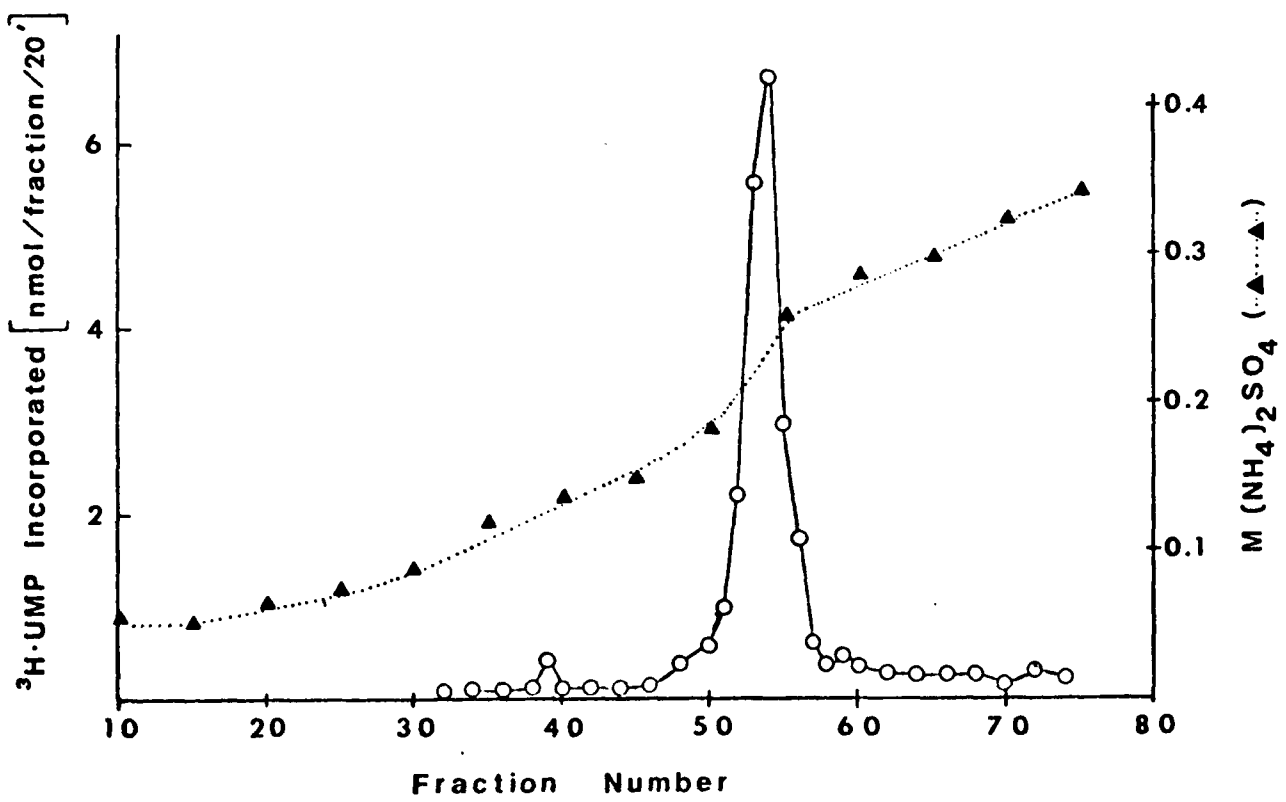

^{3}H·UMP Incorporated [nmol/fraction/20']
6
4
2
0.4
0.3
0.2
0.1
M $(NH_4)_2SO_4$ (▲···▲)
10
20
30
40
50
60
70
80
Fraction Number

Fig. 8. Results of non-denaturing gel electrophoresis of the DEAE-Sephadex activity peak fractions of figure 7. Aliquots of collected fractions were individually electrophoresed on 5% polyacrylamide gels under non-denaturing conditions according to the method of Maizel [1969]. Gels in which a band was detected after staining were scanned in a Gilford spectrophotometer. The absorbance peak of the resulting trace was cut out and weighed. In no gel was there more than one absorbance band; in each gel the band had the same relative mobility. The weight of the absorbance peak was corrected to a full scale absorbance of 0.5 and the values (mg paper) were plotted with the RNA polymerase activity against fraction number. This procedure was repeated under slightly different conditions as described below.

(-■-·-·-■-) Aliquots of 150 ul from fractions 49, 51, 53, 55 and 58 were run on 5% acrylamide gels under non-denaturing conditions at 3 mA/tube; electrophoresis was carried out in a cold room at 4°C. Electrophoresis was discontinued when the tracking dye, bromphenol blue, reached the end of the tube. Gels were 6 cm x 0.6 cm and were stained in 0.25% Coomassie blue in a solution containing methanol, distilled water and acetic acid in the ratio 5:5:1. Destaining was accomplished by several changes of the methanol-acetic acid solution, followed by changes of 7% acetic acid. The gels were scanned at 600 nm.

(··●·····●··) Aliquots of 250 ul from fractions 49 - 59 were run on 5% acrylamide gels at 4 mA/tube as described above. The gels were stained in 0.5% acid fast green in 7% acetic acid and destained electrophoretically in 7% acetic acid. Destaining was completed by changes of 7% acetic acid. Gels were scanned at 630 nm.

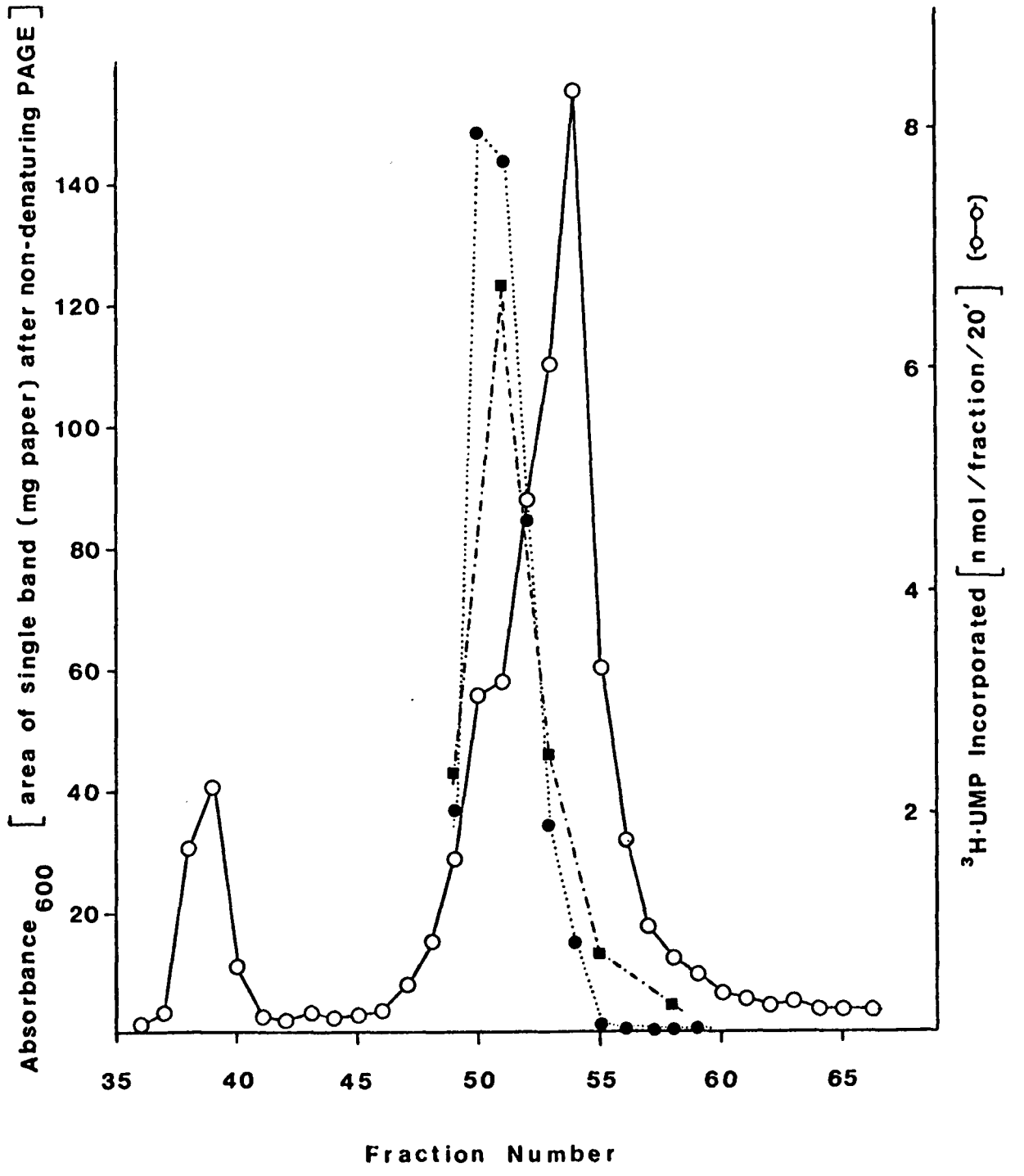

Absorbance 600 [area of single band (mg paper) after non-denaturing PAGE]
140
120
100
80
60
40
20
8
6
4
2
^{3}H-UMP Incorporated [n mol / fraction / 20'] (o—o)
35
40
45
50
55
60
65
Fraction Number

Fig. 9. The effect of increasing alpha-amanitin concentration on the activity of Artemia RNA polymerase activity from a representative DEAE-Sephadex enzyme II activity peak which had lost its amanitin-resistant activity due to several rounds of freezing and thawing in a Revco at -70°C. The standard incubation mixture contained 8.4 ug protein, 4 mM Mn^{++} and 5 ug $d(A\text{-}T)_n$. Incubation was for 20 min at 37°C. Alpha-amanitin concentration was varied as indicated.

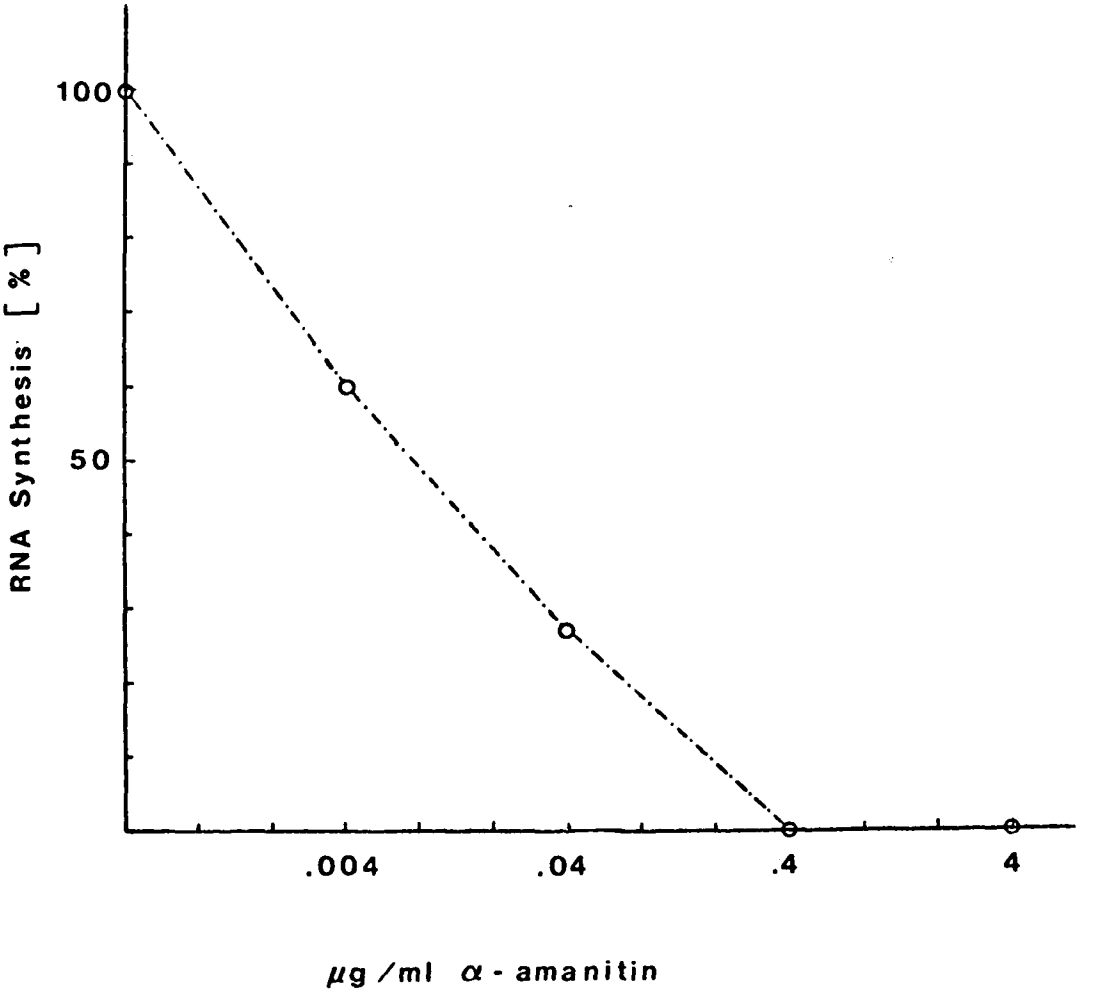

100
50
RNA Synthesis [%]
.004
.04
.4
4
µg /ml α - amanitin

Fig. 10. The effect of ammonium sulfate concentration on the activity of purified Artemia cyst RNA polymerases. The purified RNA polymerase used in this experiment was fraction 17 of the DNA-cellulose column whose activity profile is shown in figure 3. The enzyme was dialyzed against buffer containing no ammonium sulfate and then assayed in the standard reaction mix with CT DNA_{den} as template. Ammonium sulfate was added as indicated. Fraction 17 was 57% resistant to 8 ug/ml amanitin.

(-●--●-) no alpha-amanitin. Assay of a mixture of sensitive and resistant activities.

(-O--O-) assayed in the presence of alpha-amanitin at 8 ug/ml.

(-Δ--Δ-) held at -20°C overnight before dialysis and assayed in the absence of alpha-amanitin.

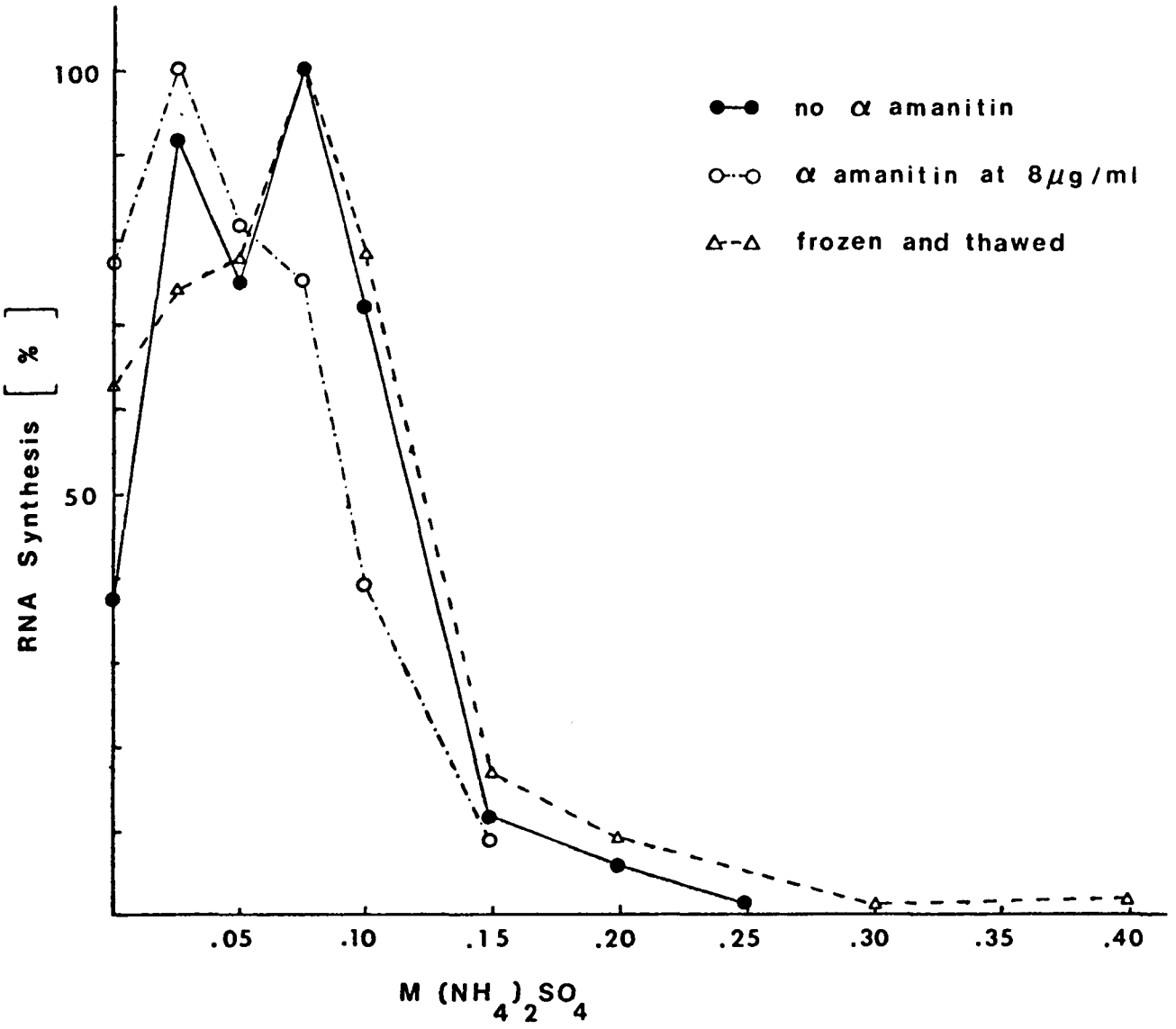
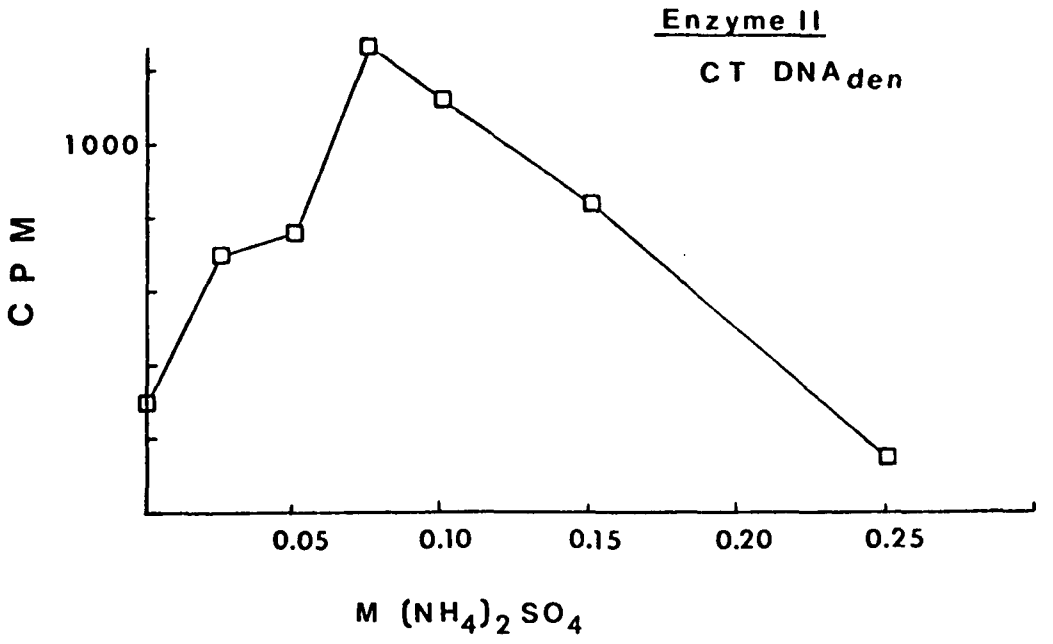

100
50
RNA Synthesis [%]
no α amanitin
α amanitin at 8μg/ml
frozen and thawed
.05
.10
.15
.20
.25
.30
.35
.40
M (NH4)2SO4

Fig. 11. Effect of ionic strength on the enzymatic activity of Artemia cyst RNA polymerase II. Enzyme II was obtained from hydrated shrimp cysts as described in the flow diagram of the purification procedure outlined in figure 1. The amanitin sensitive activity peak fractions from a DEAE-cellulose column (data not shown) were pooled and run on DEAE-Sephadex (data not shown). The activity peak from that column was pooled and dialyzed overnight against buffer A with no ammonium sulfate and no Mg^{++}. The pooled samples were concentrated from 12 ml to 7 ml by dialysis against saturated sucrose for 4 hr. The enzyme was further dialyzed to remove the sucrose. It was frozen prior to assay with ammonium sulfate and Mn^{++} in varying concentrations (figure 12).

Prior to characterization with ammonium sulfate and Mn^{++} the enzyme was shown to have no activity in the presence of 4 ug/ml alpha-amanitin. In the figure shown, 100 ul of enzyme was assayed in a double reaction mix which contained 4 mM Mn^{++} and 6.25 ug $d(A\text{-}T)_n$ (-■—■-) or 7.5 ug CT DNA_{den} (-□—□-) in a final volume of 250 ul. In the case of the assays containing $d(A\text{-}T)_n$, the data presented is the result of averaging two determinations and the range is shown. The CT DNA_{den} data is the result of a single determination.

All results are presented as cpm/30 min/100 ul enzyme in a standard reaction mix with a final volume of 250 ul. Templates were as descibed.


Enzyme II
CT DNA den
1000
CPM
0.05
0.10
0.15
0.20
0.25
M (NH4)2 SO4

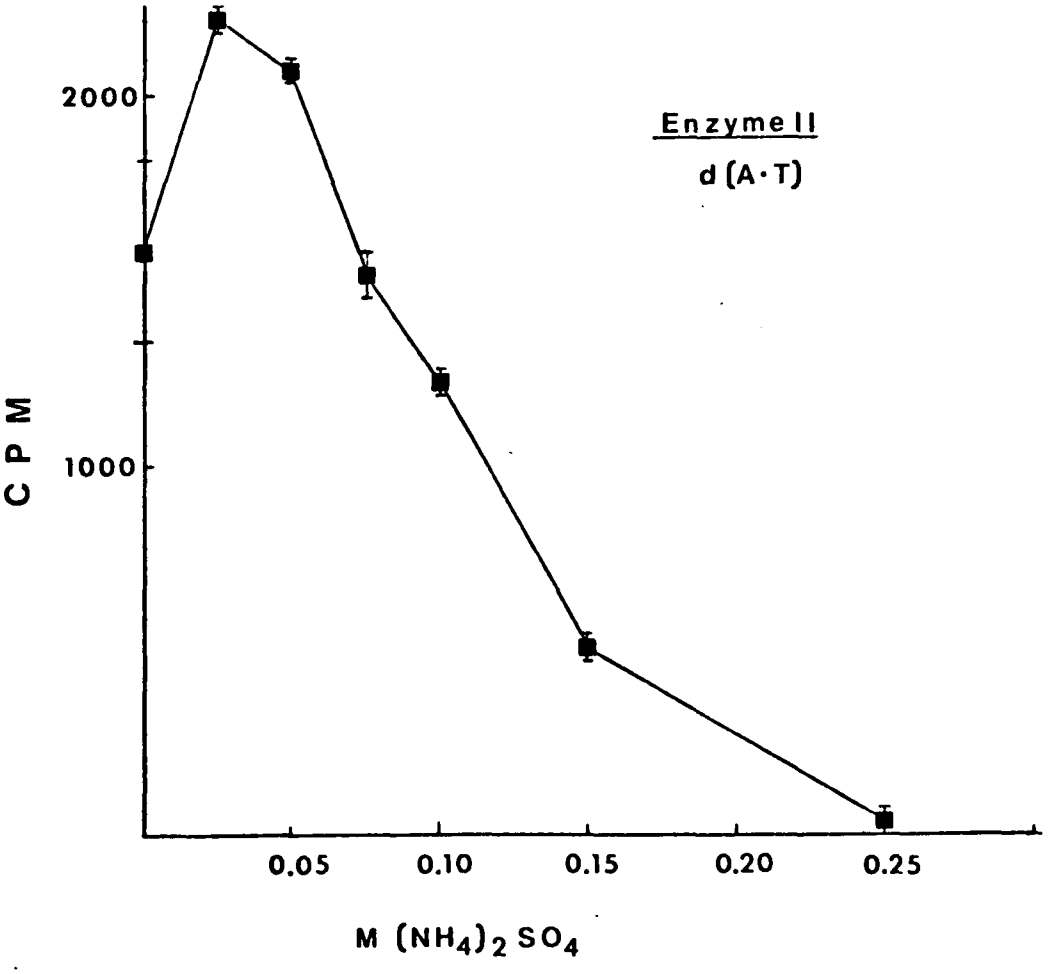

Enzyme II
d (A·T)
2000
1000
CPM
0.05
0.10
0.15
0.20
0.25
M (NH4)2 SO4

Fig. 12. Effect of Mn^{++} concentration on the enzymatic activity of Artemia RNA polymerase II. The enzyme preparation described in the legend to figure 11 was assayed at two concentrations of ammonium sulfate. The Mn^{++} concentration was varied as shown. Each point is the average of two determinations.

(-●-●-) 0.100 M ammonium sulfate and 3.75 ug $d(A\text{-}T)_n$ in a final volume of 125 ul incubated at 37°C for 25 min.

(-○-○-) 0.025 M ammonium sulfate and 7.5 ug $d(A\text{-}T)_n$ in a final volume of 250 ul incubated at 37°C for 25 min.

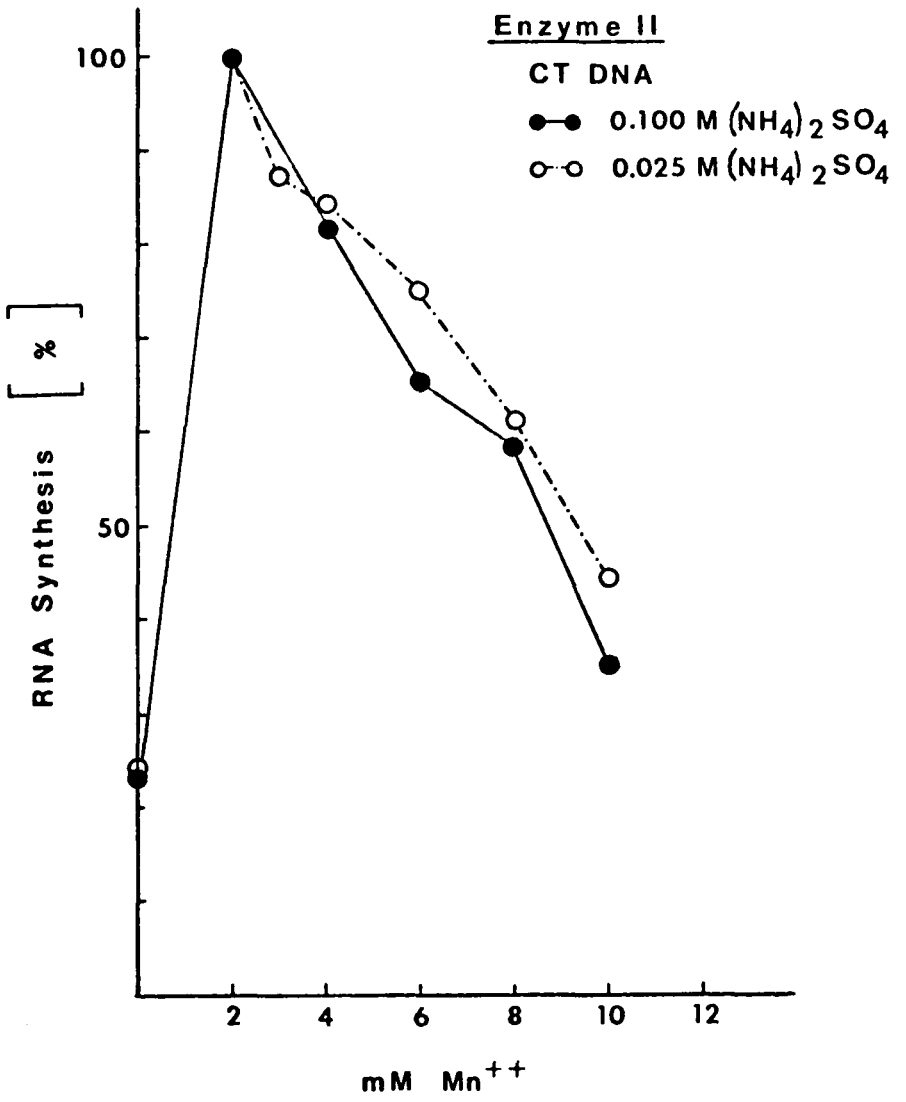

Enzyme II
CT DNA
0.100 M $(NH_4)_2SO_4$
0.025 M $(NH_4)_2SO_4$
100
50
RNA Synthesis [%]
2
4
6
8
10
12
mM Mn^{++}

Fig. 13.--Glycerol gradient centrifugation of polymerase II from Artemia salina. Polymerase II was obtained from hydrated Artemia cysts as described in the legends to figures 11 and 12. The polymerase II remaining after these assays was brought to 50% saturation with ammonium sulfate and centrifuged at 25,000 rpm in a Beckman 30 rotor for 2 hr. Pellets were resuspended in a final volume of 1.0 ml gradient buffer (4% glycerol).

This preparation required dialysis (1.5 hr at 4°C) against glycerol gradient buffer containing no glycerol in order to lower the sample density. [Dialysis against 15% glycerol glycerol gradient buffer would have been preferable since the high sample density was primarily due to ammonium sulfate.] Half of the sample was layered onto each of 2 preformed 15-30% glycerol gradients; one of the Artemia polymerase samples had been mixed with 25 ul of E. coli RNA polymerase (Sigma). Centrifugation was at 35,000 rpm for 15.4 hr in an SW41 rotor at 3°C. Gradients were fractionated as described in Methods. Fractions of 25 drops each were collected and assayed for polymerase activity in the standard reaction mix containing 2mM Mn^{++}, 0.065 M ammonium sulfate and 3.75 ug CT DNA_{den}. (-●-·-·●-)

Aliquots of 200 ul each of gradient fractions 10 - 20 were run on 5% polyacrylamide gels under non-denaturing conditions. The gels were formed and run according to the method of Kedinger et al. [1972] as described in Methods. They were stained, destained and scanned as described in the legend to figure 8. The relative absorbance of the single protein bands was plotted (··o····o··) by the method described in the legend to figure 8.

The actual gel traces of the 200 ul aliquots of gradient fractions 15 and 16-17 (inadvertantly pooled) are shown as B. and C. of figure 44.

Collapse of the centrifuge tube containing the mixture of E. coli and Artemia polymerases prevented comparison of the behavior of these enzymes.

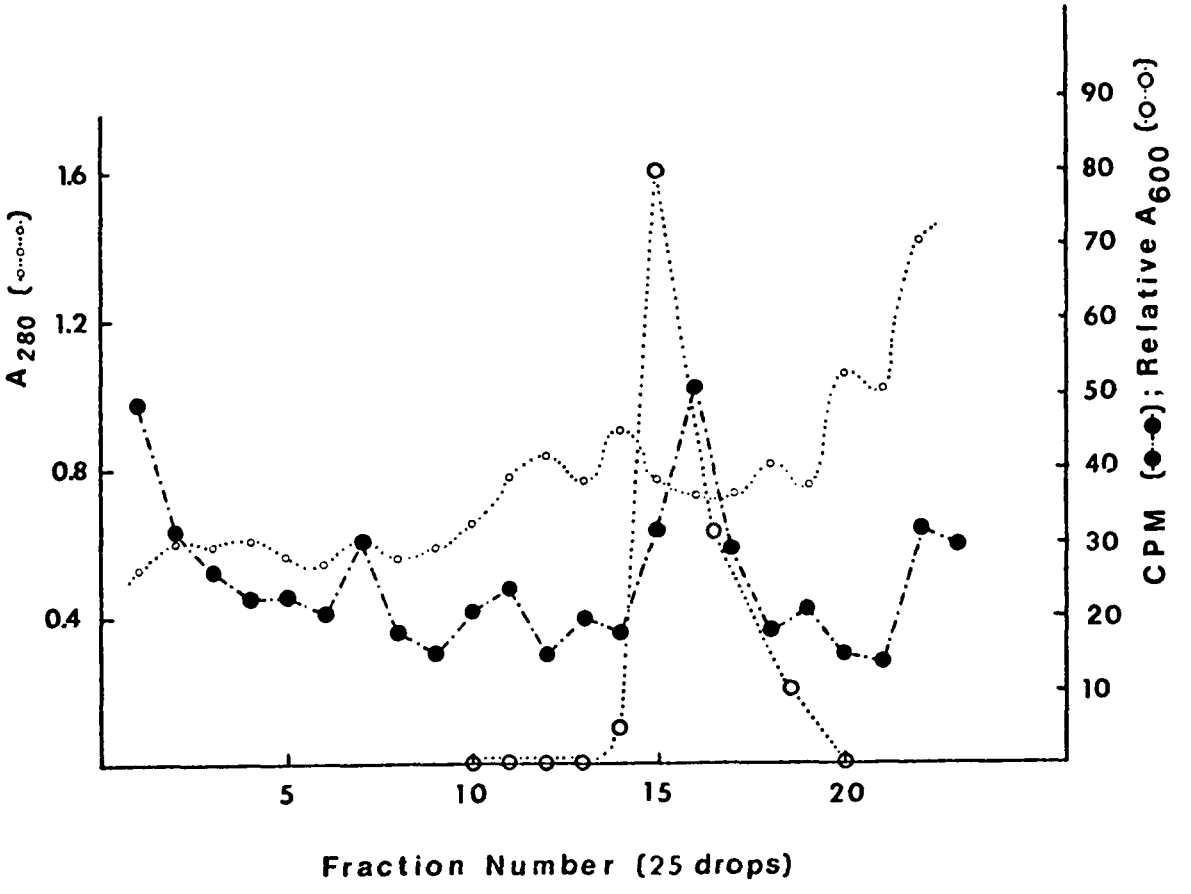
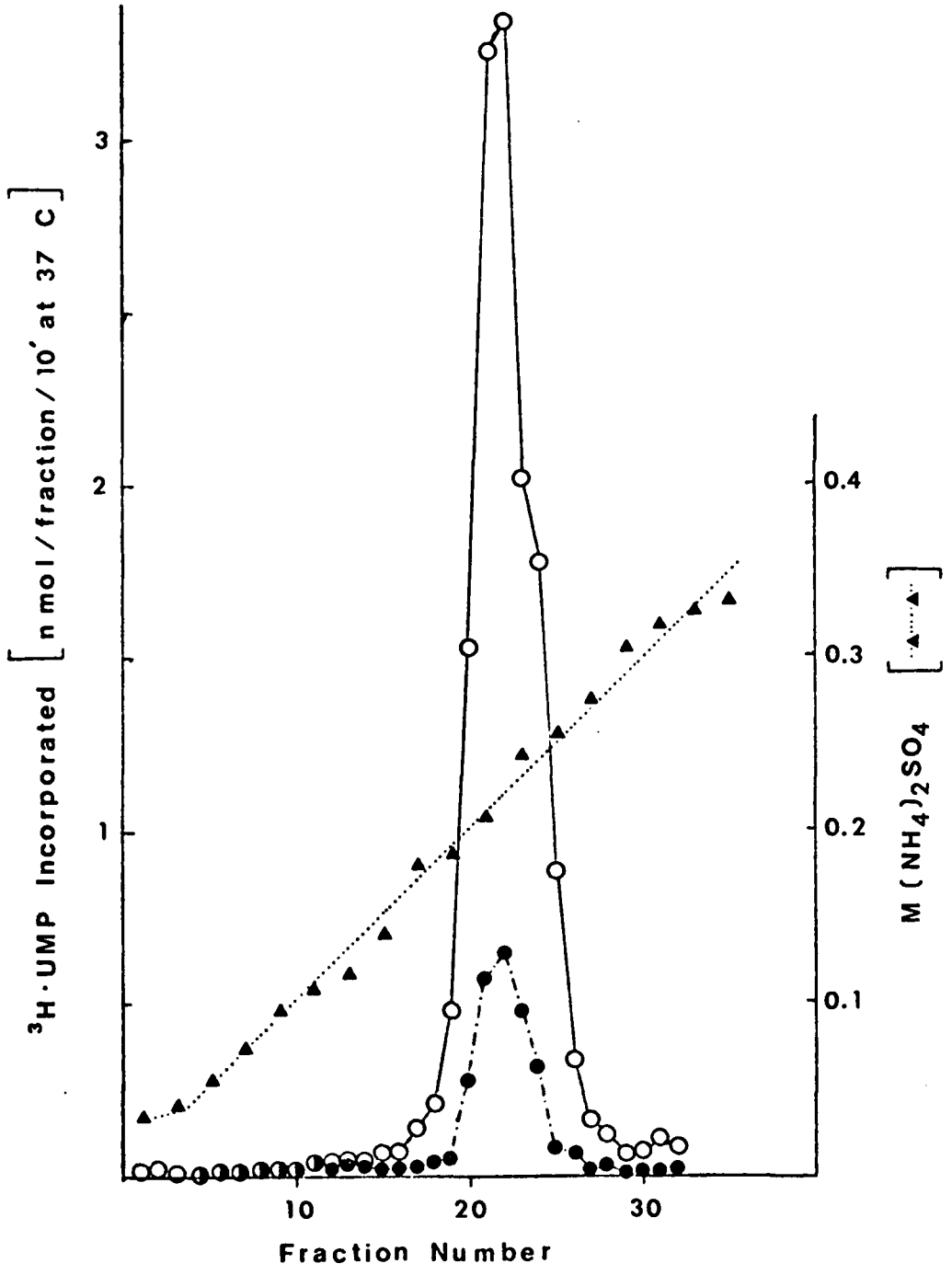

1.6
1.2
0.8
0.4
A_{280} (•···•)
90
80
70
60
50
40
30
20
10
CPM (●—●); Relative A_{600} (○···○)
5
10
15
20
Fraction Number (25 drops)

Fig. 14. Rechromatography on DEAE-Sephadex of the RNA polymerase activity from a DNA-cellulose column. The activity peak from a DNA-cellulose column was made 1 mg/ml BSA and 50% glycerol and frozen for 9 days at -70°C. Upon thawing, the enzyme activity was found to be 26% resistant to 1.3 ug/ml alpha-amanitin. The ionic strength was lowered to the equivalent of 0.05 M ammonium sulfate with buffer A and the polymerase activity was loaded onto a pre-equilibrated DEAE-Sephadex column with a bed volume of 8.8 ml. The sample was loaded in a volume of 126 ml at a flow rate of 60 ml/hr. The column was washed with 20 ml buffer A (0.05M ammonium sulfate) and the enzyme activity was eluted with a linear gradient of 25 ml each of 0.075 M and 0.35 M ammonium sulfate in buffer A. Fractions of 1.5 ml were collected and assayed in the standard reaction mix with (-●-●-) and without (-O-O-) 4 ug/ml alpha-amanitin. (··▲··▲··) M ammonium sulfate.


^{3}H·UMP Incorporated [n mol / fraction / 10' at 37 C]
3
2
1
0.4
0.3
0.2
0.1
M (NH_4)$_2$$SO_4$ [▲···▲]
10
20
30
Fraction Number

F/S fractions of experiments 1 - 3 pooled

↓

Enzyme activity adsorbed to DEAE-cellulose (at 0.05 M ammonium sulfate in buffer A)

↓

Slurry poured into Buchner funnel and washed (buffer A at 0.07 M ammonium sulfate)

↓

Pre-loaded DEAE-cellulose transferred from Buchner funnel to chromatographic column

↓

RNA polymerase activity eluted with 0.5 M ammonium sulfate (UV absorbance monitored and fractions collected)

↓

Fractions containing the UV absorbance peak pooled and precipitated with ammonium sulfate (51% saturation)

↓

Ammonium sulfate pellet resuspended in buffer B and adsorbed to DNA-cellulose at 0.15 M NaCl

↓

RNA polymerase activity eluted with buffer B at 0.5 M NaCl (figure 3)

Fig. 15. Chromatographic purification scheme for the pooled RNA polymerase activity from experiments 1 - 3.

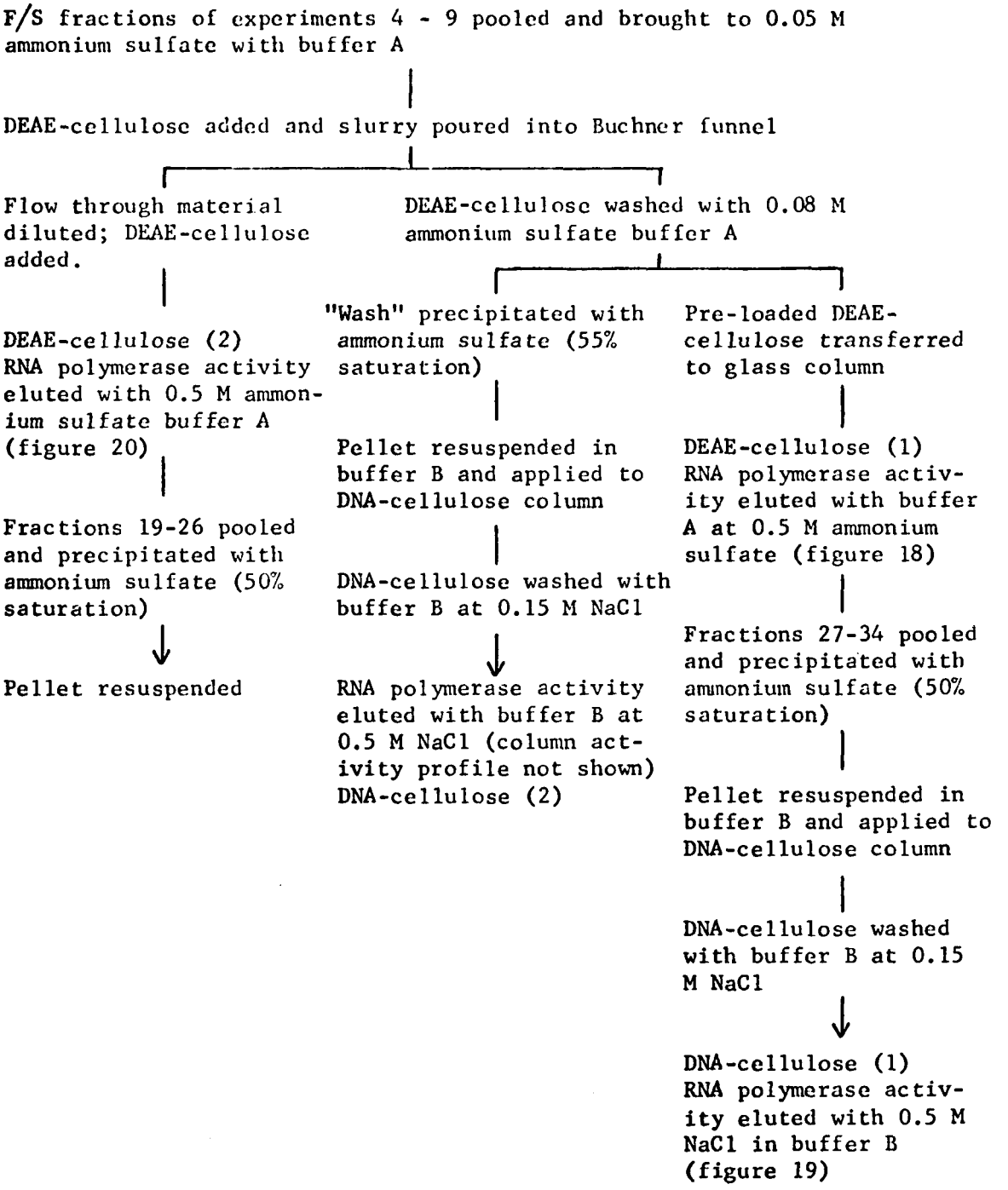


Fig. 16. Flow diagram of the chromatographic purification and partial separation of Artemia cyst RNA polymerases (pooled F/S fractions from experiments 4 - 9

Fig. 17.--Titration of RNA polymerase activity against polymin-P concentration. Data from four experiments (see also table 4). Aliquots of the HSS fraction (high speed supernatant) of Artemia cyst RNA polymerase preparations were titrated against increasing amounts of polymin-P as described in Methods. The purpose was to find the polymin concentration corresponding to maximum reduction of nucleic acids with minimum loss of enzyme activity. The optimum polymin concentration for each shrimp polymerase preparation was determined in the following manner: aliquots (either 0.5 ml or 1.0 ml) of the HSS fraction were mixed with very small amounts of 2.5% (or 5%) polymin and allowed to stand on ice for 15 - 20 min. As described, the nucleic acid-polymin complexes were removed by centrifugation and the supernatant was assayed for RNA polymerase activity in the standard reaction mix. The remaining nucleic acid content was determined from the relative absorbance at 280 and 260 nm after dilution of a measured amount of the supernatant in 1 M NaCl.

Results of four experiments are given; although the scale is changed, the units as presented for D. are the same for the four graphs. (-●-●-) RNA polymerase activity of the supernatant after precipitation of the n nucleic acid-polymin complex. (--▲--▲--) A_{280}/A_{260} measured in the presence of 1 M NaCl. The abscissa is the amount of polymin (at 5% w/v) added to 0.5 ml HSS.

A. Titration of aliquots of HSS from experiment 2

B. Titration of aliquots of HSS from experiment 3

C. Titration of aliquots of HSS from experiment 9

D. Titration of aliquots of HSS from experiment 6

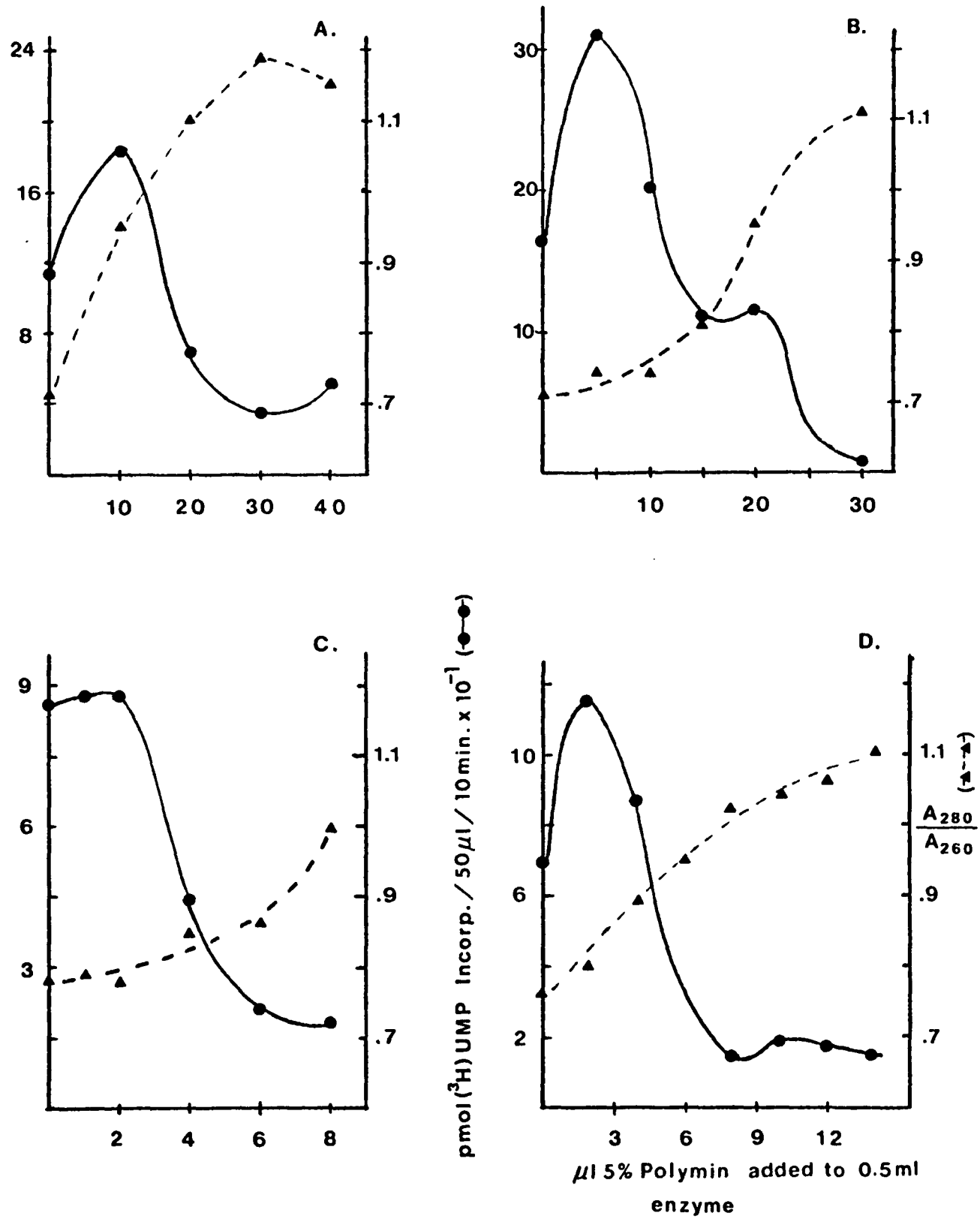

A.
B.
C.
D.
24
16
8
30
20
10
9
6
3
2
1.1
.9
.7
10
20
30
40
4
6
8
12
pmol(^{3}H)UMP Incorp./50µl/10min. x 10^{-1}
$\frac{A_{280}}{A_{260}}$
µl 5% Polymin added to 0.5ml enzyme

Fig. 18. DEAE-cellulose column chromatography of RNA polymerase activity remaining bound to DEAE-cellulose after stepwise elution with low ionic strength ammonium sulfate buffer A. (DEAE-cellulose 1). F/S fractions from six experiments (4 - 9) in which Artemia cysts were hydrated and the polymerase activity was extracted as outlined in figure 1, were pooled for preliminary separation of alpha-amanitin-resistant activity and enzyme II on DEAE-cellulose. Enzymatic activity of each fraction was assayed upon thawing to determine the loss of activity during storage (table 5).

A total of 15.1 g protein containing 4,756 units of activity was mixed with 84 g (dry weight) DEAE-cellulose pre-equilibrated with buffer A at 0.05 M ammonium sulfate. After 45 min the slurry was poured into a large Buchner funnel. The DEAE-cellulose was washed with two liters of buffer A containing 0.08 M ammonium sulfate and transferred to a chromatography column measuring 6 cm x 40 cm. The DEAE-cellulose was allowed to settle and the column was washed with 0.5 M ammonium sulfate buffer A. Fractions of 350 drops were collected and the protein concentration of the effluent determined by an LKB UV monitor and chart recorder. The protein peak corresponded to the activity peak (data not shown). The conductivity of collected fractions was measured using a radiometer conductivity meter and ionic strength was determined from a standard curve. Enzymatic activity of fractions was determined by assay in the standard reaction mix containing 4 mM Mn^{++} and $d(A\text{-}T)_n$ as template in the absence of alpha-amanitin. All procedures were carried out in a cold room at 4°C.

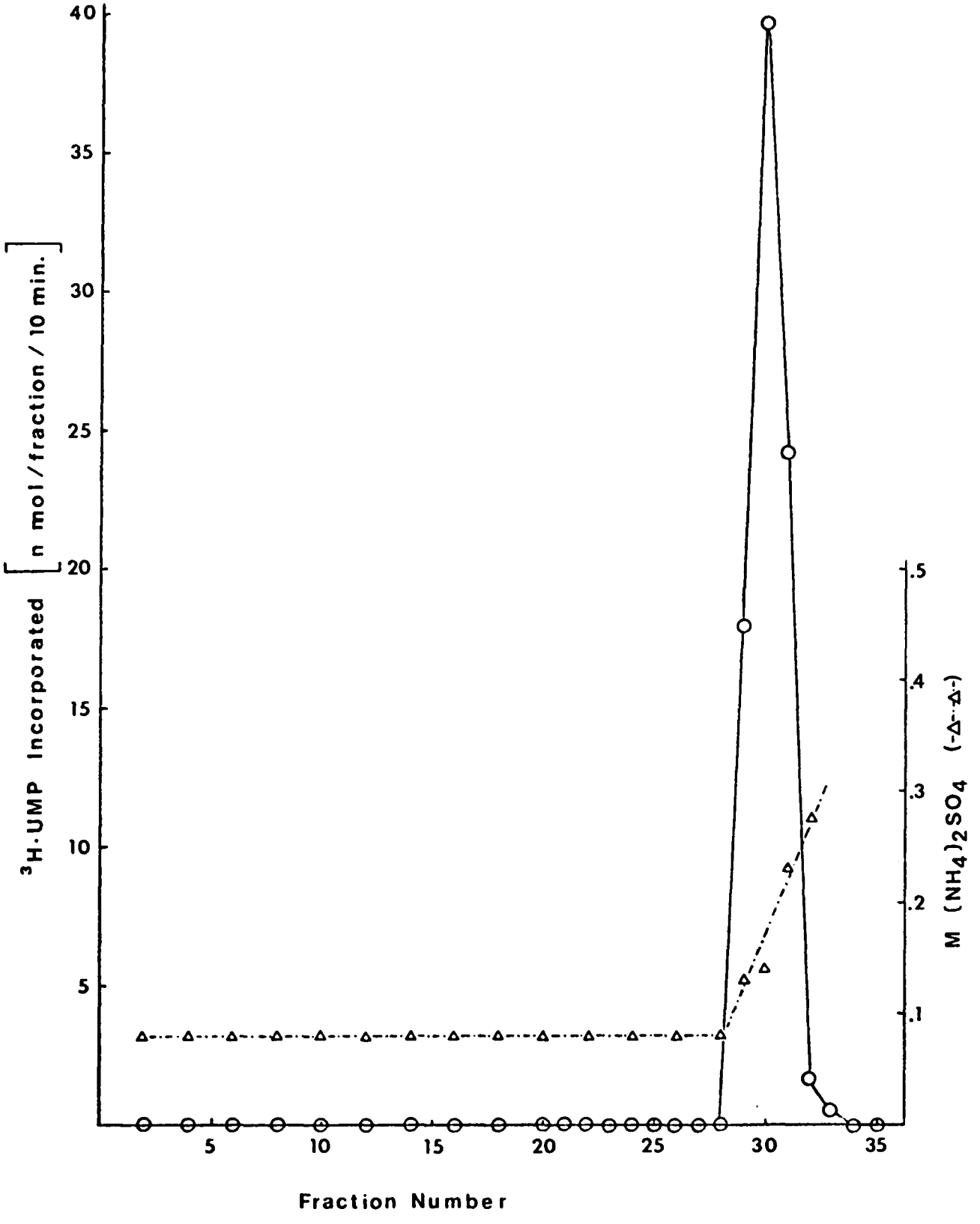

40
35
30
25
20
15
10
5
^{3}H-UMP Incorporated [n mol / fraction / 10 min.]
.5
.4
.3
.2
.1
M $(NH_4)_2SO_4$ (-Δ--Δ-)
5
10
15
20
25
30
35
Fraction Number

Fig. 19. Rechromatography on DNA-cellulose of the Artemia cyst RNA polymerase activity remaining bound to DEAE-cellulose at 0.08 M ammonium sulfate. Fractions 27 - 32 of the DEAE-cellulose column described in the legend to figure 18 were pooled and made 50% saturated with ammonium sulfate. After overnight precipitation at 4°C and centrifugation at 30,000 rpm in a Beckman 35 rotor for 20 min, the pellet was resuspended in buffer B (50% glycerol, no $MgCl_2$, no NaCl) and frozen at -20°C.

The sample contained 400 mg protein and was loaded overnight onto a DNA-cellulose column measuring 3 cm x 27.5 cm and containing 0.37 mg DNA/mg cellulose. The column was washed with 200 ml buffer B (0.15 M NaCl) and the enzyme activity was eluted with buffer B (0.5 M NaCl). Fractions were collected and 30 ul aliquots were assayed for RNA polymerase activity in the standard reaction mix containing 4 mM Mn^{++} and $d(A\text{-}T)_n$ in the presence (-●-●-) and absence (-○-○-) of 8 ug/ml alpha-amanitin. Incubation was at 37°C for 15 min. (·o·o·o·o·) protein concentration as determined by the Lowry method. (·▲·▲·) M NaCl.

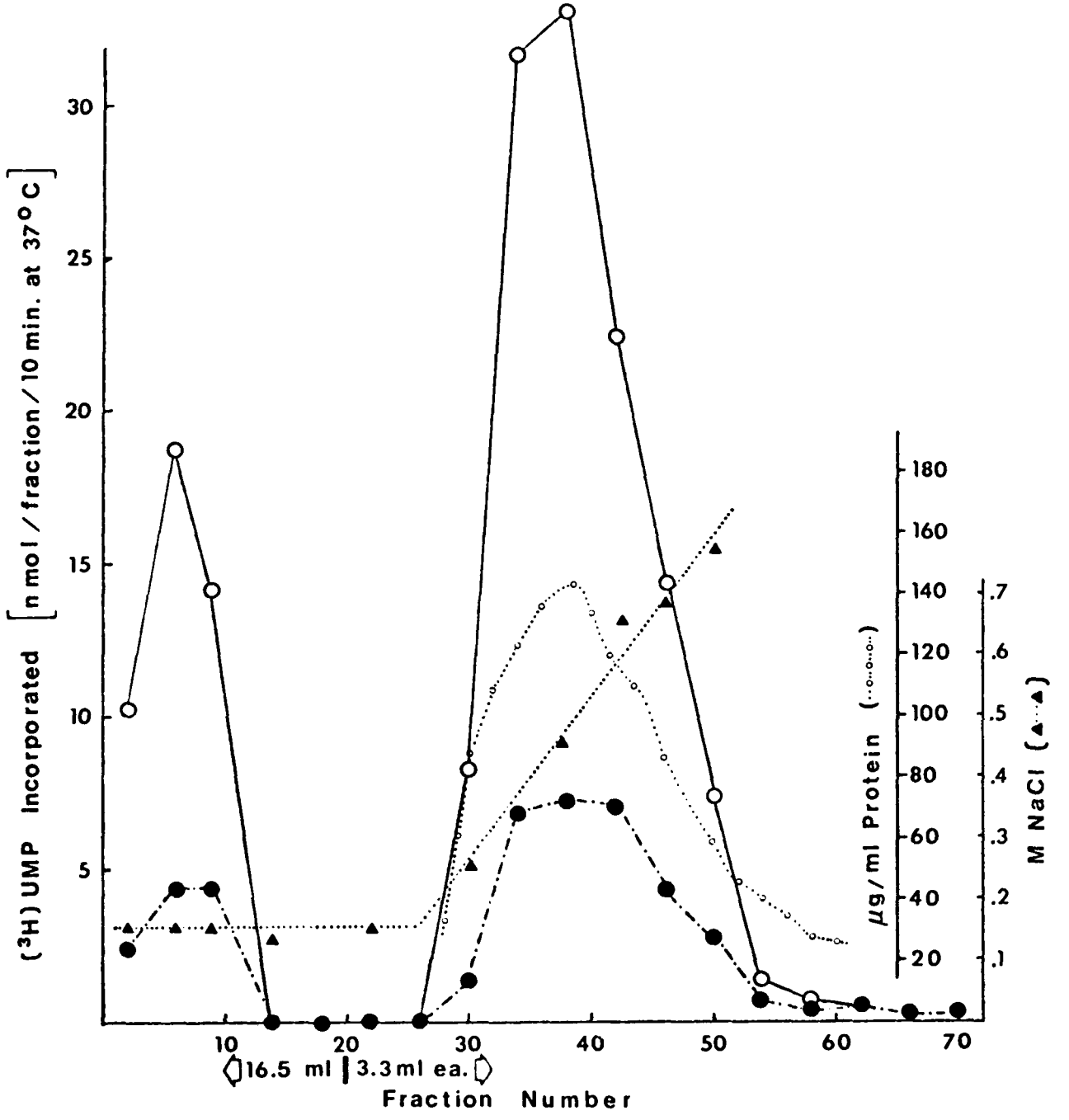
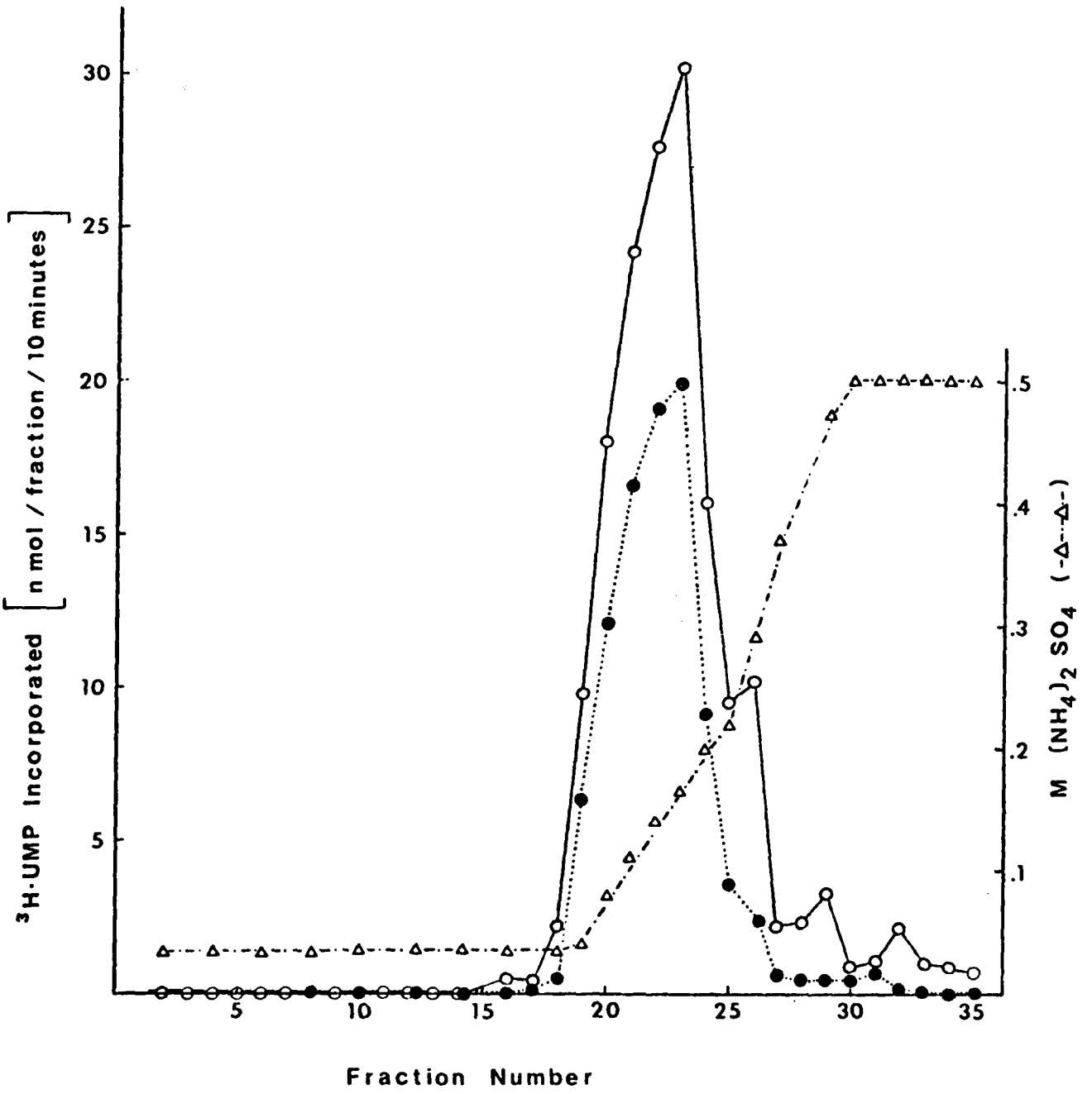

(³H) UMP Incorporated [n mol / fraction / 10 min. at 37° C]
30
25
20
15
10
5
180
160
140
120
100
80
60
40
20
μg/ml Protein (·-o-·-o-·)
.7
.6
.5
.4
.3
.2
.1
M NaCl (▲-·-▲)
10
20
30
40
50
60
70
16.5 ml | 3.3 ml ea.
Fraction Number

Fig. 20. DEAE-cellulose column chromatography of RNA polymerase activity not bound to DEAE-cellulose at low ionic strength. (DEAE-cellulose 2). The pooled F/S fractions of experiments 1 - 3 were mixed with DEAE-cellulose and poured onto a Buchner funnel as described in the text. The flow-through volume was 5.1 liters and had a conductivity indicating 0.045 M ammonium sulfate. This material was found to contain 556 units of polymerase activity with 72% resistance to 8 ug/ml alpha-amanitin.

Buffer A containing no ammonium sulfate was added to lower the ionic strength. When the flow-through fraction volume was 7.8 liters, 83.8 g (dry weight) pre-equilibrated DEAE-cellulose at 0.7 meq/g was stirred into it. After a slow mixing overnight in the cold room, the supernatant contained no enzyme activity. Supernatant conductivity indicated an ionic strength of 0.04 M ammonium sulfate. The DEAE-cellulose was collected in a Buchner funnel and transferred to a column which was washed with buffer A at 0.05 M ammonium sulfate. The polymerase activity was batch eluted with 0.5 M ammonium sulfate buffer A.

Fractions of 18.3 ml/tube were collected and 30 ul aliquots were assayed for RNA polymerase activity in the standard reaction mix with $d(A\text{-}T)_n$ as the template in the presence (··●····●··) and absence (-o——o-) of 8 ug/ml alpha amanitin. (-△-··-△-) indicates the ionic strength of the ammonium sulfate buffer. Conductivity was measured directly using a Radiometer conductivity meter with a flow-through probe. Ionic strength was determined by comparing sample conductivity to a standard curve. All procedures were carried out at 4°C.


30
25
20
15
10
5
5
10
15
20
25
30
35
.5
.4
.3
.2
.1
^{3}H·UMP Incorporated [n mol / fraction / 10 minutes]
M $(NH_4)_2$ SO_4 (-Δ--Δ-)
Fraction Number

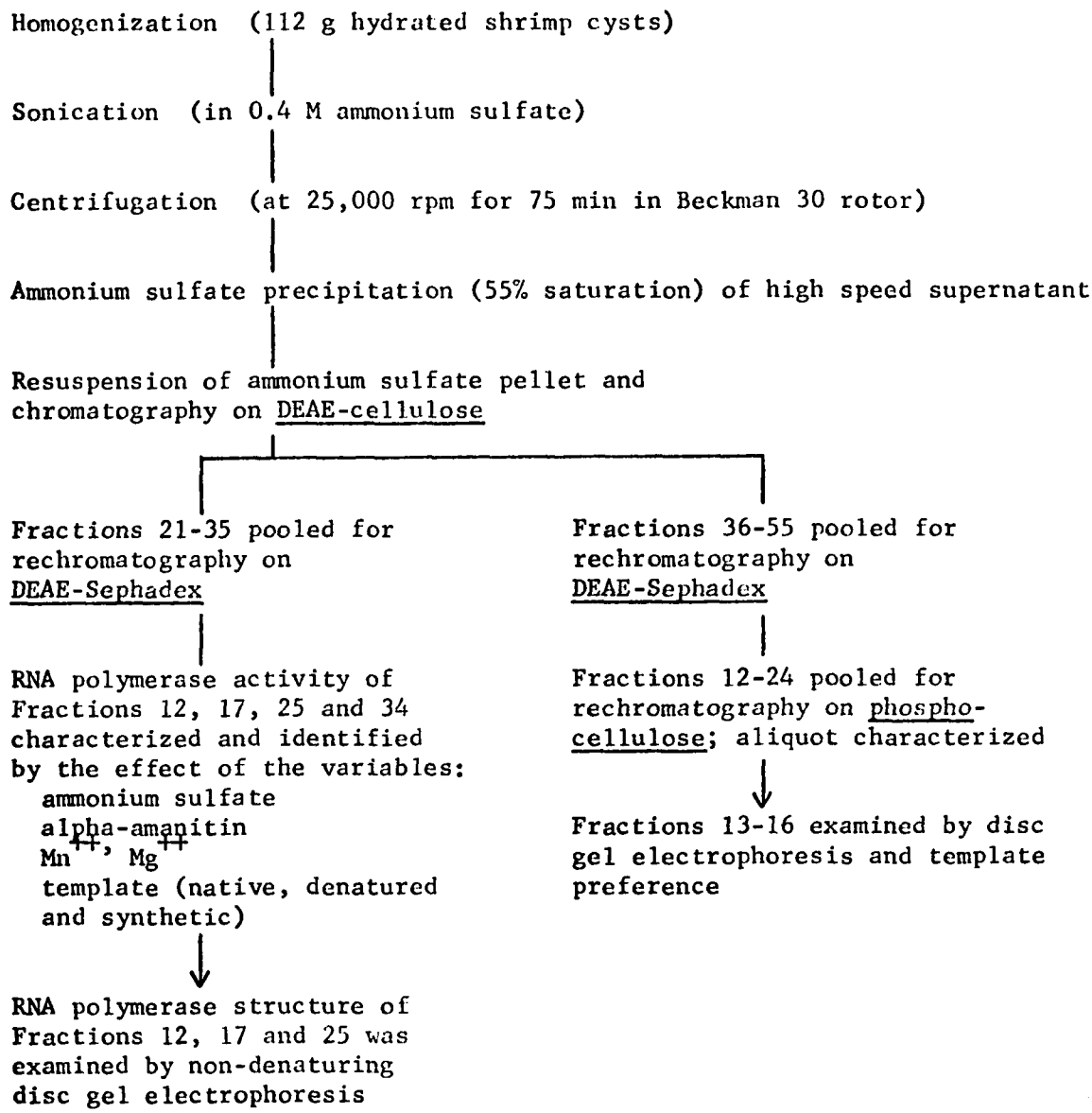


Fig. 21.--Flow diagram of chromatographic separation and subsequent characterization of RNA polymerases from hydrated Artemia cysts

Fig. 22.--DEAE-cellulose chromatography of RNA polymerases from hydrated Artemia cysts. The ammonium sulfate-precipitated protein pellet from the HSS (high speed supernatant) of 112 g (wet weight) hydrated shrimp cysts was resuspended in buffer A with no ammonium sulfate and diluted to 500 ml. At this point, the ammonium sulfate concentration was less than 0.05 M, the total protein was 882 mg and the nucleic acid content was 7.5%.

To eliminate the delay of loading the sample by running it onto the column, DEAE-cellulose (Whatman, fine, 0.7 meq/h, 37 g dry weight) equilibrated with buffer A (0.05 M ammonium sulfate) was mixed into the sample. After 30 min with occasional stirring, the mixture was spun at 5,000 rpm in a Sorvall RC-2b SS-34 rotor for 5 min. The DEAE-cellulose pellets were collected and resuspended in buffer A at 0.05 M ammonium sulfate and spun as described four times before the slurry was transferred to a glass column. The DEAE-cellulose was packed by gravity while the column was running. The bed volume was 74 ml.

The enzyme activity was eluted with buffer A containing a linear gradient of ammonium sulfate. The flow rate was 30 ml/hr and the gradient consisted of 200 ml each of buffer A at 0.05 M and 0.35 M respectively.

Fractions of 5.25 ml each were collected and assayed in the standard reaction mix containing 3 mM Mn^{++} and 3.8 ug CT DNA_{den} in the presence (-●-●-) and absence (-○-○-) of 8 ug/ml alpha-amanitin. Fifty microliter aliquots were incubated for 20 min at 37°C in a final volume of 125 ul. (-o-o-o-o-) A_{280}. (··▲···▲··) M ammonium sulfate.

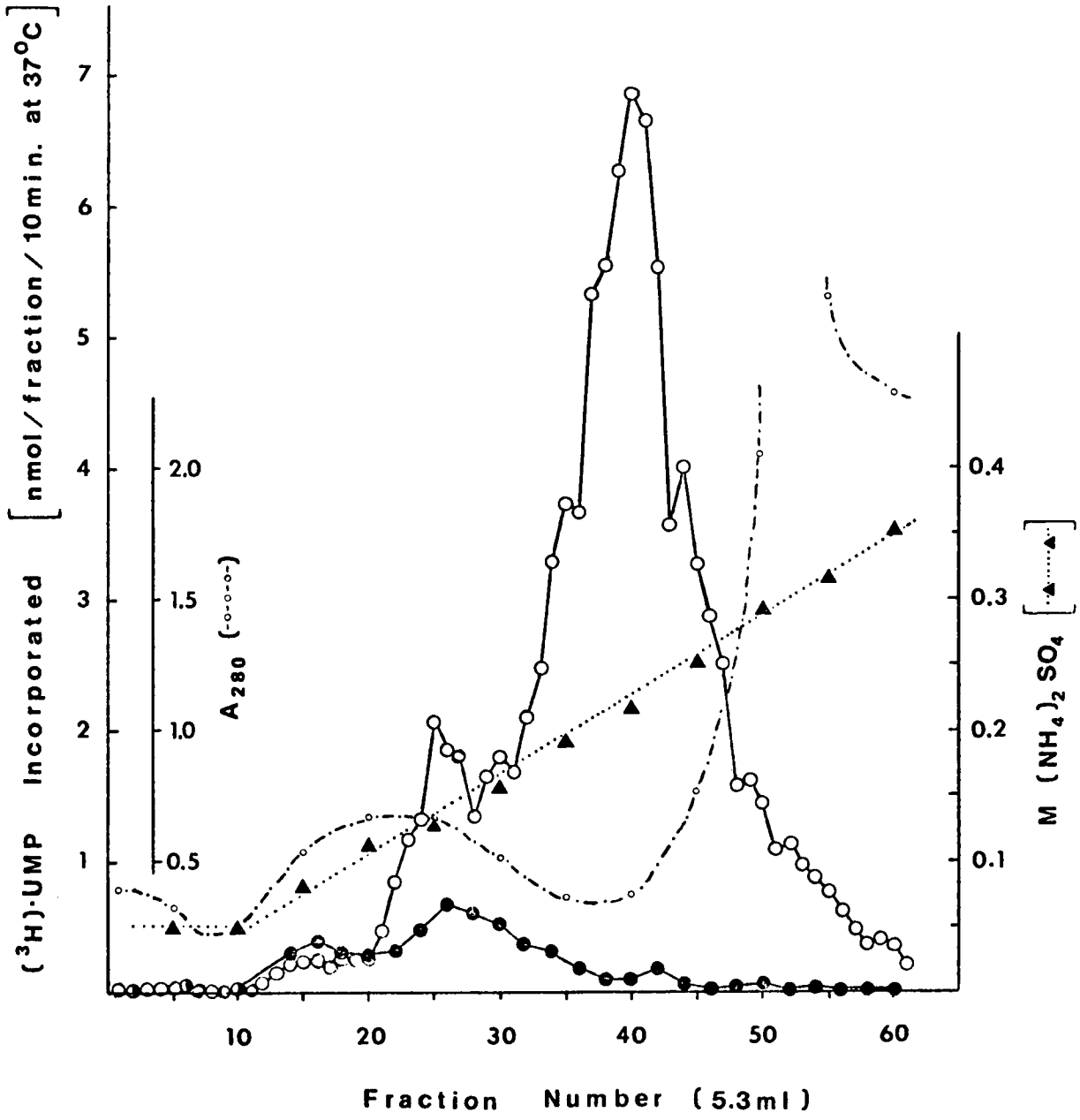

(³H)-UMP Incorporated [nmol/fraction/10min. at 37°C]
A280 (-o-o-o-)
M (NH4)2 SO4
Fraction Number (5.3ml)
1
2
3
4
5
6
7
0.5
1.0
1.5
2.0
0.1
0.2
0.3
0.4
10
20
30
40
50
60

Fig. 23. Rechromatography on DEAE-Sephadex of hydrated Artemia cyst RNA polymerase eluting early from DEAE-cellulose chromatography. Fractions 21 through 35 were pooled (figure 22). The volume was 75 ml, the total protein was 30.8 mg, the A_{280}/A_{260} ratio was 1.13 and the ammonium sulfate concentration was 0.15 M. To reduce the ammonium sulfate concentration to less than 0.05 M the volume was brought to 300 ml with buffer A. Forty milliliters (packed volume) of DEAE-Sephadex equilibrated with 0.05 M ammonium sulfate buffer A was mixed with the sample and, after one and a half hours on ice with occasional stirring, the mixture was poured into a column measuring 1.5 cm inner diameter. The Sephadex was allowed to settle while the column was running. The height of the packed DEAE-Sephadex was 22 cm and the bed volume was 40 ml. The column was washed with 30 ml of buffer A at 0.05 M ammonium sulfate and then the enzyme was eluted with 220 ml of a linear gradient of 0.05 M to 0.50 M ammonium sulfate in buffer A.

Fractions of 4.1 ml were collected and assayed in the presence (-●-●-) and absence (-○-○-) of 4 ug/ml alpha-amanitin. Ammonium sulfate concentration of the fractions was determined by measuring the conductivity of 25 ul aliquots diluted in 5 ml distilled water at 0°C and comparing these values with the same dilutions of known ammonium sulfate concentrations.

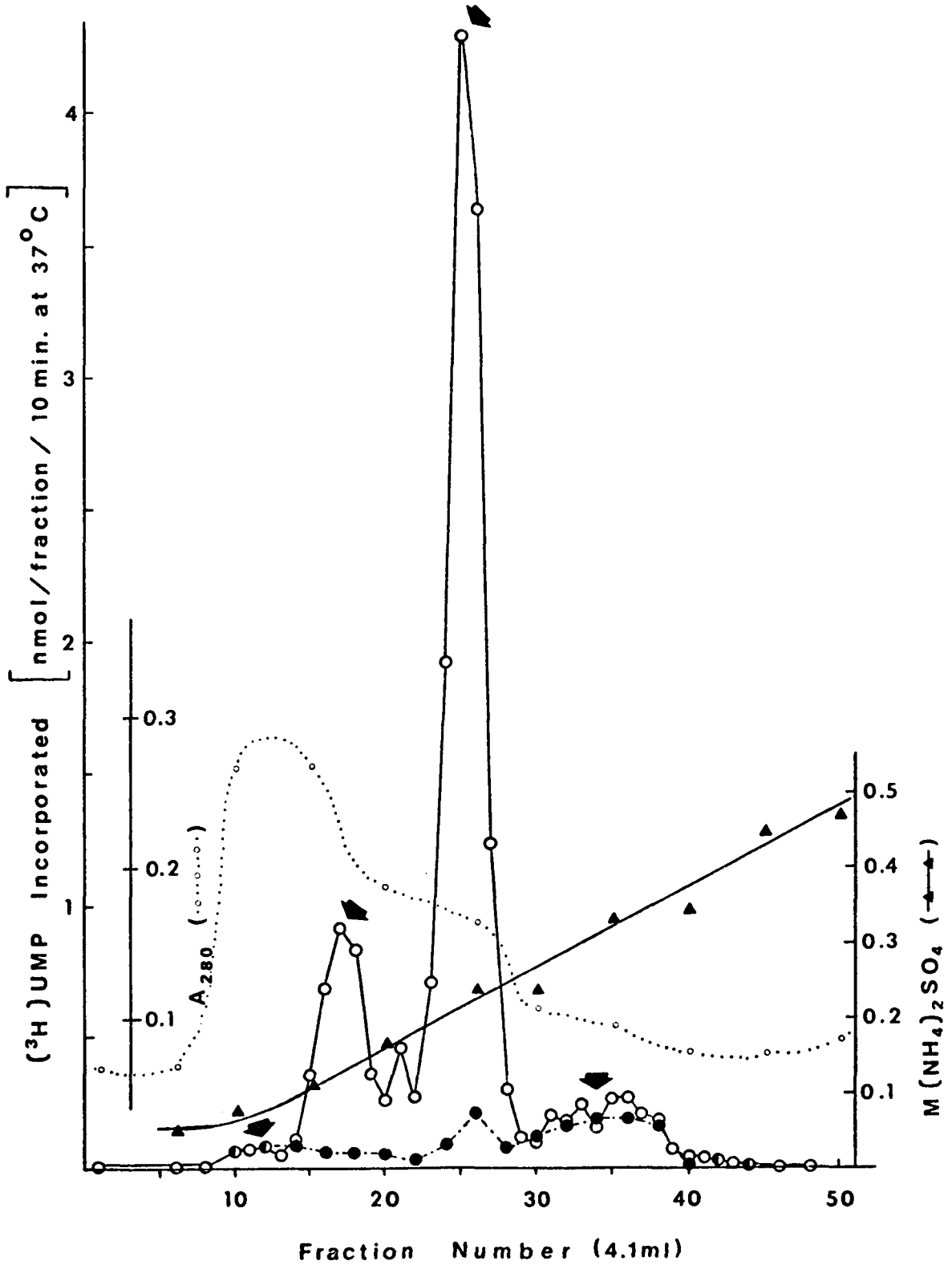

(³H)UMP Incorporated [nmol/fraction / 10 min. at 37°C]
A280 (o.....o)
M (NH4)2SO4 (▲—▲)
Fraction Number (4.1ml)
0.1
0.2
0.3
0.4
0.5
1
2
3
4
10
20
30
40
50

Fig. 24. Rechromatography on DEAE-Sephadex of pooled fractions obtained after DEAE-cellulose chromatography of RNA polymerase activity from hydrated Artemia cysts. The pooled fractions 36 - 55 (the polymerase II region) from the DEAE-cellulose column whose activity profile is shown in figure 22 had a volume of 110 ml and an A_{280} = 1.616 and an A_{260} = 2.778. After overnight dialysis, the sample was mixed with DEAE-Sephadex equilibrated with buffer A at 0.05 M ammonium sulfate. After an hour on ice with occasional stirring, the sample-DEAE-Sephadex mixture was poured into a column with a 1.5 cm inner diameter. The DEAE-Sephadex was packed by gravity with the column running. The height of the packed DEAE-Sephadex was 22 cm and the bed volume was 40 ml. After washing with 40 ml buffer A with 0.05 M ammonium sulfate, a 220 ml linear gradient from 0.05 M to 0.50 M ammonium sulfate in buffer A was applied to the column at the rate of 30 ml/hr. Forty-seven fractions of 4.6 ml each were collected and assayed for RNA polymerase as described in Methods in the presence (-●-●-) and absence (-O-O-) of 4 ug/ml alpha-amanitin.

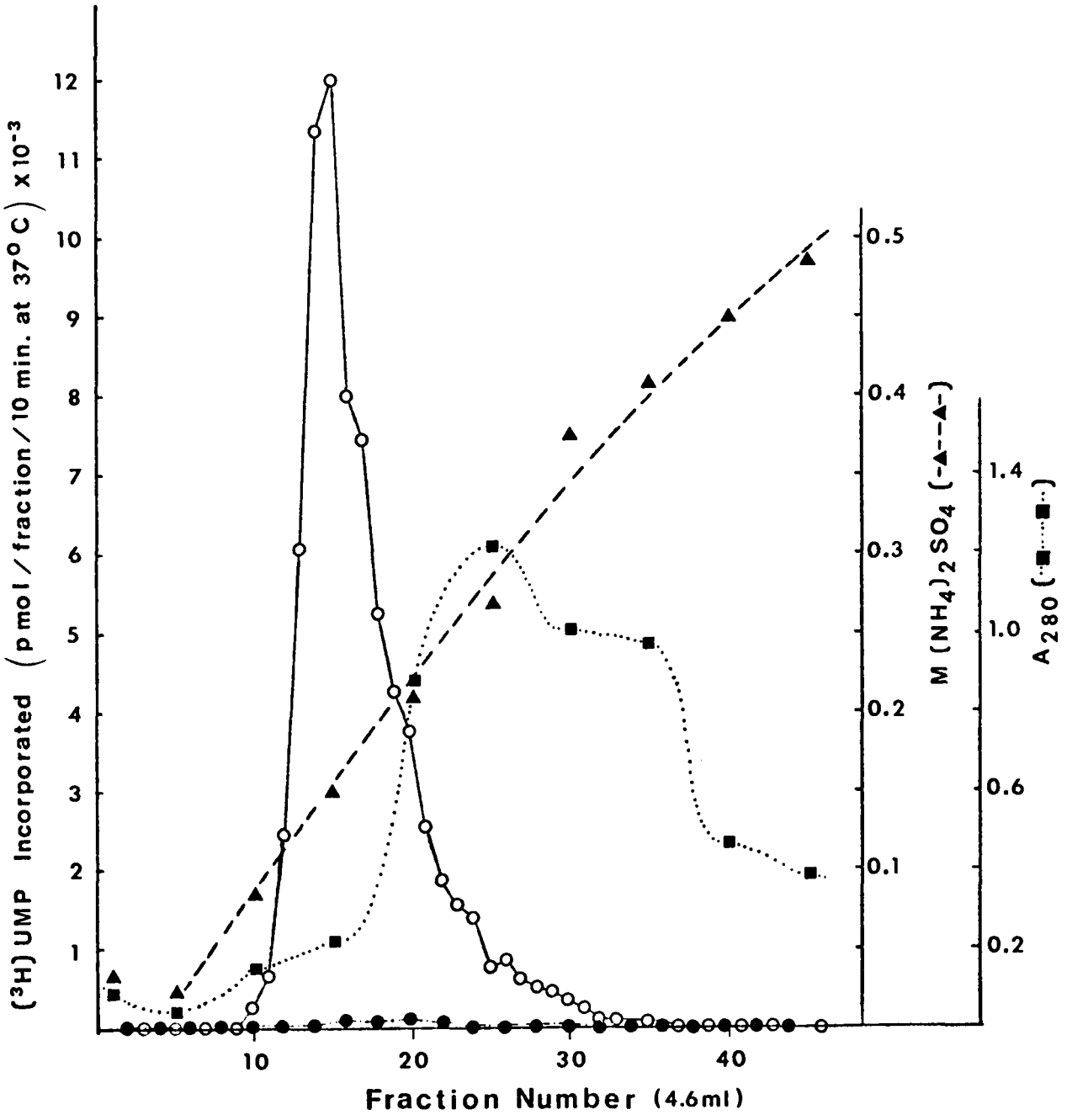

(³H) UMP Incorporated (p mol / fraction / 10 min. at 37° C) x 10⁻³
M (NH4)2 SO4 (-▲--▲-)
A280 (■·····■)
Fraction Number (4.6ml)
10
20
30
40
1
2
3
4
5
6
7
8
9
10
11
12
0.1
0.2
0.3
0.4
0.5
0.2
0.6
1.0
1.4

Fig. 25. Phosphocellulose chromatography of enzyme II activity. Fractions 12 through 24 from the DEAE-Sephadex column whose activity profile is given in figure 24 were pooled and had a volume of 60.5 ml. This material was dialyzed overnight against buffer A with 0.05 M ammonium sulfate and no Mg^{++}. This step reduced the ammonium sulfate concentration as well as removing Mg^{++} which would interfere with the binding of the enzyme to the phosphocellulose. The dialyzed enzyme was mixed with phosphocellulose (Whatman P-11) equilibrated at 0.05 M ammonium sulfate and loaded directly into a column. After settling, the matrix dimensions were 1.5 cm x 12 cm. The column was washed with 5 volumes of buffer A at 0.08 M ammonium sulfate and the enzyme activity was eluted with 0.15 M ammonium sulfate (flow rate throughout was 60 ml/hr). Fractions of 3.3 ml each were collected and assayed for enzyme activity as described. Assays in the presence of alpha-amanitin were not performed since the polymerase activity showed no alpha-amanitin resistance prior to loading on this column.

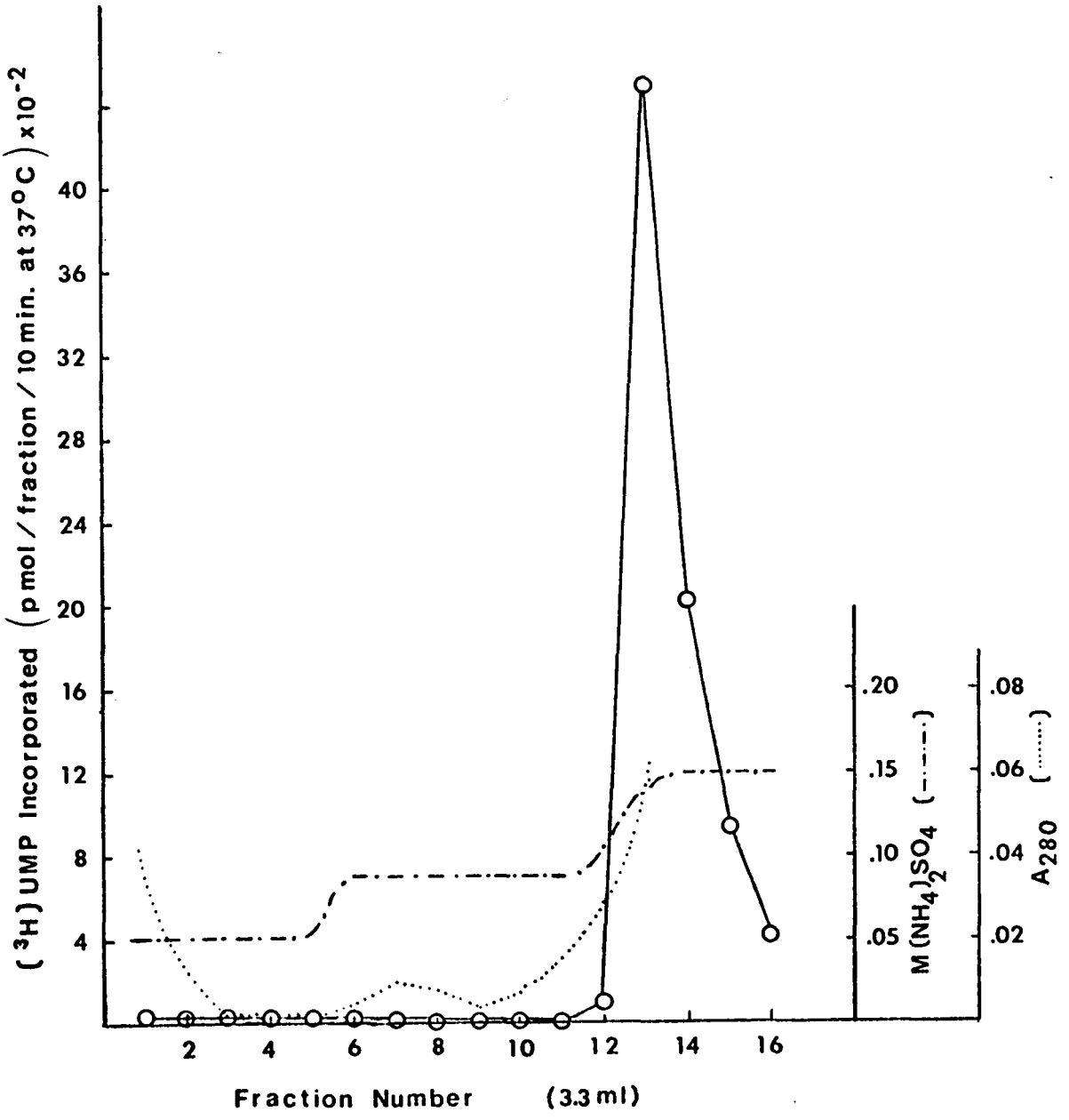

(³H)UMP Incorporated (p mol / fraction / 10 min. at 37°C) x10⁻²
40
36
32
28
24
20
16
12
8
4
2
4
6
8
10
12
14
16
Fraction Number (3.3 ml)
.20
.15
.10
.05
M (NH4)2SO4 (-·-·-)
.08
.06
.04
.02
A280 (·····)

Fig. 26. Alpha-amanitin resistance of individual fractions collected after DEAE-Sephadex column chromatography. The individual fractions obtained by DEAE-Sephadex column chromatography (figure 23) of the pooled fractions 21-35 from the DEAE-cellulose column whose activity profile is presented as figure 22 were assayed for RNA polymerase activity in the absence and presence of 4 ug/ml alpha-amanitin. In this figure, the percent resistance represents the percent of the total RNA polymerase activity remaining in the presence of this concentration of inhibitor.

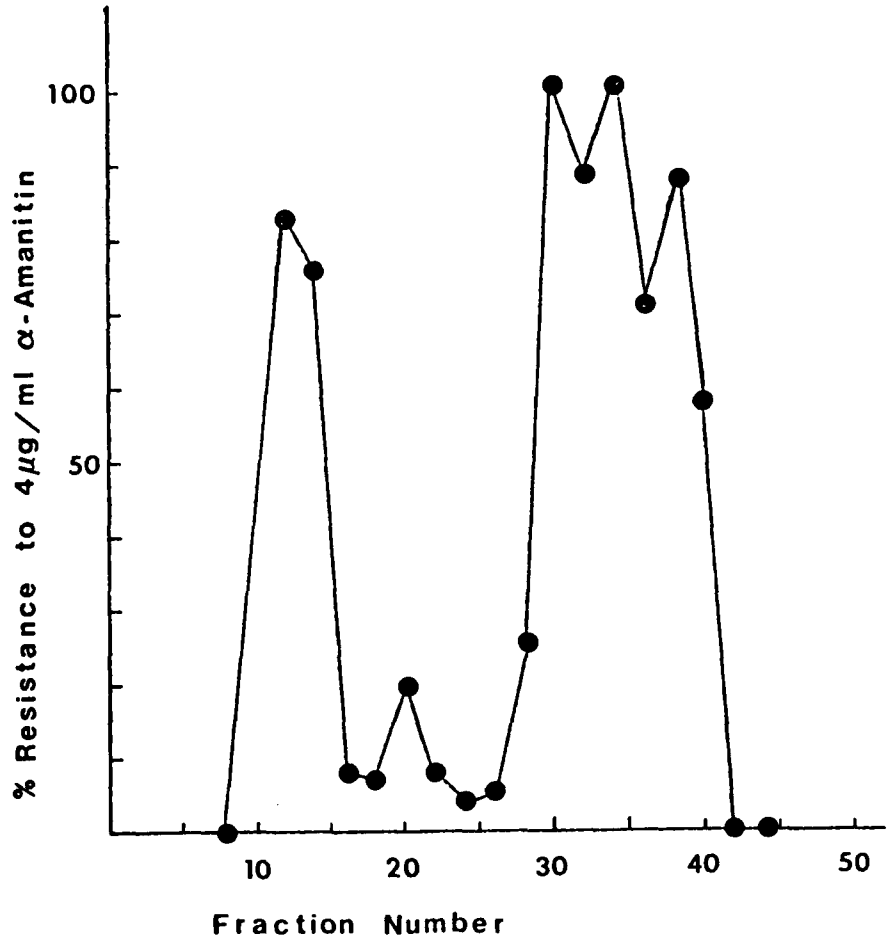

100
50
% Resistance to 4µg/ml α-Amanitin
10
20
30
40
50
Fraction Number

Fig. 27.--Effect of alpha-amanitin on the RNA polymerase activity of fraction 17 of the DEAE-Sephadex column shown in figure 23. RNA polymerase activity was measured in the standard reaction mix containing 50 ul enzyme, 2 mM Mn^{++}, 0.025 M ammonium sulfate and 3 ug CT DNA_{den}. The concentration of alpha-amanitin was varied as indicated. Incubation was for 20 min at 37°C. The results are presented as the percentage of the activity measured without alpha-amanitin. Each point is the average of two determinations. 100% activity corresponds to 373 cpm.

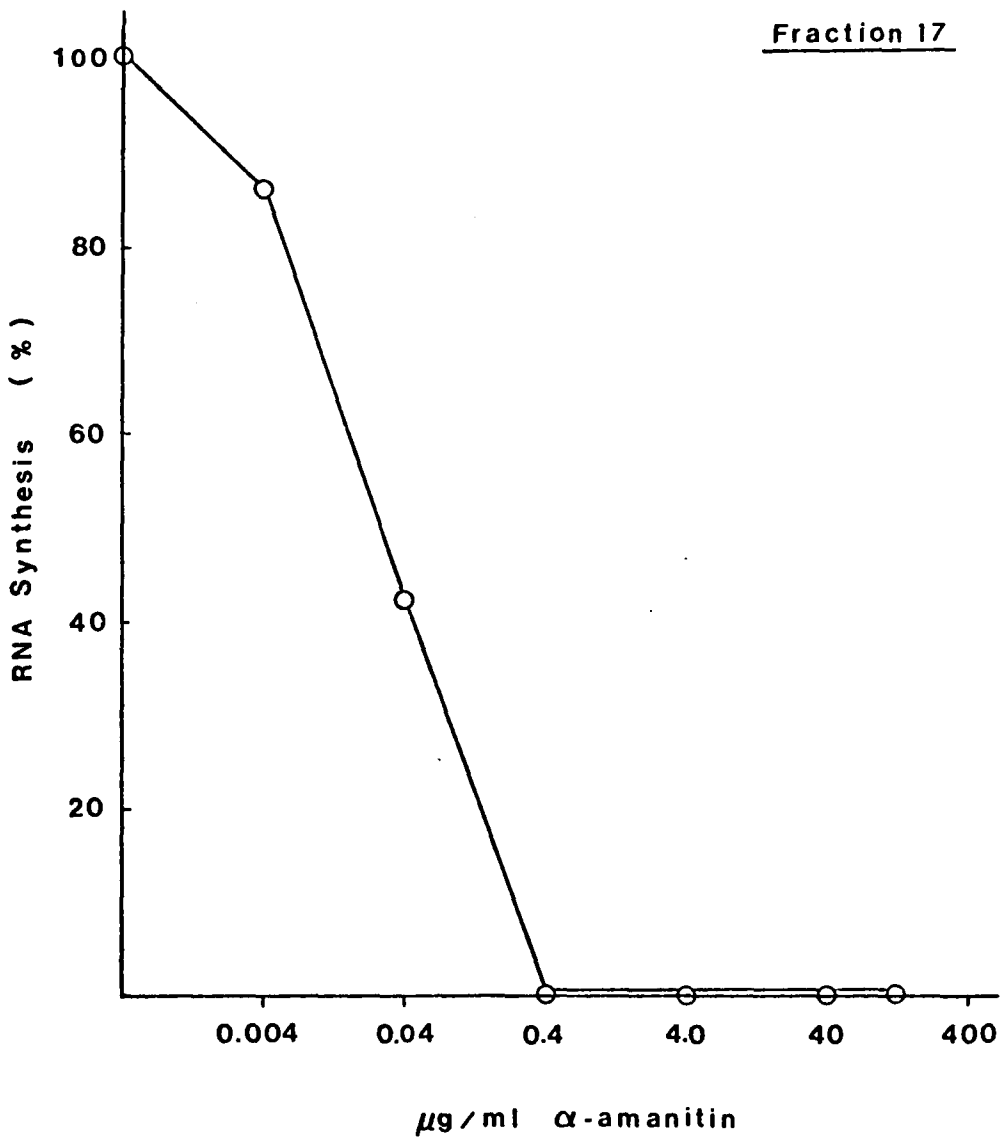

Fraction 17
100
80
60
40
20
RNA Synthesis (%)
0.004
0.04
0.4
4.0
40
400
µg/ml α-amanitin

Fig. 28.--Effect of alpha-amanitin on the RNA polymerase activity of fraction 25 of the DEAE-Sephadex column shown in figure 23. RNA polymerase activity was measured in the standard reaction mix containing 50 ul enzyme, 2 mM Mn^{++}, 0.025 M ammonium sulfate and 3 ug CT DNA_{den}. The concentration of alpha-amanitin was varied as indicated. Incubation was for 20 min at 37°C. The results are presented as the percentage of the activity measured without alpha-amanitin. Each point is the average of two determinations. 100% activity corresponds to 880 cpm.

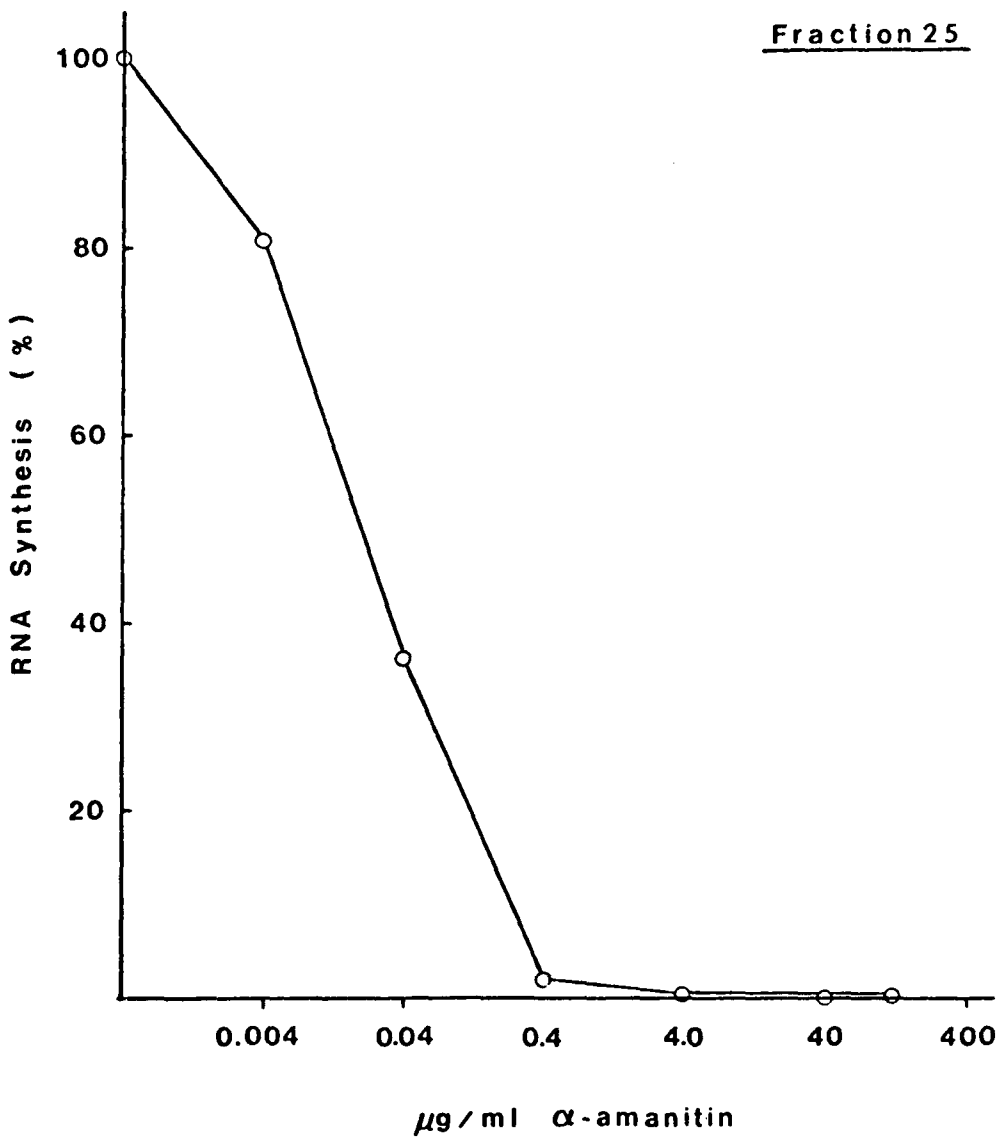

Fraction 25
100
80
60
40
20
RNA Synthesis (%)
0.004
0.04
0.4
4.0
40
400
μg / ml α-amanitin

Fig. 29. Effect of alpha-amanitin on the RNA polymerase activity of fraction 34 of the DEAE-Sephadex column shown in figure 23. RNA polymerase activity was measured in the standard reaction mix containing 100 ul enzyme, 2 mM Mn^{++} and 10 ug $d(A\text{-}T)_n$ in a final volume of 250 ul. Since these assays were performed on an undialyzed aliquot of this fraction, and the column elution position was at 0.32 M ammonium sulfate, the final concentration in the reaction mixture was 0.13 M ammonium sulfate. The concentration of alpha-amanitin was varied as indicated. Incubation was for 20 min at 37°C. The results are presented as the percentage of the activity measured without alpha-amanitin. Each point is the result of a single determination. 100% activity corresponds to 264 cpm.

Fraction 34

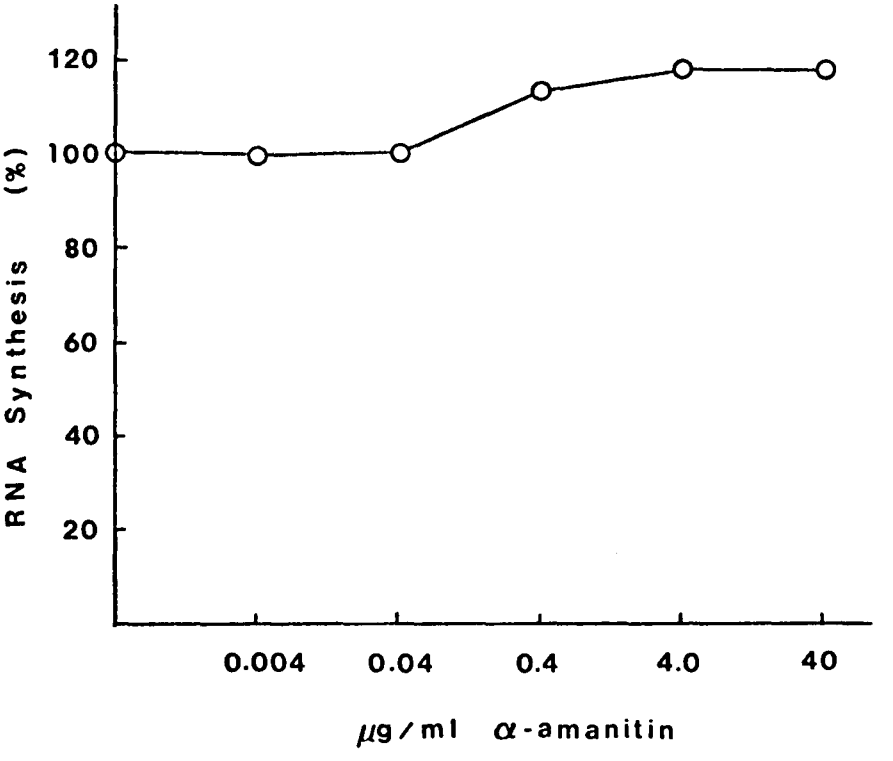

120
100
80
60
40
20
RNA Synthesis (%)
0.004
0.04
0.4
4.0
40
μg / ml α-amanitin

Fig. 30. Comparison of the alpha-amanitin inhibition curves of Artemia salina polymerases IIA and IIB with the inhibition curves of polymerase II from HeLa cells, Drosophila melanogaster and Saccharomyces cerevisiae and polymerase III from HeLa cells. 100% activity corresponds to 373 cpm in the case of Artemia salina polymerase IIA and 1,019 cpm for Artemia salina polymerase IIB.

(■—■) HeLa cell polymerase II. [Hossenlopp, Wells and Chambon 1975]

(□—□) HeLa cell polymerase II. [Seifart and Benecke 1975]

(·■··■·) HeLa call polymerase III. [Hossenlopp, Wells and Chambon 1975]

(·□··□·) HeLa cell polymerase III. [Seifart and Benecke 1975]

(△—△) Drosophila polymerase II. [Greenleaf and Bautz 1975]

(▲—▲) Saccharomyces polymerase II. [Greenleaf and Bautz 1975]

(●—●) Artemia polymerase IIA. Fraction 17 from figure 23.

(○—○) Artemia polymerase IIB. Fraction 25 from figure 23.

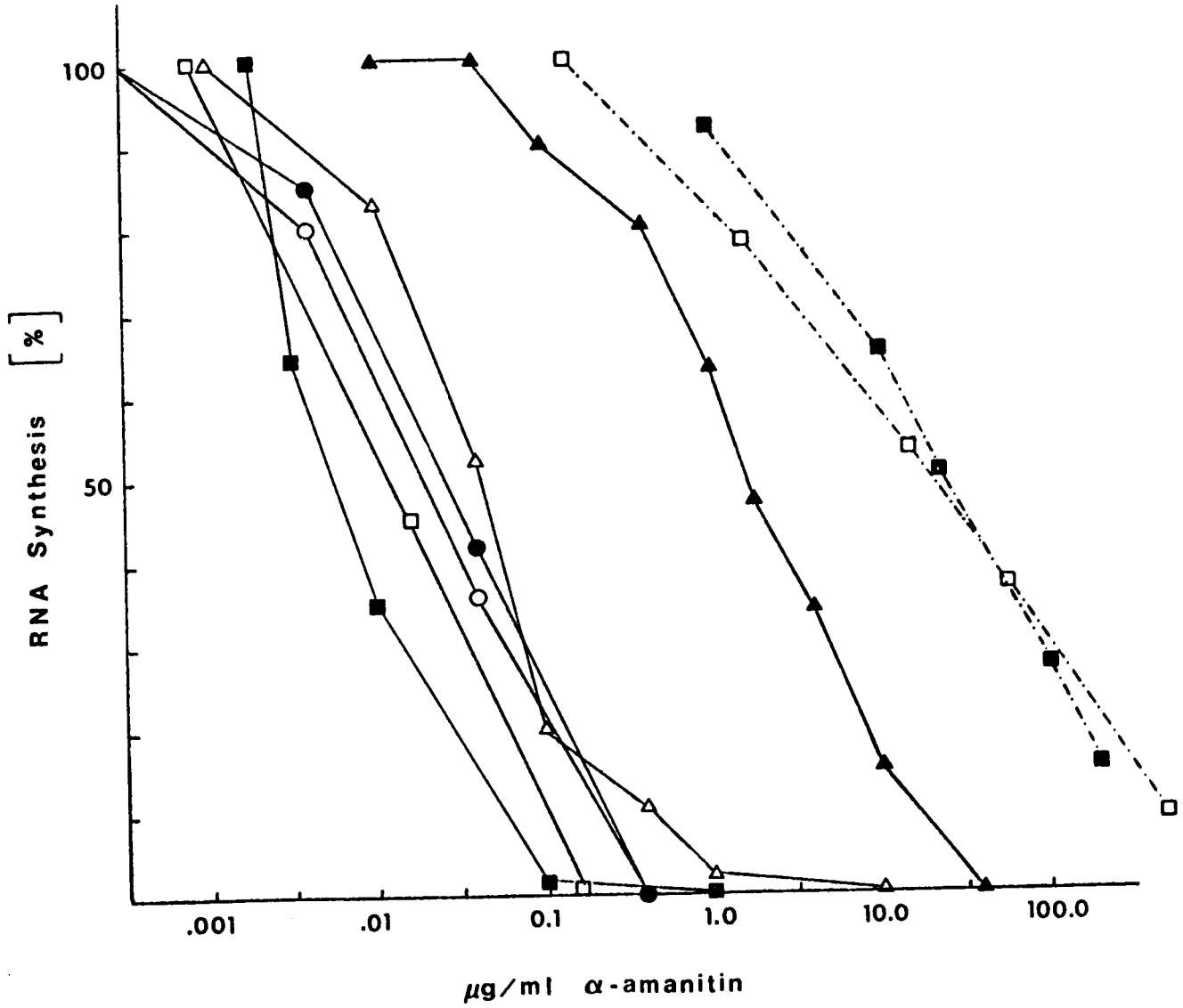

100
50
RNA Synthesis [%]
.001
.01
0.1
1.0
10.0
100.0
μg/ml α-amanitin

Fig. 31. Effect of ammonium sulfate concentration on the RNA polymerase activity of fraction 12 from the DEAE-Sephadex column shown in figure 23. The standard reaction mix contained 50 ul enzyme and either 5 ug $d(A\text{-}T)_n$ (-■-■-) or 3 ug CT DNA_{den} (-□-□-). Mn^{++} concentration was 2 mM and the ionic strength was varied as indicated. Incubation was for 20 min at 37°C. Each point was the result of a single determination.

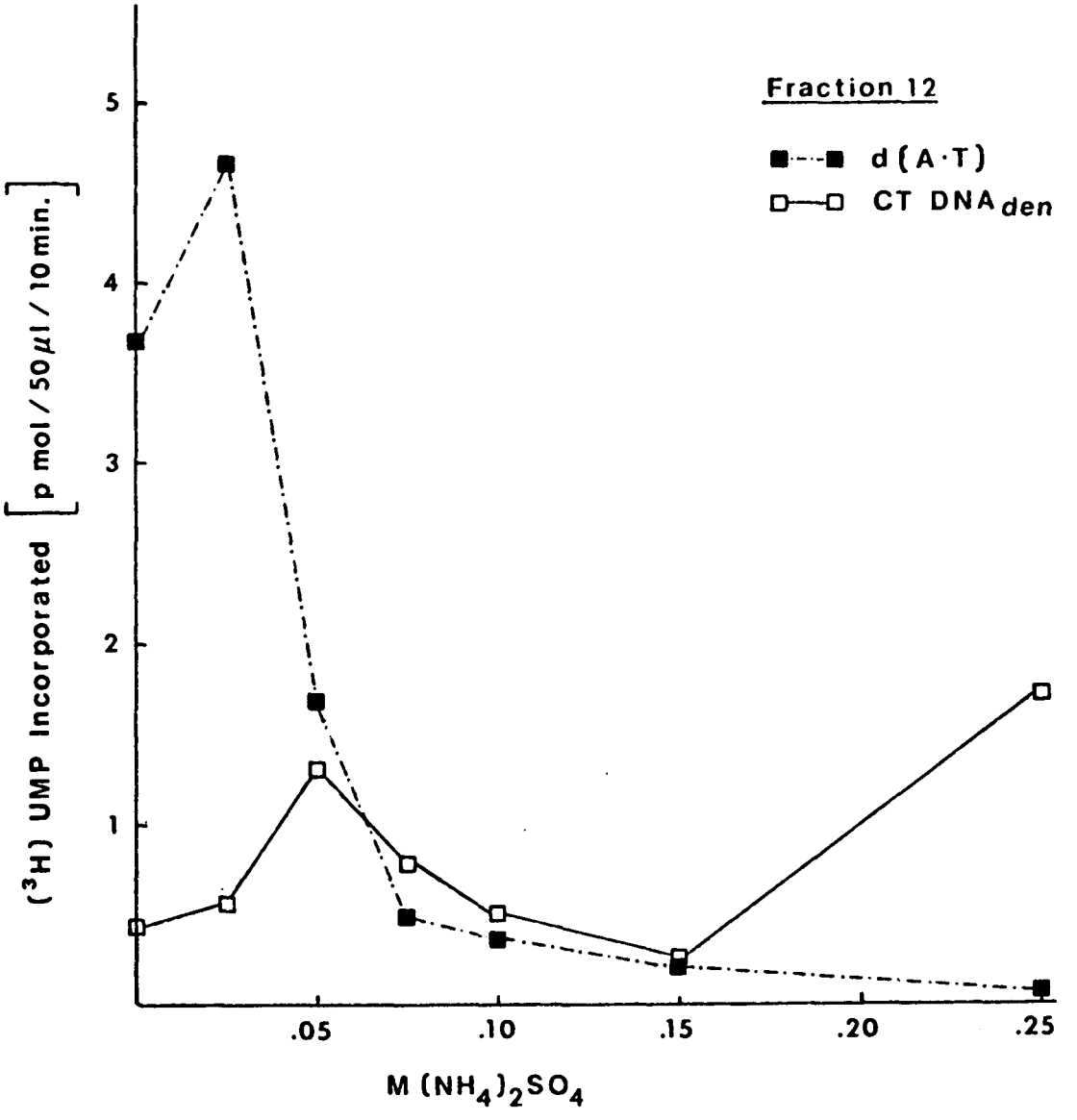

Fraction 12
d(A·T)
CT DNA den
(³H) UMP Incorporated [p mol / 50 µl / 10 min.]
5
4
3
2
1
.05
.10
.15
.20
.25
M (NH4)2SO4

Fig. 32. Effect of ammonium sulfate concentration on the RNA polymerase activity of fraction 17 from the DEAE-Sephadex column shown in figure 23. The standard reaction mix contained 50 ul enzyme and 3 ug CT DNA_{den}. Mn^{++} concentration was 2 mM and the ionic strength was varied as indicated. Incubation was for 20 min at 37°C. Each point was the result of two determinations.

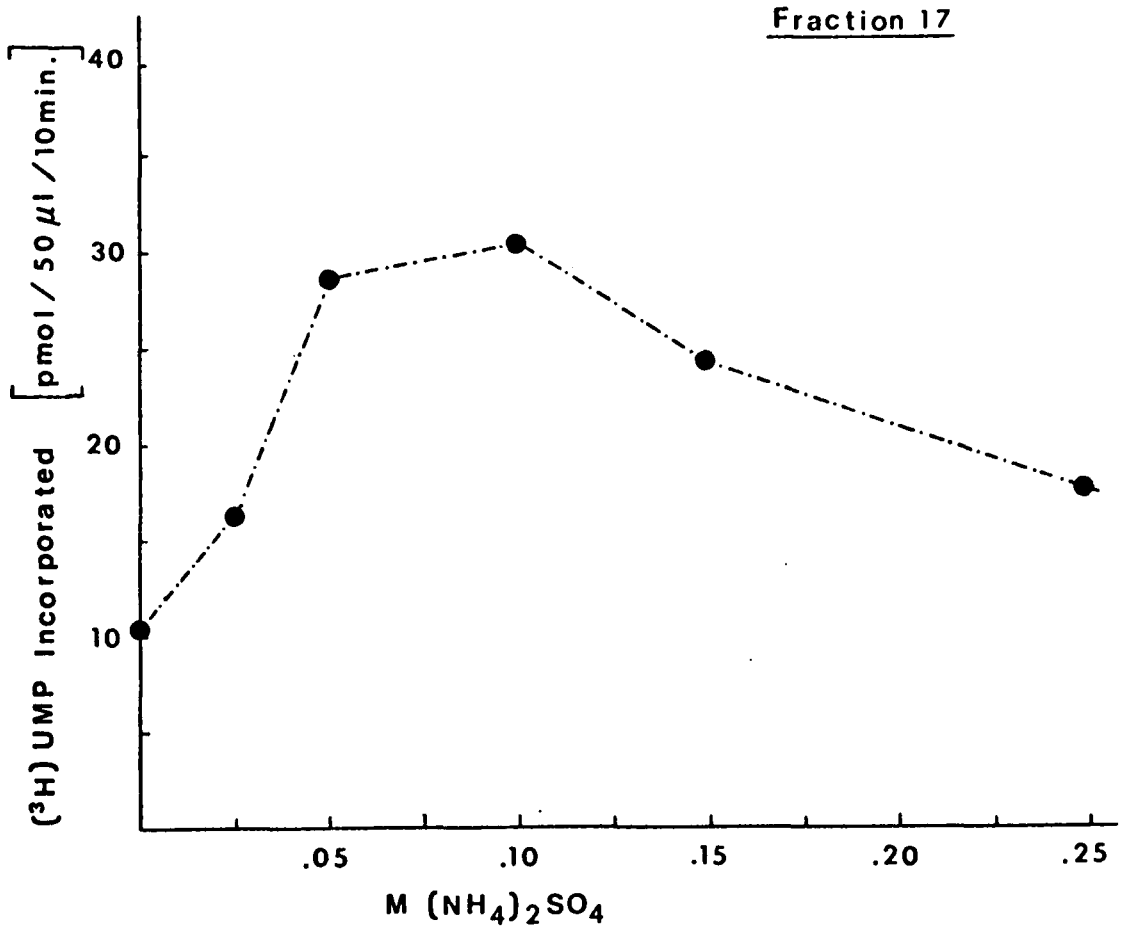

Fraction 17
(³H) UMP Incorporated [pmol / 50 µl / 10min.]
40
30
20
10
.05
.10
.15
.20
.25
M (NH4)2SO4

Fig. 33. Effect of ammonium sulfate concentration on the RNA polymerase activity of fraction 25 from the DEAE-Sephadex column whose activity profile is given in figure 23. The standard reaction mix contained 50 ul enzyme, 3 ug CT DNA_{den} and 2 mM Mn^{++}. Ionic strength was varied as indicated. Incubation was for 20 min at 37°C. Each point is the average of two determinations.

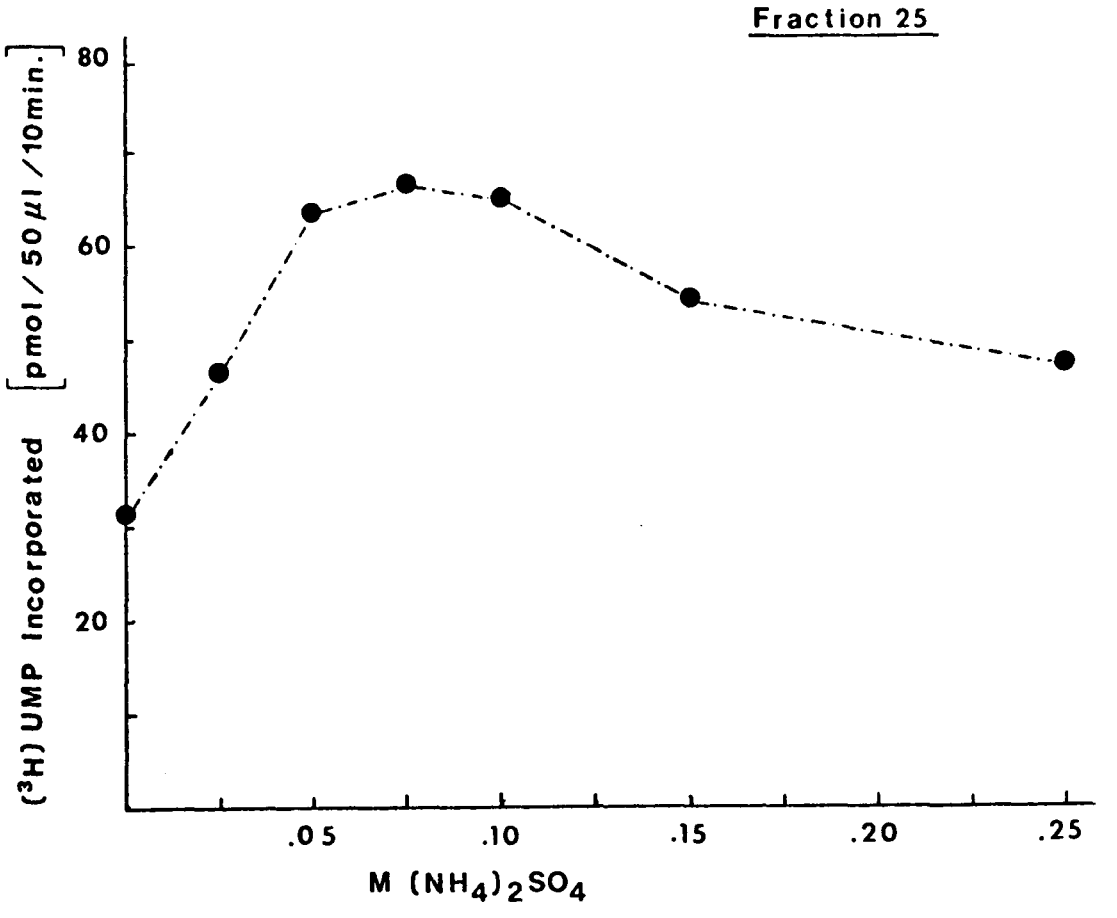

Fraction 25
(³H) UMP Incorporated [pmol / 50 µl / 10min.]
80
60
40
20
.05
.10
.15
.20
.25
M (NH4)2SO4

Fig. 34. Effect of ammonium sulfate concentration on the RNA polymerase activity of fraction 34 from the DEAE-Sephadex column shown in figure 23. The standard reaction mix contained 50 ul enzyme and either 5 ug $d(A\text{-}T)_n$ (-■-■-) or 3 ug CT DNA_{den} (-□-□-). Mn^{++} concentration was 2 mM and the ionic strength was varied as indicated. Incubation was for 20 min at 37°C. Each point is the result of a single determination.

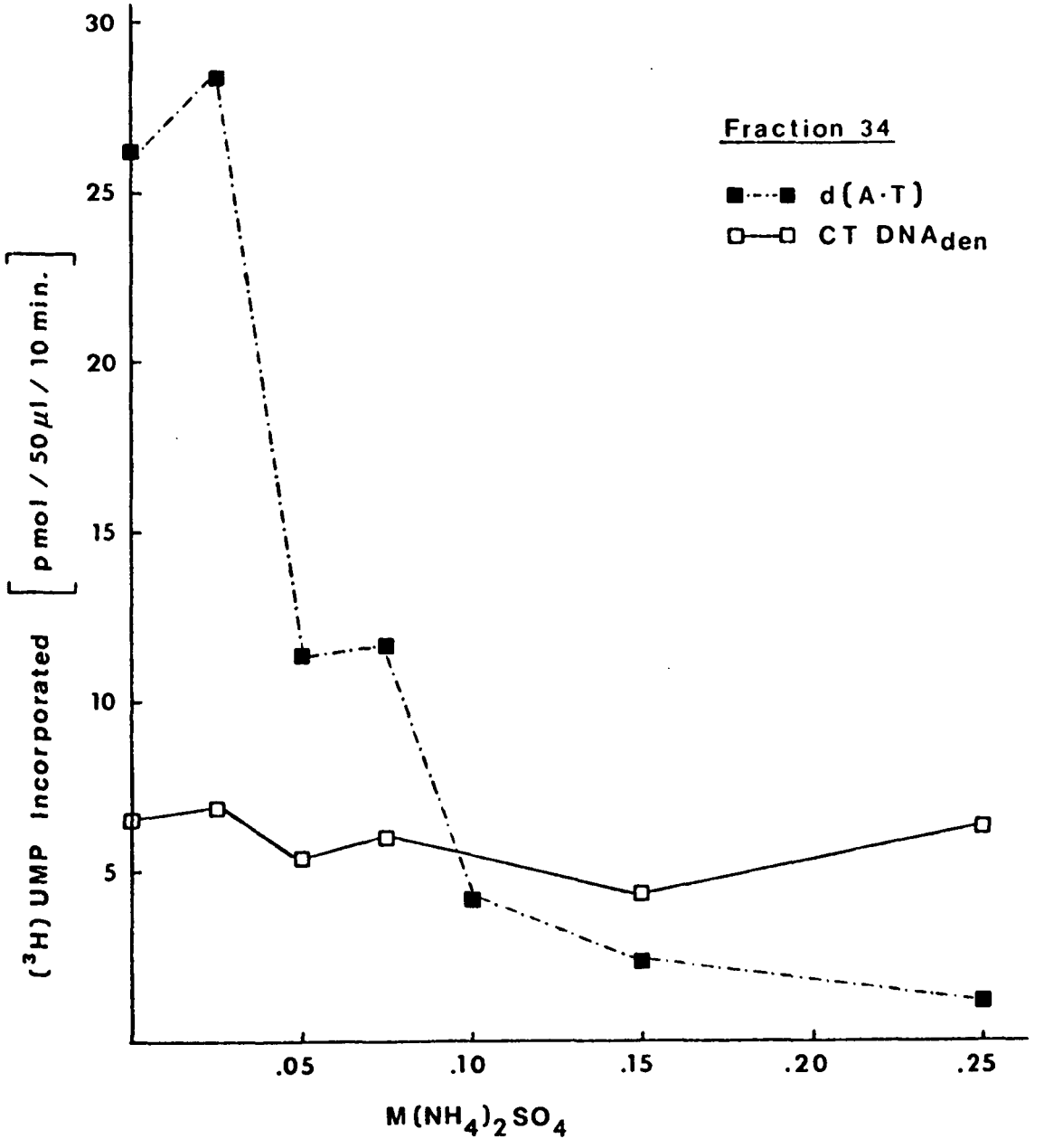
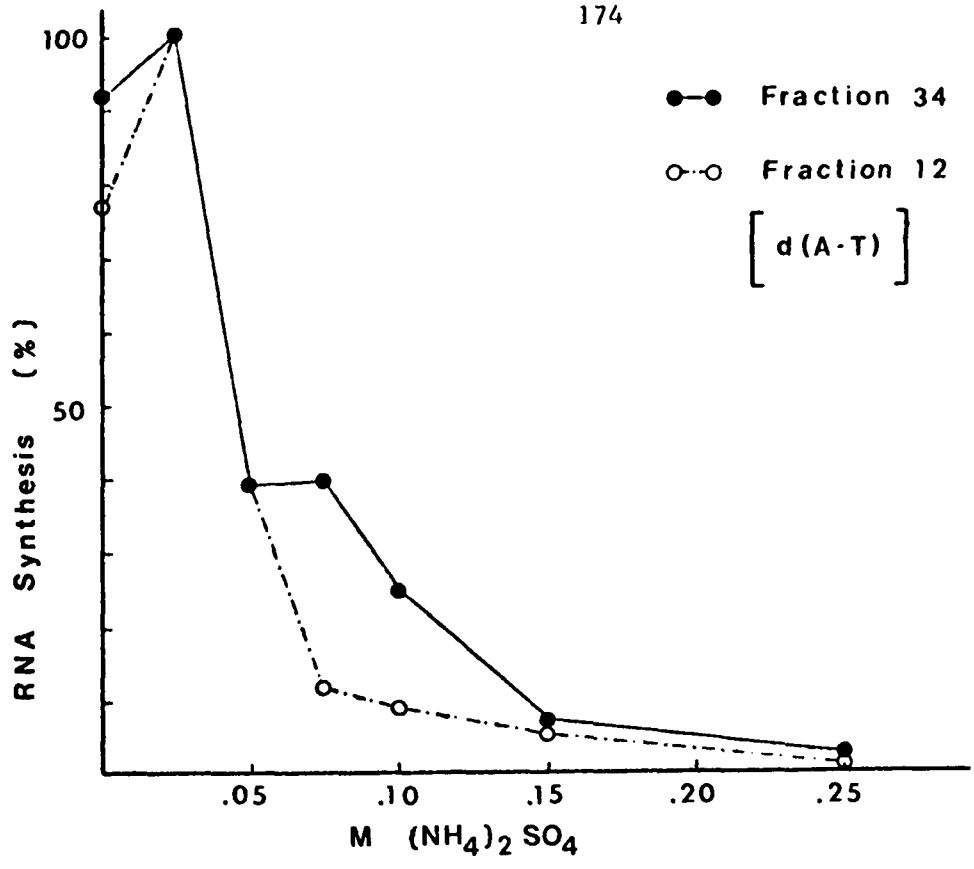

Fraction 34
d(A·T)
CT DNAden
(3H) UMP Incorporated [p mol / 50 µl / 10 min.]
30
25
20
15
10
5
.05
.10
.15
.20
.25
M (NH4)2 SO4

Fig. 35. A comparison of the effects of ionic strength on fractions 12, 17, 25 and 34 from the DEAE-Sephadex column shown in figure 23. A. A comparison of the effect of ammonium sulfate concentration on the RNA polymerase activity of fraction 12 (-O-O-) and fraction 34 (-●—●-). In this comparison, the optimum ammonium sulfate concentration is 0.025 M so the RNA polymerase activity at this ionic strength is taken to be 100%. The activity of these enzyme fractions at other ionic strengths are presented in terms of the optimum (0.025 M) ionic strengths. (See figures 31 and 34) B. A comparison of the effect of ammonium sulfate concentration on the RNA polymerase activity of fraction 17 (-Δ-Δ-) and 25 (-▲—▲-). In this comparison, the activities at various ionic strengths are presented in terms of the activity at 0.075 M ammonium sulfate which is taken to be 100% activity. (See figures 32 and 33)


100
50
RNA Synthesis (%)
Fraction 34
Fraction 12
[d(A-T)]
.05
.10
.15
.20
.25
M (NH4)2 SO4

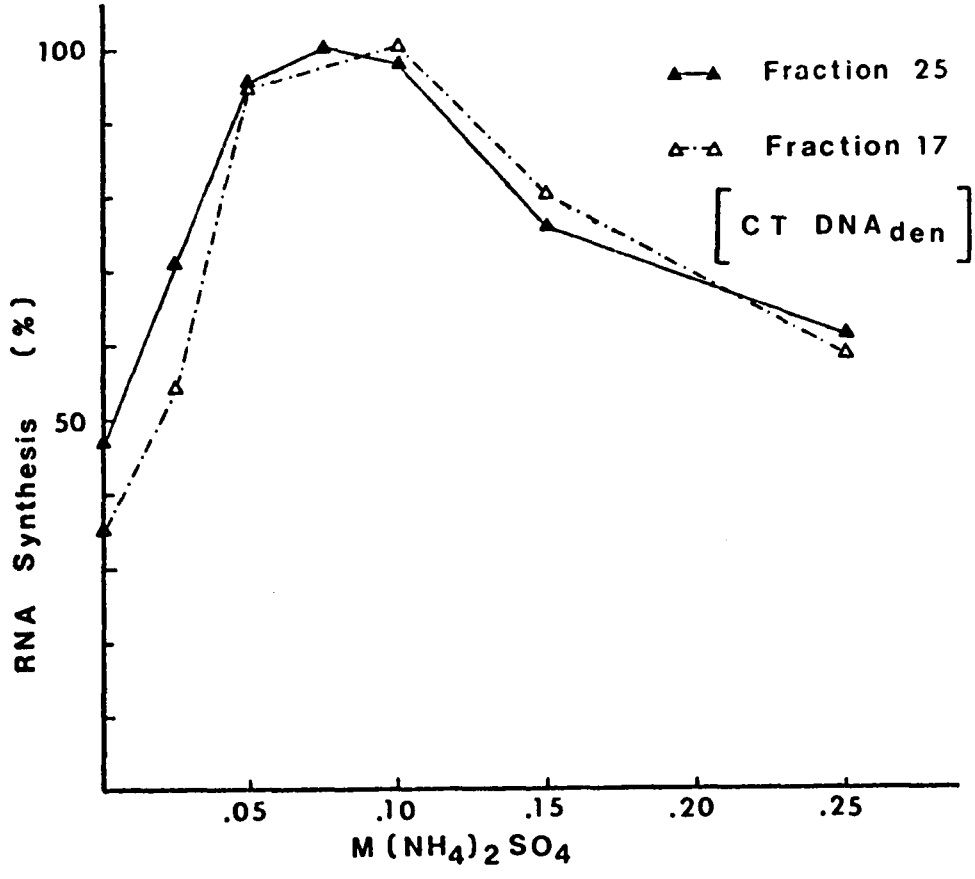

100
50
RNA Synthesis (%)
Fraction 25
Fraction 17
[CT DNA den]
.05
.10
.15
.20
.25
M (NH4)2 SO4

Fig. 36. The effect of metal ion cofactor concentration and template on the RNA polymerase activity of fraction 12 of the DEAE-Sephadex column shown in figure 23. The RNA polymerase activity of fraction 12 was measured in the standard reaction mix containing 50 ul enzyme and either 3 ug CT DNA_{den} or 5 ug $d(A\text{-}T)_n$. The metal ion cofactor was Mn^{++} or Mg^{++} and the concentrations were varied as shown. The RNA polymerase activity was tested under the four combinations of template and cofactor, and since low concentrations of Mn^{++} in the presence of $d(A\text{-}T)_n$ had the most stimulating effect, all data is presented in terms of percent of the optimum activity. This optimum was obtained with $d(A\text{-}T)_n$ and 2 mM Mn^{++}. Ammonium sulfate concentration was 0.025 M. Each point is the result of a single determination. 100% activity corresponds to 1,093 cpm.

(-●—●-) Mn^{++}, CT DNA_{den}

(-·O-·O-·) Mn^{++}, $d(A\text{-}T)_n$

(-▲—▲-) Mg^{++}, CT DNA_{den}

(-·Δ-·Δ-·) Mg^{++}, $d(A\text{-}T)_n$

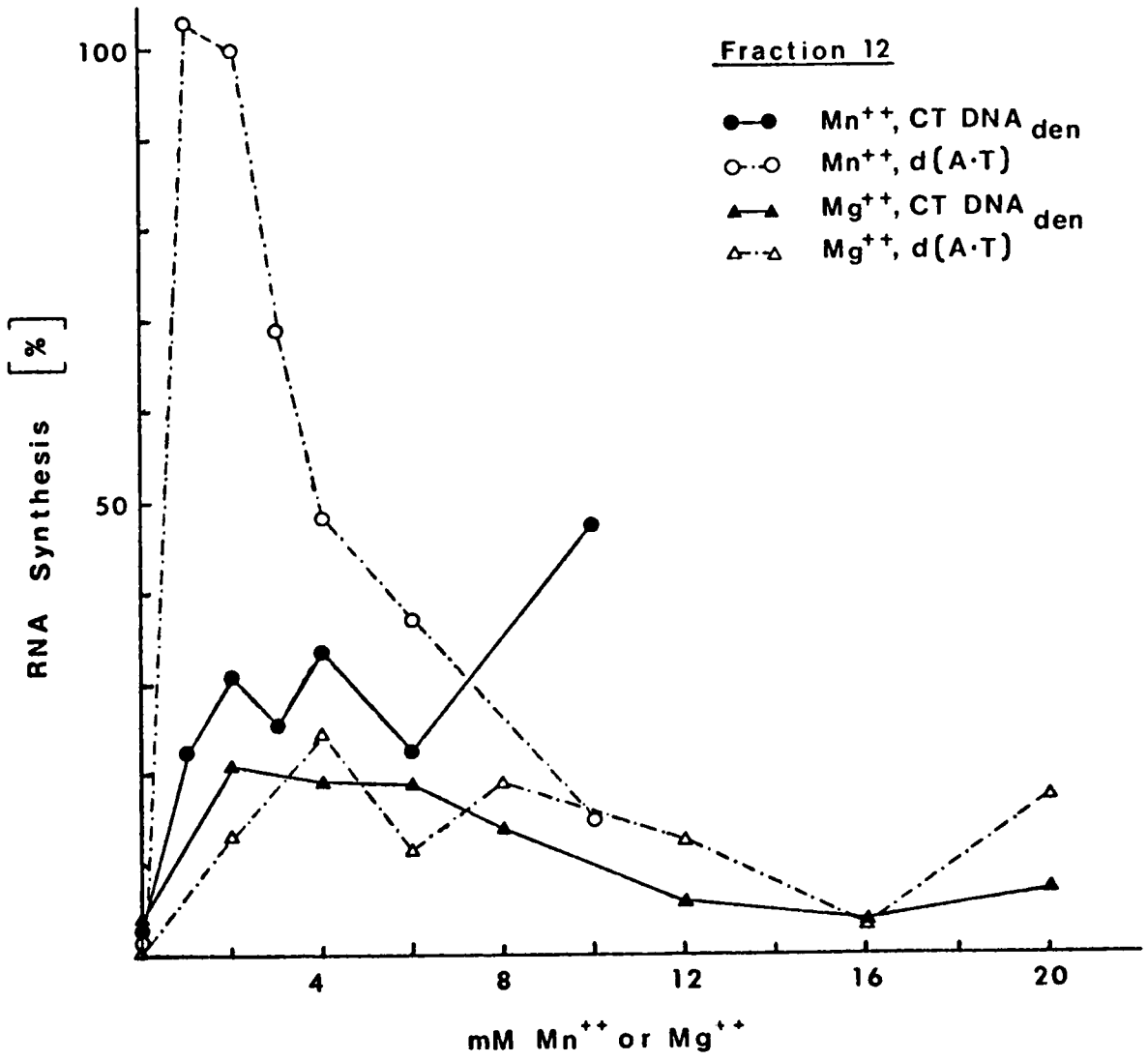

Fraction 12
Mn++, CT DNA den
Mn++, d(A·T)
Mg++, CT DNA den
Mg++, d(A·T)
RNA Synthesis [%]
100
50
4
8
12
16
20
mM Mn++ or Mg++

Fig. 37. The effect of metal ion cofactor concentration on the RNA polymerase activity of fraction 17 from the DEAE-Sephadex column shown in figure 23. The RNA polymerase activity of fraction 17 was measured in a standard reaction mix containing 50 ul enzyme and 3 ug CT DNA_{den}. The metal ion cofactor was either Mg^{++} (▲—▲) or Mn^{++} (●—●) and the concentrations of each were varied as indicated. Data is presented in terms of optimum activity. Optimum activity was achieved with 2 mM Mn^{++}. Ammonium sulfate concentration was 0.025 M and the incubation was for 20 min at 37°C. Each point is the average of two determinations. 100% activity corresponds to 3,735 cpm.

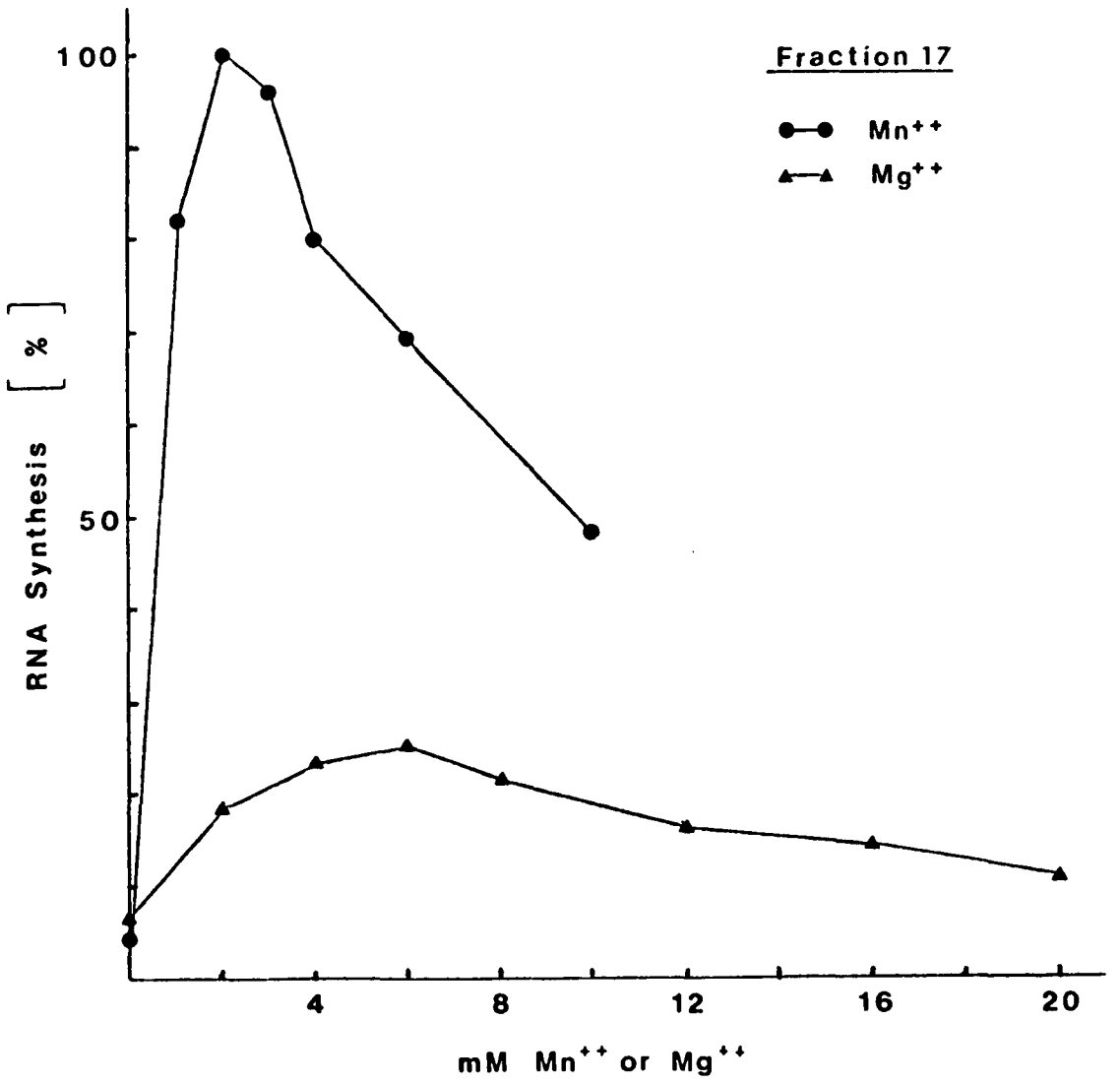

Fraction 17
Mn++
Mg++
100
50
RNA Synthesis [%]
4
8
12
16
20
mM Mn++ or Mg++

Fig. 38. The effect of metal ion cofactor concentration on the RNA polymerase activity of fraction 25 from the DEAE-Sephadex column shown in figure 23. The RNA polymerase activity of fraction 25 was measured in a standard reaction mix containing 50 ul enzyme and 3 ug CT DNA_{den}. The metal ion cofactor was either Mg^{++} (-▲—▲-) or Mn^{++} (-●—●-) and the concentrations of each were varied as indicated. Data is presented in terms of optimum activity. Optimum activity was achieved with 2 mM Mn^{++}. Ammonium sulfate concentration was 0.025 M and the incubation was for 20 min at 37°C. Each point is the average of two determinations. 100% activity corresponds to 8,001 cpm.

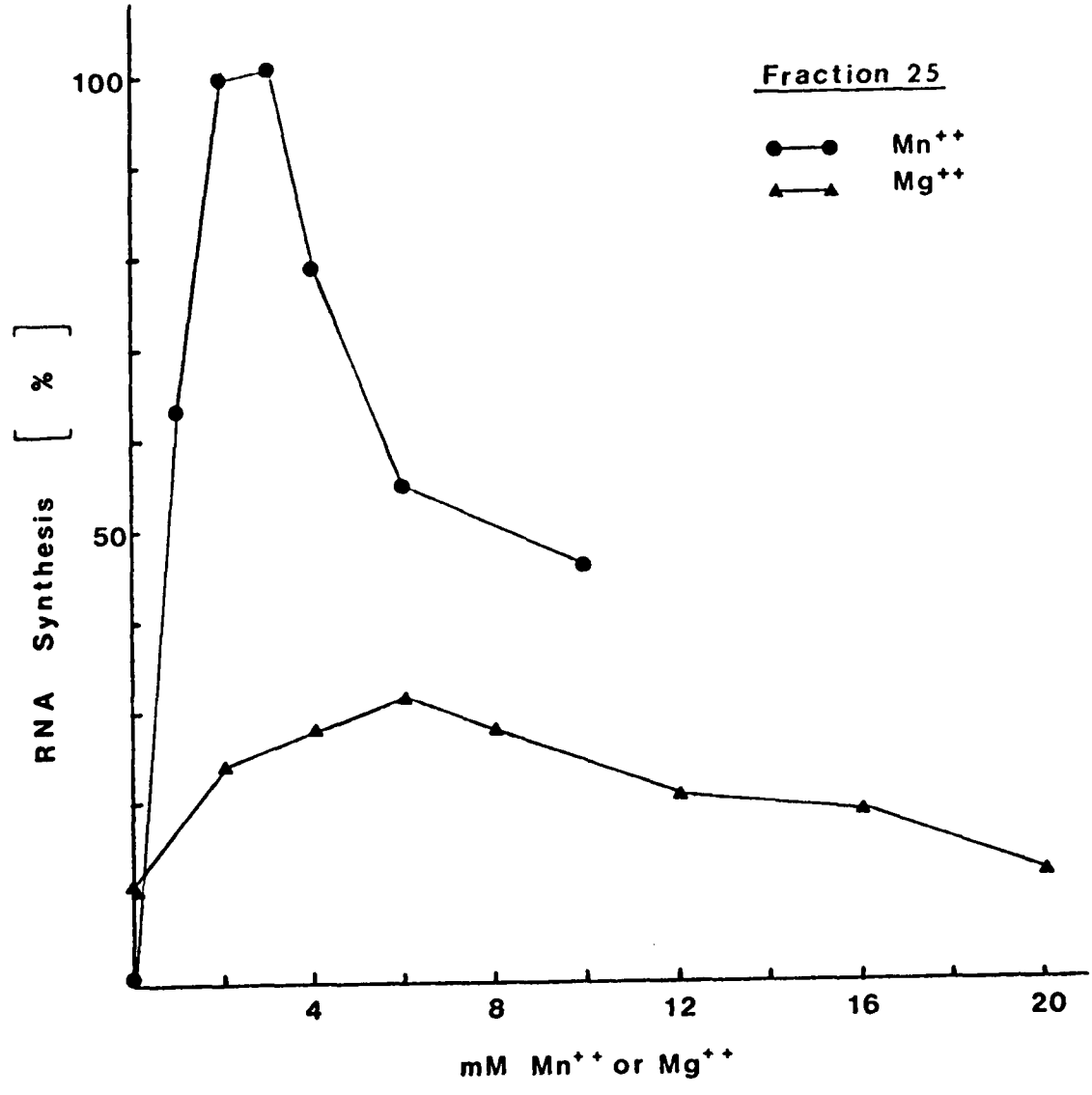

Fraction 25
Mn++
Mg++
100
50
RNA Synthesis [%]
4
8
12
16
20
mM Mn++ or Mg++

Fig. 39. Effect of metal ion cofactor concentration and template on the RNA polymerase activity of fraction 34 of the DEAE-Sephadex column shown in figure 23. The RNA polymerase activity of fraction 34 was measured in the standard reaction mix containing 50 ul enzyme and either 3 ug CT DNA_{den} or 5 ug $d(A\text{-}T)_n$. The metal ion cofactor was either Mn^{++} or Mg^{++} and the concentrations were varied as shown. The RNA polymerase activity was tested under the four combinations of template and metal ion cofactor. Data is presented in terms of the activity at 2 mM Mn^{++} with CT DNA_{den} with both the Mn^{++} and Mg^{++} cofactors. With $d(A\text{-}T)_n$ as the template, both Mn^{++} and Mg^{++} results are presented in terms of the activity achieved with 2 mM Mn^{++} and $d(A\text{-}T)_n$. Incubation was for 20 min at 37°C. Ammonium sulfate concentration was 0.025 M. Each point represents a single determination. 100% activity corresponds to 5,418 cpm.

(-●—●-) Mn^{++}, CT DNA_{den}

(-O--O-) Mn^{++}, $d(A\text{-}T)_n$

(-▲—▲-) Mg^{++}, CT DNA_{den}

(-Δ--Δ-) Mg^{++}, $d(A\text{-}T)_n$

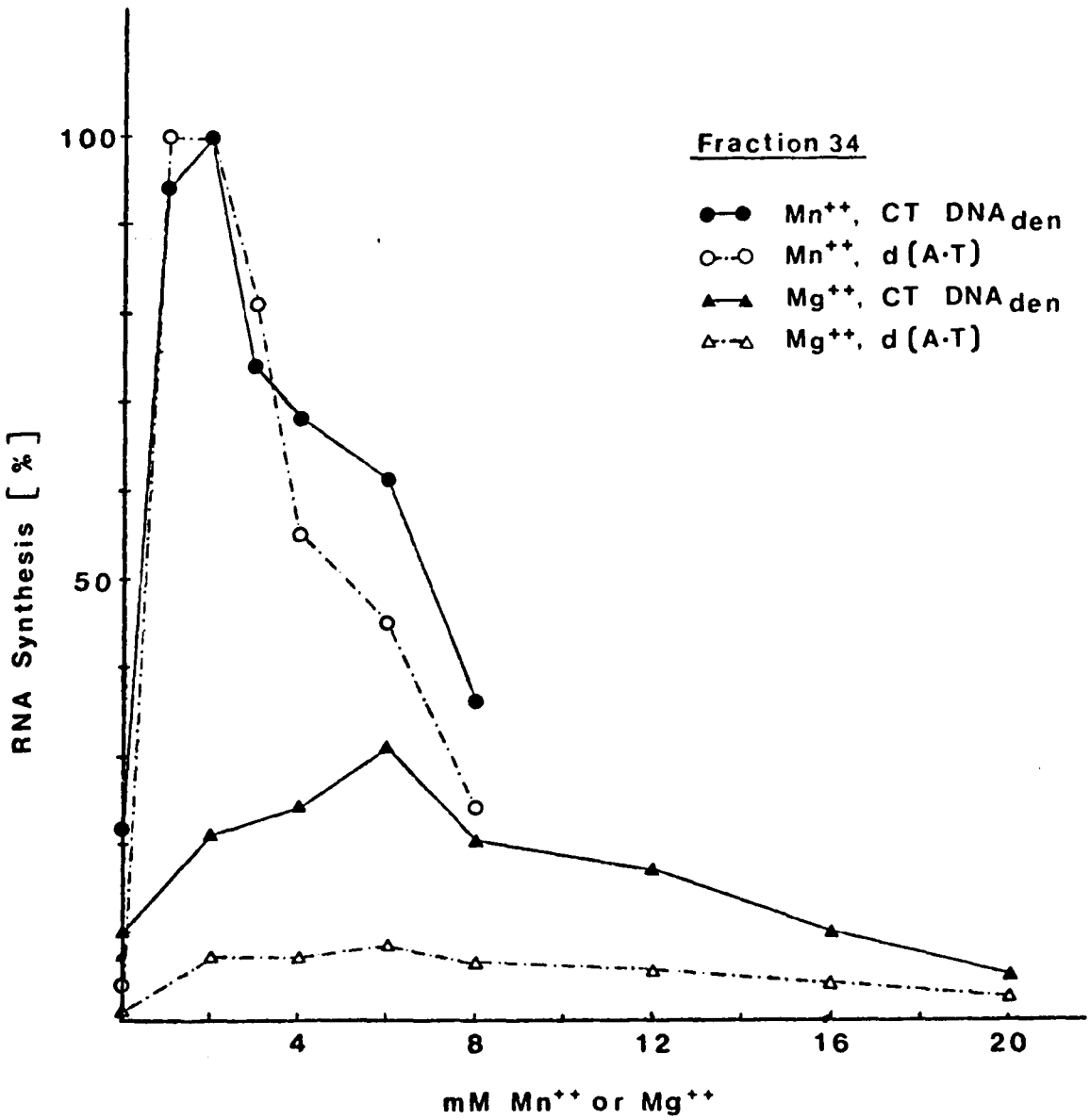
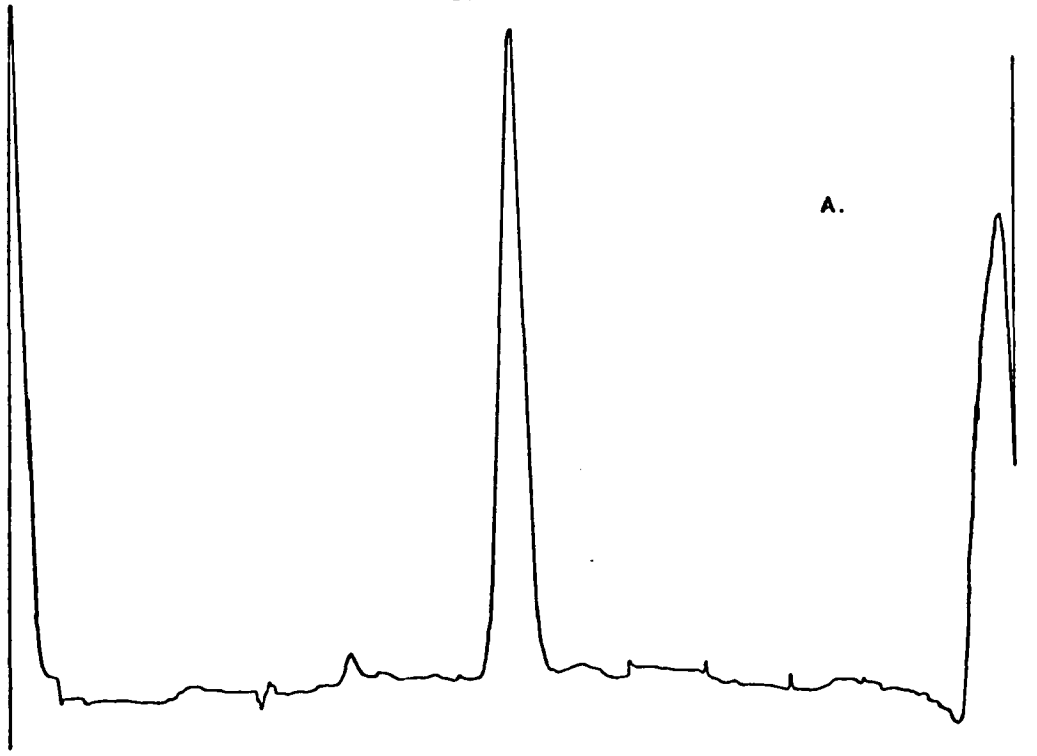

Fraction 34
Mn++, CT DNA den
Mn++, d(A·T)
Mg++, CT DNA den
Mg++, d(A·T)
RNA Synthesis [%]
100
50
4
8
12
16
20
mM Mn++ or Mg++

Fig. 40.--Absorbance profile of fraction 12 from the DEAE-Sephadex column shown in figure 23 and E. coli RNA polymerase after electrophoresis on 5% polyacryamide gels under non-denaturing conditions. Five percent polyacrylamide gels were prepared and electrophoresis was carried out as described in Methods. Gels were run at 4°C in a cold room and scanned at 600 nm in a Gilford spectrophotometer. A. 60 ul fraction 12, full absorbance = 2.5. B. 45 ul fraction 12 mixed with 30 ul E. coli RNA polymerase (6.3 ug protein), full absorbance = 2.5. Migration was toward right.


A.

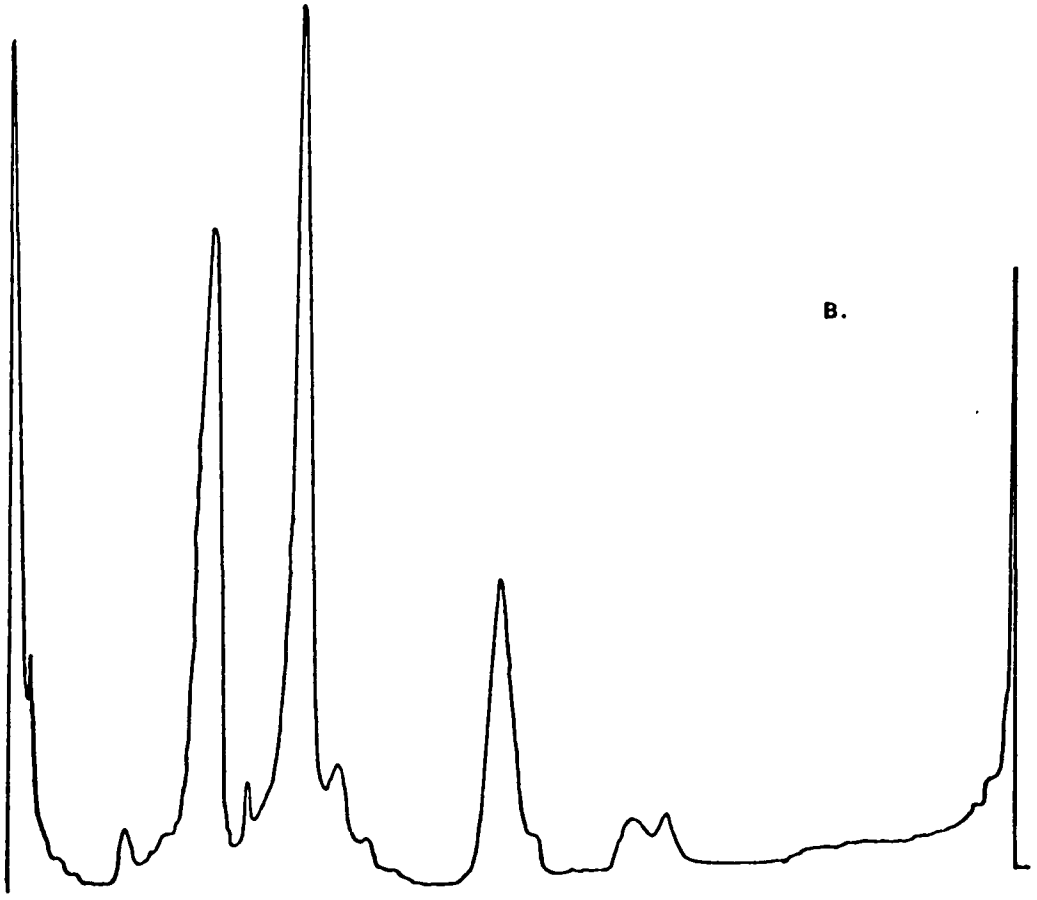
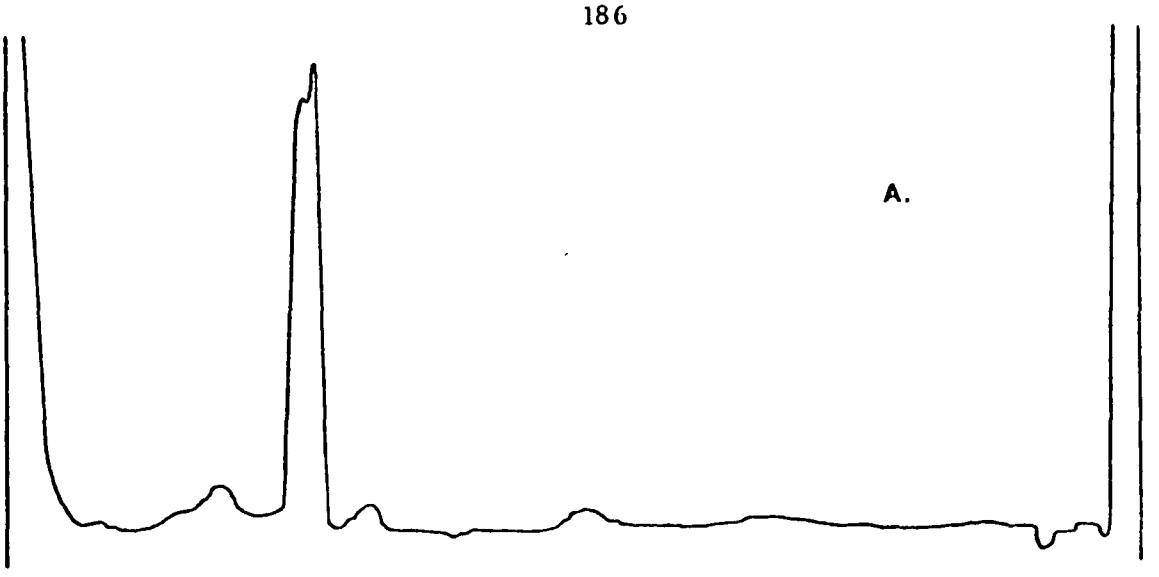

B.

Fig. 41.--Absorbance profile of fraction 17 from the DEAE-Sephadex column shown in figure 23 and E. coli RNA polymerase after electrophoresis on 5% polyacrylamide gels under non-denaturing conditions. Five percent polyacrylamide gels were prepared and electrophoresis was carried out as described in Methods. Gels were run at 4°C in a cold room and scanned at 600 nm in a Gilford spectrophotometer. A. 60 ul fraction 17, full scale absorbance = 2.5. B. 45 ul fraction 17 mixed with 6.3 ug E. coli RNA polymerase, full scale absorbance = 2.5. Migration was toward the right.


A.

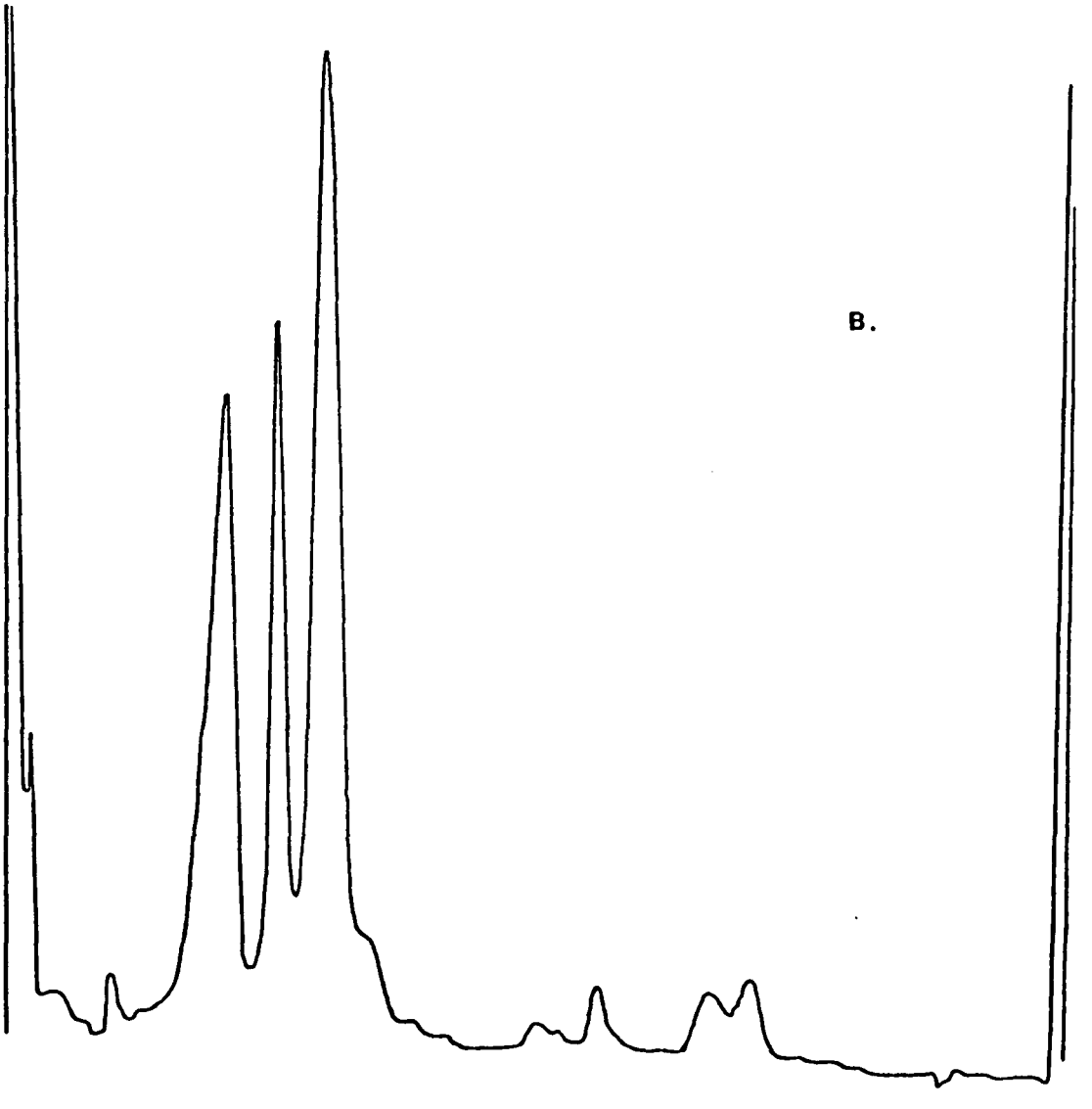

B.

Fig. 42.--Absorbance profile of fractions 24 and 26 from the DEAE-Sephadex column shown in figure 23 and E. coli RNA polymerase after electrophoresis on 5% polyacrylamide gels under non-denaturing conditions. Five percent polyacrylamide gels were prepared and electrophoresis was carried out as described in Methods. Gels were run at 4°C in a cold room and scanned at 600 nm in a Gilford spectrophotometer. A. 5.3 ug E. coli RNA polymerase, full scale absorbance = 1.5. B. 50 ul mixture of fraction 24 and 26, full scale absorbance = 1.5. C. 25 ul E. coli RNA polymerase (5.3 ug), full scale absorbance = 1.5. Migration was toward the right.

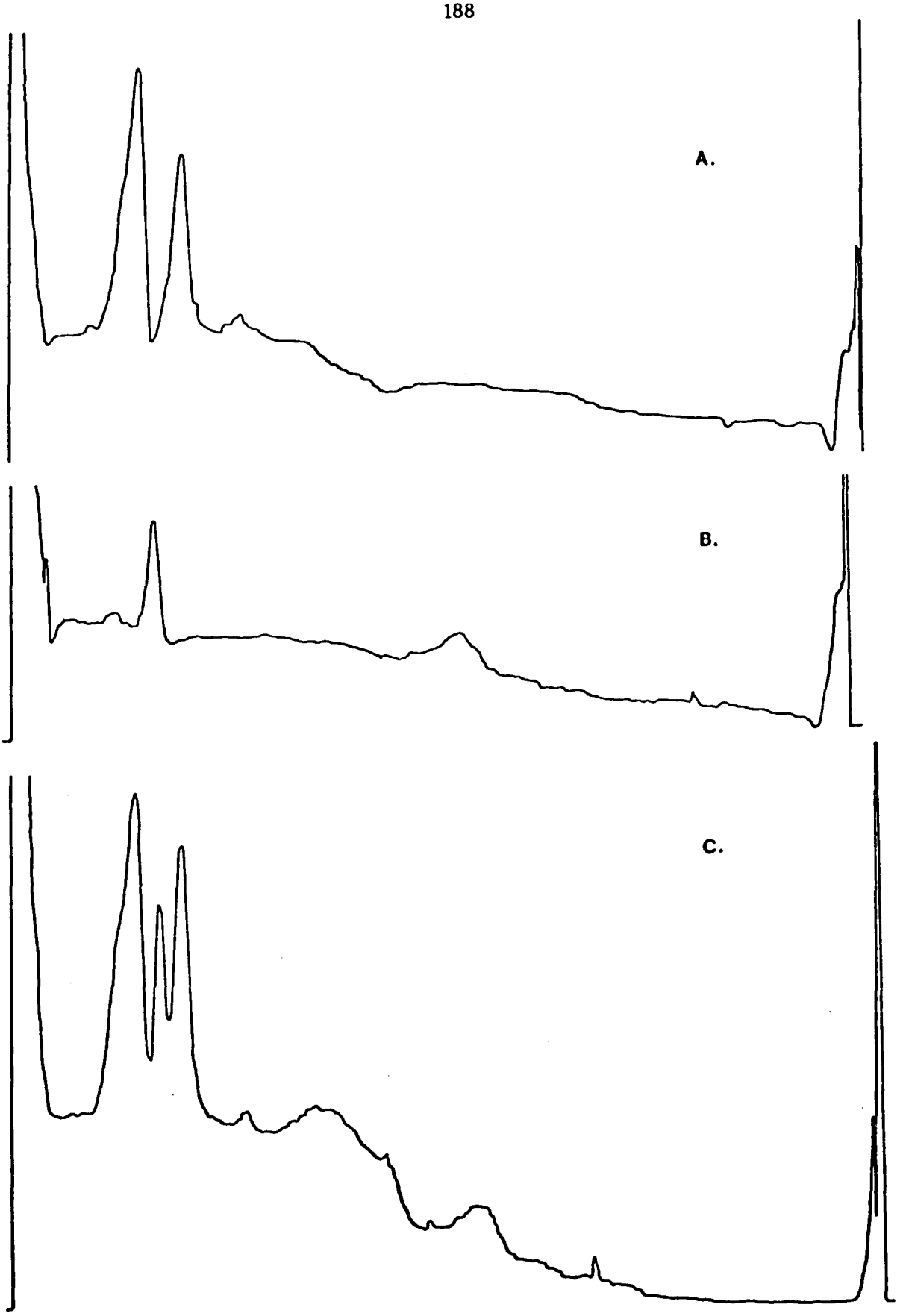

A.
B.
C.

Fig. 43.--Absorbance profile of fractions 24 and 26 from the DEAE-Sephadex column shown in figure 23 and Artemia RNA polymerase II from figure 24 after electrophoresis on 5% polyacryamide gels under non-denaturing conditions. Five percent polyacrylamide gels were prepared and electrophoresis was carried out as described in Methods. Gels were run at 4°C in a cold room and scanned at 600 nm in a Gilford spectrophotometer. A. 50 ul fractions 24 and 26 mixed, full scale absorbance = 1.5. B. 175 ul Artemia RNA polymerase II from figure 24, full scale absorbance = 1.5. C. 25 ul mixture of fractions 24 and 26 mixed with 175 ul Artemia RNA polymerase II from figure 24 (pooled fractions 12 - 24), full absorbance = 1.5. Migration was toward the right.

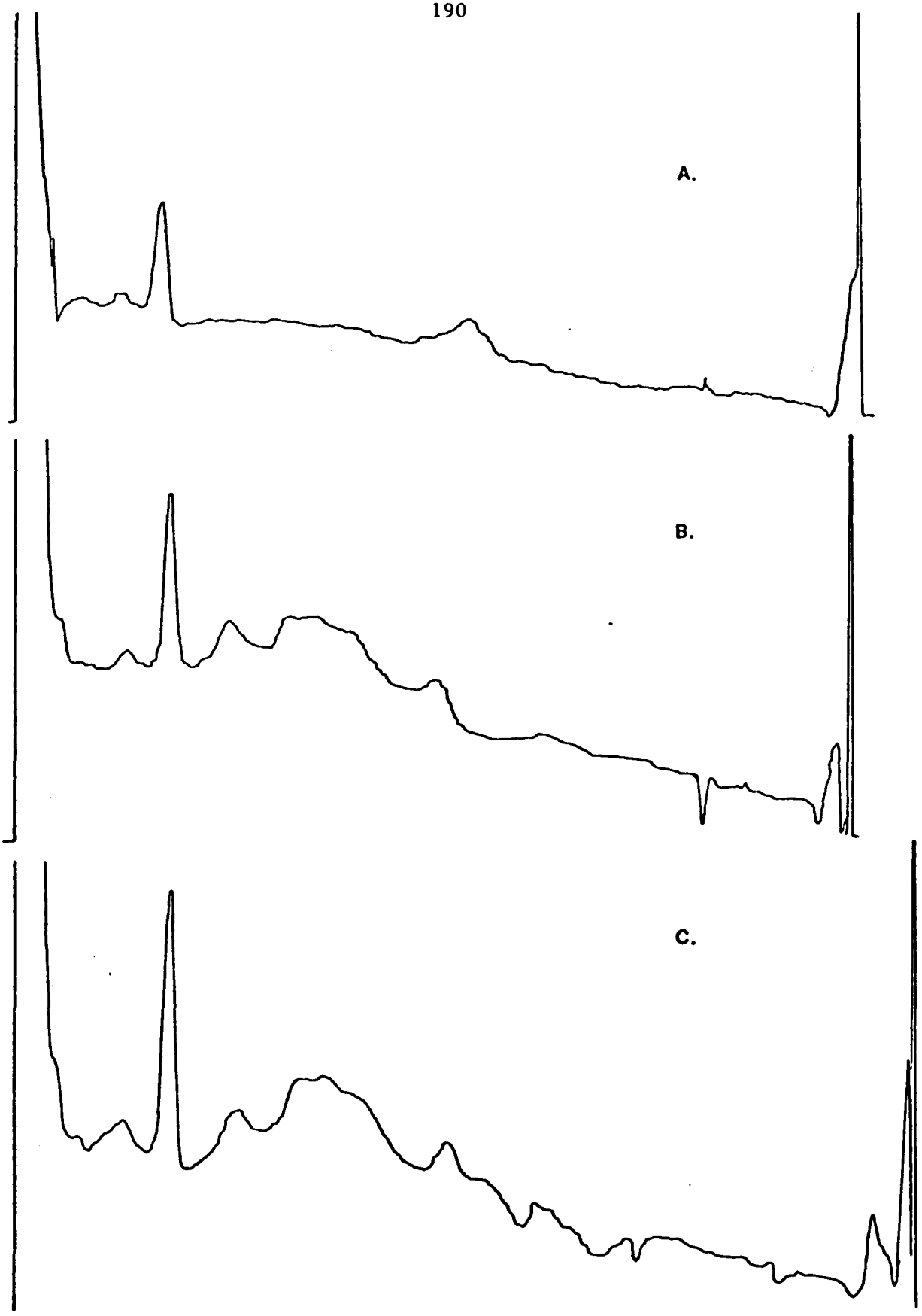
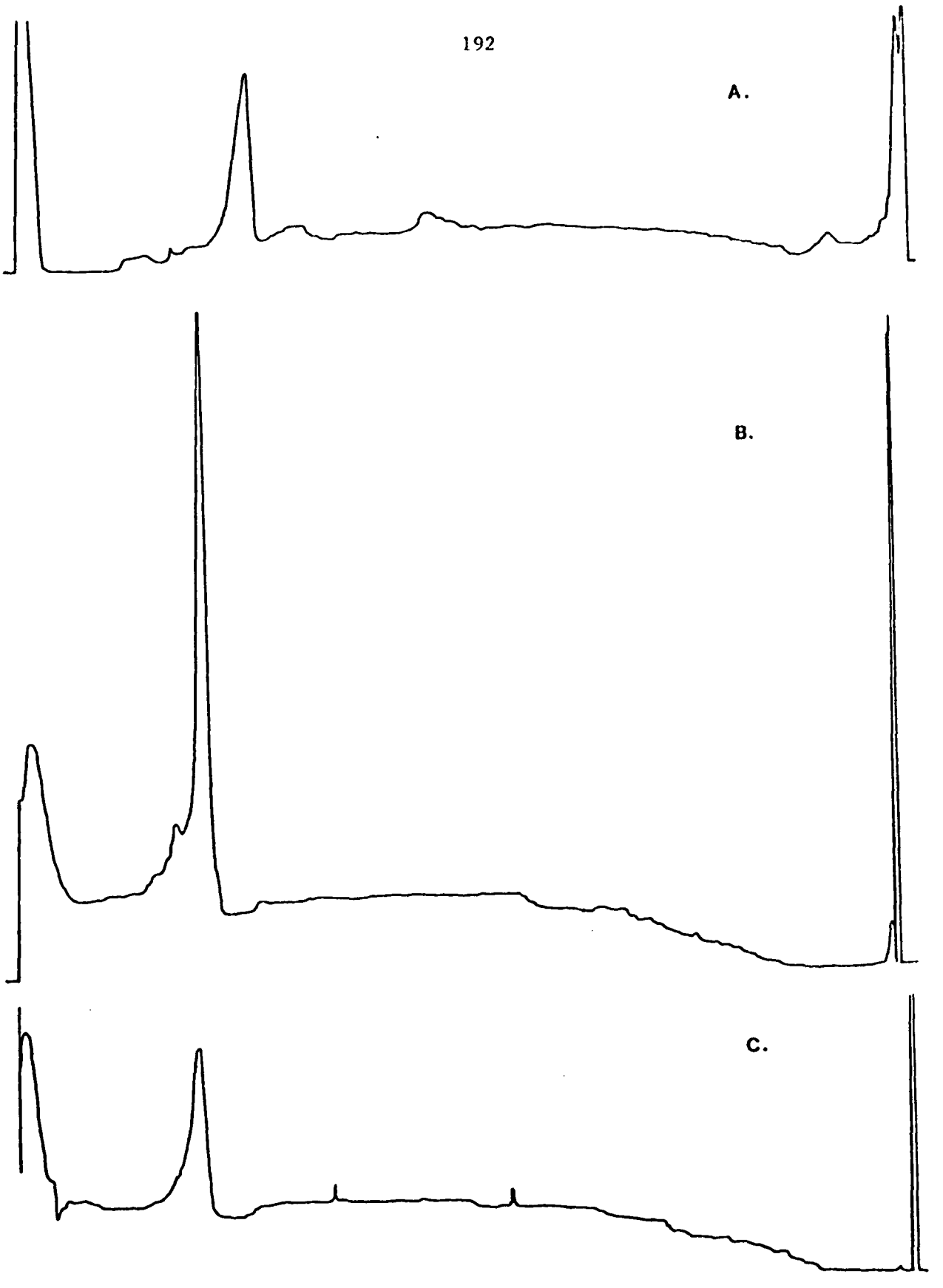

A.
B.
C.

Fig. 44.--Absorbance profile of Artemia RNA polymerase II and E. coli RNA polymerase electrophoresed on 5% polyacrylamide gels under non-denaturing conditions after glycerol gradient centrifugation. Five percent polyacrylamide gels were prepared and electrophoresis was carried out as described in Methods. Gels were run at 4°C in a cold room and scanned at 600 nm in a Gilford spectrophotometer. A. 100 ul E. coli RNA polymerase from fraction 14 of the glycerol gradient activity profile shown in Appendix 2. B. 200 ul of Artemia RNA polymerase II activity from fraction 15 of the glycerol gradient whose activity profile is shown in figure 13. C. 200ul Artemia RNA polymerase II activity from fraction 16/17 (inadvertantly pooled after centrifugation) of the glycerol gradient shown in figure 13. Full scale absorbance = 1.5 for A., B. and C. Migration was toward the right.

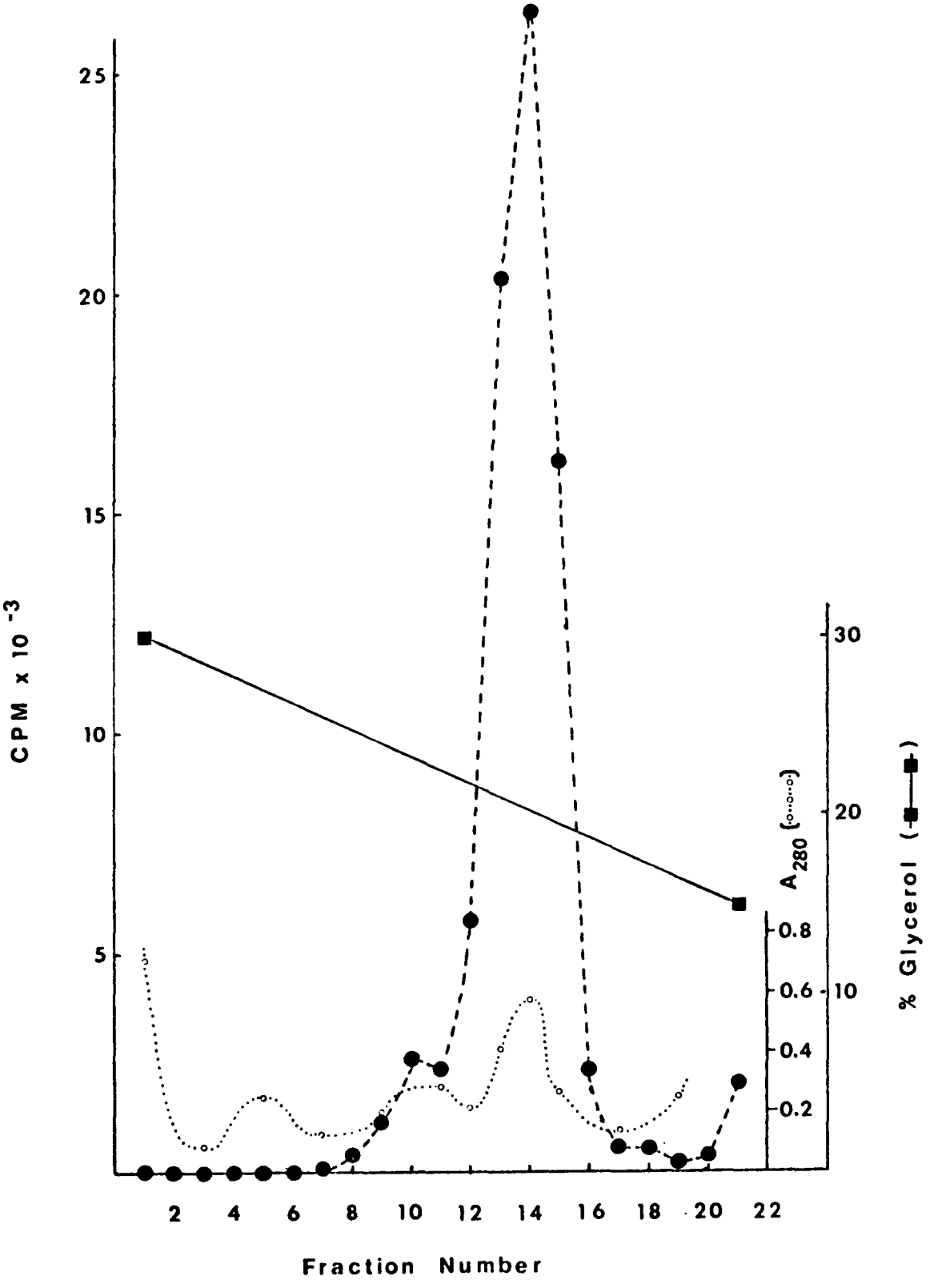

A.
B.
C.

APPENDIX 1.--Stock Concentrations and Dilutions Used to Achieve Various Final Concentrations of RNA Polymerase Reaction Mix Substances

Test Substance	Stock Solution Concentration	Dilution	Final Concentration
	(mM)		(mM)
Mn^{++}	25	5 ul/125 ul	1
	50	"	2
	75	"	3
	100	"	4
	125	"	5
	150	"	6
	175	"	7
	200	"	8
	125	10 ul/125 ul	10
Mg^{++}	50	5 ul/125 ul	2
	100	"	4
	150	"	6
	200	"	8
	250	"	10
	300	"	12
	400	"	16
	500	"	20
	(M)		(M)
Ammonium Sulfate	0.5	5 ul/125ul	0.020
	1.0	"	0.040
	2.0	"	0.080
	0.625	5 ul/125 ul	0.025
	1.250	"	0.050
	1.875	"	0.075
	2.50	"	0.100
	3.125	"	0.125
	3.75	"	0.150
	3.125	10 ul/125 ul	0.250
	ug/ml		ug/ml
Alpha-amanitin	1,000	5 ul/125 ul	40
	100	"	4
	10	"	0.4
	1	"	0.04
	0.1	"	0.004

Appendix 2.--Glycerol gradient centrifugation of E. coli RNA polymerase. Seventy-five microliters of E. coli RNA polymerase (Sigma) containing 158 ug protein in 50% glycerol buffer was mixed with 300 ul of the light glycerol gradient buffer (4% glycerol) for a final volume of 375 ul at 13% glycerol. This was layered onto the top of a preformed (15 - 30% v/v) glycerol gradient in an SW41 rotor tube. The total volume was 13.2 ml.

After centrifugation at 30,000 rpm in an SW41 rotor for 20.9 hr at 3°C, the tube was punctured from the bottom and fractions of 25 drops each were collected. Fractionation was carried out at 4°C in a cold room. Fractions were assayed for RNA polymerase activity in the standard reaction mix containing 50 ul enzyme, 4 mM Mn^{++} and 5 ug $d(A\text{-}T)_n$. Incubation was for 20 min at 37°C. Absorbance was measured at 280 nm. The results are presented with the bottom of the gradient at the left.


CPM x 10 -3
25
20
15
10
5
A280 (o...o...o)
0.8
0.6
0.4
0.2
30
20
10
% Glycerol (■—■—■)
2
4
6
8
10
12
14
16
18
20
22
Fraction Number

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