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ISOLATION AND CHARACTERIZATION OF MITOCHONDRIAL RNA
POLYMERASE FROM DROSOPHILA MELANOGASTER

City University of New York

PH.D. 1981

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ISOLATION AND CHARACTERIZATION OF MITOCHONDRIAL RNA
POLYMERASE FROM DROSOPHILA MELANOGASTER

by

Michael Joseph Goldenthal

A dissertation submitted to the Graduate Faculty in
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University of New York

1981

Michael Goldenthal

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

ISOLATION AND CHARACTERIZATION OF MITOCHONDRIAL RNA POLYMERASE FROM DROSOPHILA MELANOGASTER

Adviser: Dr. J. Nishiura

A DNA-dependent RNA polymerase was solubilized from sucrose-gradient fractionated, DNase-treated mitochondria isolated from Drosophila melanogaster. These fractionated mitochondria were shown to be free of nuclear or bacterial contamination by the analysis of the DNA of these organelles by CsCl buoyant density gradient centrifugation, and PLK chromatography. The polymerase is solubilized most efficiently by non-ionic detergents (0.2% NP-40 or 3% Triton X-100). The same concentration of detergent also solubilized cytochrome c oxidase which suggests an inner membrane site for the mitochondrial RNA polymerase. This RNA polymerase has the following properties: 40-50% inhibition by 50 ug/ml rifampicin, an $(\text{NH}_4)_2\text{SO}_4$ optima of 0.025 M, Mg^{++} optima of 15 mM, and a Mn^{++} optima of 2 mM. Stimulation of polymerase activity by Mg^{++} is roughly 2 fold that with Mn^{++} . There is no significant inhibition of enzyme activity by 100 ug/ml alpha-amanitin.

Gel filtration of the mitochondrial RNA polymerase on Sephacryl S-200 columns resolves multiple peaks of apparent molecular weights of 60,000, 110,000, 160,000, 210,000,

and a peak > 250,000. These activities share most of the properties listed above with some differences in the extent of Mn^{++} stimulation, and rifampicin sensitivity. The lowest apparent molecular weight activity is greatly enriched when the Sephacryl column is eluted in high salt. These results suggest that the peak of lowest apparent molecular weight is capable of aggregation, more so in low than in high salt to form dimers, trimers, etc. This is further supported by glycerol gradient centrifugation of the low molecular weight activity which yields multiple slow and fast sedimenting polymerase activities, the distribution of which depends on salt concentration. Alternative explanations involving endogenous DNA-binding, and associated protein factors can not be entirely precluded at present, although they are made less probable by the following results. DNase treatment, PEG precipitation or pre-chromatography of the mitochondrial extracts on heparin-Sepharose have no effect on the heterogenous profile from Sephacryl S-200 columns. In addition, SDS gel electrophoresis shows that the fastest and slowest sedimenting RNA polymerase activities have a single major protein component of 60,000 d.

A similar heterogeneity is observed with ion-exchange chromatography of the mitochondrial RNA polymerase. A correspondence is noted between distinct ion-

exchanges peaks from DEAE Sephadex and phosphocellulose columns, and various sized activities as resolved on Sephacryl S-200. One explanation of this data is that the different sized molecular aggregates of mitochondrial RNA polymerase have different charge properties, which may in turn have physiological significance, e.g., in the binding of polymerase to the membrane, or DNA template.

Two preparative schemes for purifying mitochondrial RNA polymerase from isolated mitochondria are presented which are similar in enzyme yields (15-20%), specific activity (800-1000 units/mg protein) and amount of enzyme protein recovered (25-60 ug/200 g Flies).

This enzyme can also be prepared from whole fly homogenates without mitochondrial isolation. This procedure involves PEG precipitation, $(\text{NH}_4)_2\text{SO}_4$ fractionation, batch phosphocellulose, gel filtration and heparin-Sepharose chromatography resulting in a low molecular weight RNA polymerase with the same catalytic properties and single subunit structure as that found with the enzyme from purified mitochondria.

In summary, a low molecular weight RNA polymerase can be isolated from mitochondria with demonstrably different properties from the nuclear or bacterial RNA polymerases.

To Rachel and Sarah

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This work has been brought to fruition with the aid and support of a number of people.

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ABBREVIATIONS

NP-40	Nonidet P-40
DEAE-S	Diethylaminoethyl Sephadex
PMSF	Phenylmethyl sulfonyl fluoride
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
MW	Molecular weight
PPO	2,5 - Diphenyloxazole
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
STE	0.3 M Sucrose, 0.05 M Tris pH 7.9, 1mM EDTA
TGED	0.05 M Tris pH 7.9, glycerol (15%%)v/v, 1 mM EDTA, 0.5 mM DTT
Tris	Tris (hydroxymethyl)aminomethane
BSA	Bovine serum albumin
ds	Double-stranded
ss	Single-stranded
mtDNA	Mitochondrial DNA
NAD	Nicotinamide adenine dinucleotide
PLK	Polylysine Kieselguhr
CO	Cytochrome oxidase
MDH	Malate dehydrogenase
ADH	Alcohol dehydrogenase
RNAP	RNA polymerase
PC	Phosphocellulose

HS	Heparin Sepharose
EB	Ethidium bromide
PEG	Polyethylene glycol

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INTRODUCTION

One major rationale for studying mitochondrial transcription is its possible use as a model system for in vitro studies of the molecular interactions involved in transcription. The small, less complex mitochondrial genome offers several advantages compared to the nuclear genome. The easy isolation of the mitochondrial DNA from most higher eucaryotes in intact closed circular form, its small number of increasingly well-characterized gene products, and the relatively few proteins that surround and possibly regulate the mitochondrial DNA and its expression, all these make for a situation where in vitro events might be better compared with in vivo events.

The application of recombinant DNA technology has aided greatly in the identification and mapping of the mitochondrial transcripts, as well as the cloning of genes for sequence-study and template use. The isolation of a mitochondrial transcription complex, and a unique mitochondrial RNA polymerase have made possible a "purified" components approach for probing conditions of DNA-RNA polymerase binding and the initiation of RNA synthesis, as well as reconstitution studies to search for putative specificity factors. Questions concerning the location, number and importance of transcriptional promoter regions on the mitochondrial genome can be addressed. Lastly, an in

vitro analysis of mitochondrial transcription should be free of the rapid processing of RNA which appears to complicate interpretation of in vivo transcriptional events.

Another rationale for studying mitochondrial transcription and the mitochondrial RNA polymerase specifically comes from the role it plays in mitochondrial biogenesis and development. Elucidation of transcriptional control mechanisms might tell us how and when the mitochondrial polypeptides are produced, and whether this is coordinated with nuclear transcription. Also the question of how a nuclear-coded protein (such as mitochondrial RNA polymerase) is specified by, and localized inside organelles such as mitochondria is a current problem of great interest to cell biologists.

To understand the context of these experiments more clearly, the introduction presents a description of mitochondrial DNA, the organization of the genome, and results from both in vivo and in vitro studies of mitochondrial transcription. Included in this background information is a brief sketch of mitochondrial DNA replication, because the enzyme of transcription under study in this thesis might well be involved in the initiation of mitochondrial DNA synthesis. The last part of the introduction delineates the emerging picture of a mitochondrial chromosome, and the description of previously characterized mitochondrial RNA polymerases.

Mitochondrial DNA

Mitochondrial DNAs are small duplex molecules varying in size between 5u (animal genomes) and 30u (fungi). The size appears to decrease as a function of evolution: animal cells having $9-12 \times 10^6$ (between 15-18,500 base pairs) with Drosophila having 12.5×10^6 d. The fungi such as yeast and Neurospora have the largest mitochondrial genomes in the range of $40-50 \times 10^6$ d. The smaller genomes are more easily isolated in intact duplex circles; the larger genomes are not recoverable in circular form.

Other forms of mitochondrial DNA are routinely isolated in all species. Circular concatenated multimers are often observed, the in vivo function of which is not understood (Borst, 1972). In addition, a number of replicative intermediates have been isolated and visualized by electron microscopy. Still another frequently observed, but not understood phenomena is the presence of variable numbers of these circles in each organelle. For example, in rat liver cells, there are 2-10 circles/mitochondria. Hybridization experiments have shown that these circles are repeats of each other, and contain equivalent information (Dawid, et al., 1967).

Although the mitochondrial DNA can represent as little as 1% of total cellular DNA in a number of organisms, its closed circular topology permits quantitative isolation

using CsCl-dye buoyant density gradients and subsequent characterization. Dyes, e.g., ethidium bromide allow the separation of nicked, linear and closed circular forms of mitochondrial DNA. In organisms with particularly A-T rich mitochondrial DNA such as yeast and Drosophila, mitochondrial DNA is also easily separated from nuclear DNA in CsCL or using polylysine Kieselguhr chromatography (Ayad, 1970). Complementary strands may be isolated in alkaline CsCl density gradients. These techniques demonstrate that mitochondrial DNA is relatively easily handled. Single-strands, restriction fragments or intact duplexes in a variety of conformations are available as templates to probe mitochondrial DNAs transcription.

Organization of the Mitochondrial Genome

The mitochondrial DNA of all organisms studied codes for two rRNAs, a set of tRNAs that are involved in mitochondrial protein synthesis as well as a number of messenger RNAs some which have been observed to direct the synthesis of mitochondrial specific polypeptides. Figure 1 depicts four representative mitochondrial genomes which illustrate diagrammatically a variety of points.

1) The tandem arrangement of 13S and 16S rRNA cistrons, as shown here for Drosophila, Xenopus and HeLa genomes, seems to be a general feature of higher metazoans. The more primitive fungi represented here by yeast, have widely separated ribosomal cistrons.

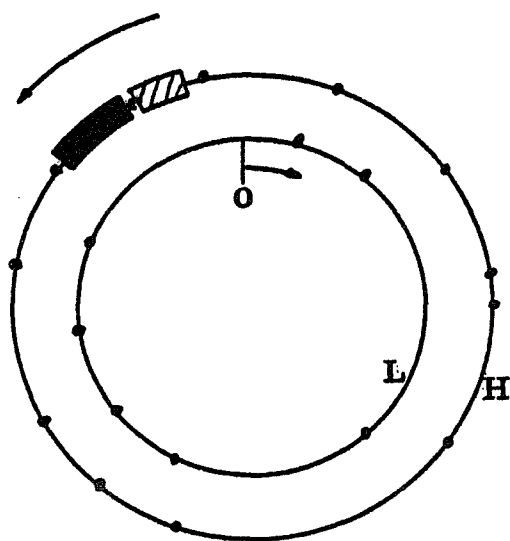
2) The number and distribution of mitochondrial-coded tRNAs seems to vary along similar lines. That is, the higher metazoans show a pattern of fewer tRNAs cistons (19-Xenopus, 21-Hela) which are widely spaced around the genome (Dawid et al., 1979). The fungal mitochondrial genome have a larger number of tRNA cistrons (25-30), most of which are clustered in 1/4 of the genome (Borst and Grivell, 1978).

3) The kinds of information that exists about each mitochondrial genome is limited by the methodologies that can be employed with each organism.

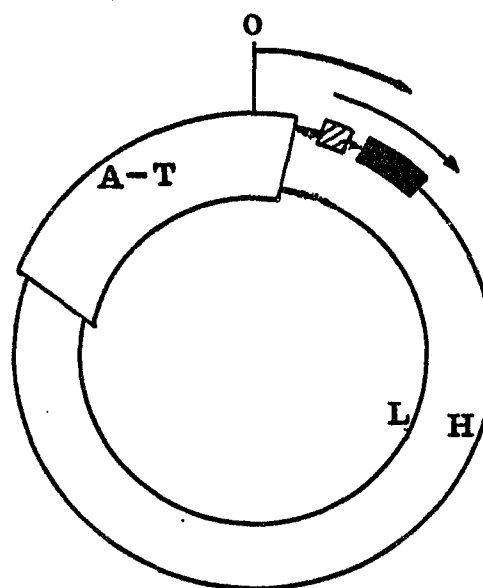
Inhibitor studies have been used with all these organisms to show that 8-12 polypeptides coded for by mitochondrial DNA are translated in the mitochondria (Schatz et al., 1974). However, only in yeast, which as a facultative anaerobe can grow in the absence of mitochondrial function, has a genetic analysis been possible. Loci coding for the synthesis of mitochondrial coded peptides of three subunits of cytochrome oxidase, one subunit of cytochrome b and four subunits of mitochondrial ATPase have been mapped. These genetic loci as well as several mutations concerning resistance to antibiotics--erythromycin and oligomycin--have been localized as shown for yeast. Similar genetic mapping studies for these apparently well-conserved polypeptides (Mahler et al., 1979) have not yet been done in the higher metazoans which are not amenable to such genetic analysis.

Figure 1. Organization of the mitochondrial genomes of Hela, Drosophila melanogaster, Sacchromyces cerevisiae, and Xenopus laevis.

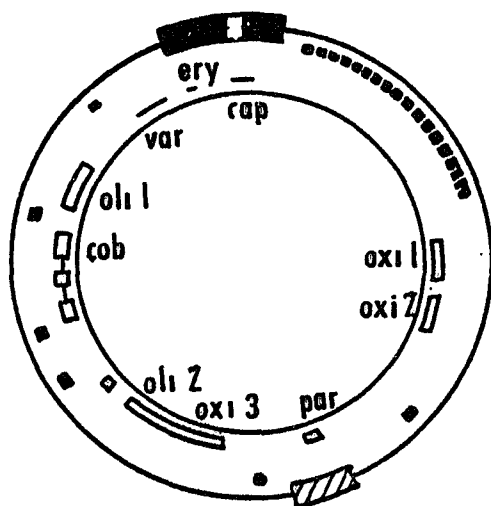
These mt DNA maps are based on hybridization of restriction fragments with small rRNA (), large rRNA () and tRNAs (•). The origin (O) and direction () of mt DNA replication are indicated, as are the H and L strands. Direction of transcription (), and the A-T region in Drosophila () are shown. Major genetic loci which have been mapped in yeast are shown as well.



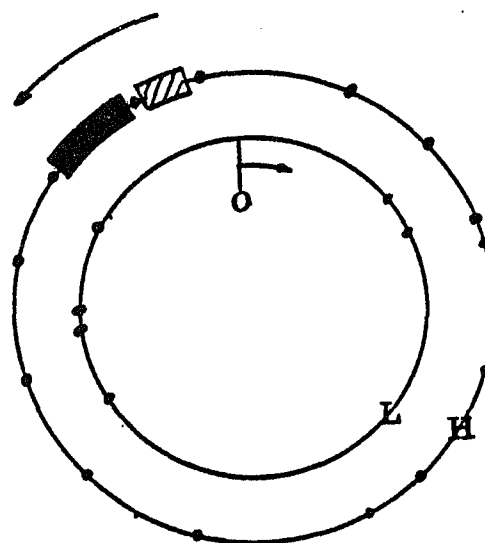
Hela
15-16 kb



Drosophila melanogaster
18.4 kb



Yeast
(*S. cerevisiae*)
76 kb



Xenopus laevis
15-16 kb

On the other hand, the organization of the mitochondrial genome from higher eucaryotes has been greatly elucidated by recombinant DNA technology. The isolation of DNA strands, and replicative intermediates has allowed the origins of DNA replication to be assigned to a specific region (as shown in Figure 1). Such techniques have aided in establishing the direction of transcription, and the hybridization of putative RNA transcripts with different strands (O'Brien, 1976). This kind of information is lagging thus far with yeast, since the methodology has been dependent on the isolation of intact mitochondrial DNA strands.

4) A number of unusual aspects of mitochondrial DNA sequence should be noted in that they are interesting both from an evolutionary standpoint, as well as possibly having some relevance to the study of transcription.

While mitochondrial DNA is found and apparently contains most of the same genes in all eucaryotes, it shows considerable divergence in base composition, and sequence content (Mahler et al., 1975). Gene order is apparently conserved among higher metazoans like human, Xenopus laevis and mouse, as well as amongst the mitochondrial genomes of different yeast strains and Neurospora. A non-random distribution in sequence variation has been noted in studies using both hybridization and restriction enzyme patterns to compare mitochondrial DNAs between closely-related

species. Such studies with Xenopus (Dawid et al., 1967) and sheep/goat (Upholt et al., 1977) have shown that a major area of sequence divergence was found to be within the extreme boundaries of the D loop region containing the origin(o) of replication. A similar variability in the mitochondrial DNA between different species of Drosophila (Goddard et al., 1978) has been found to be due to different sizes of the A-T rich region which contains the origin of replication. The studies with yeast (Sanders et al., 1977) show that variability between strains is due to the presence of not one but multiple A-T rich inserted fragments, one of which is found in the large ribosomal subunit.

There are a number of possible regulatory functions of these A-T rich regions. In higher eucaryotes while A-T sequence variability in mito DNA has been correlated with replication regions, this has not yet been observed in yeast. The variability in size of A-T rich regions has led some to claim that they are without function analogous to "spacer" DNA (Tzagaloff et al., 1979). However, in one instance in yeast, the presence of an inserted region (omega) has been correlated with recombinational polarity (Strausberg et al., 1978). This has led Bernardi (1976) to propose that these inserted sequences are sites of unequal crossing-over. Recently, Van Ommen et al. (1979) has proposed that these might play a role in RNA processing.

It should be indicated that the presence of intervening sequences and recombination may represent particular anomalies of yeast mitochondrial DNA since no such intervening sequences have been found in the large and small rRNAs of Hela and Xenopus (Dawid et al., 1979, Ojala et al., 1980a).

One model that might integrate these observations is that these A-T rich regions might aid in self unwinding that is necessary for both H strand replication, and transcriptional initiation. Procaryotic promotor regions have been found to be A-T rich (Rosenberg and Court, 1979). High affinity binding of RNA polymerase to such A-T rich regions may well be involved in promoter selection and efficiency (Chamberlin, 1974).

Such a relationship has been suggested in studies of the trypanosome's large mitochondria (Brack et al., 1975) or kinetoplast. A-T rich clusters are found to preferentially bind berenil, a drug which specifically inhibits both mitochondrial DNA and RNA synthesis. In addition, these clusters appear to specifically bind RNA polymerase. Though this binding may be fortuitous and not physiologically significant, future studies might address the possible function of such A-T rich regions of mitochondrial DNA in replication and transcription in vitro.

Mitochondrial DNA Replication

Replication of mitochondrial DNA has been extensively studied by analyzing isolated replication intermediates of discrete size and frequency. In addition, restriction enzyme sites and A-T rich ('early melting') regions are used as "markers" to establish the origin, extent and direction of replication. Experiments primarily with mouse L cell (Berk et al., 1974) and rat liver mitochondria (Kasamatsu et al., 1973) have shown that mitochondrial DNA replication is unidirectional, discontinuous and asynchronous. That is, one strand is approximately 60% replicated before replication of the complement strand begins. Replication of a closed circular duplex DNA begins with the initiation of H Strand synthesis at a specific origin on the L strand. This synthesis may pause after polymerization of approximately 600 dnT, creating a molecule with a partially displaced parental H strand. This structure is termed D-loop mitochondrial DNA, and is the most frequently observed replication intermediate. Replication of the H strand then proceeds yielding an increasingly larger D loop.

The initiation of light-strand synthesis does not occur until synthesis of the H strand is 2/3 completed and has extended past the light strand origin. Synthesis of the nascent L strand begins at a single-stranded site, and

proceeds in the opposite direction to that of H strand synthesis. (See Figure 2.)

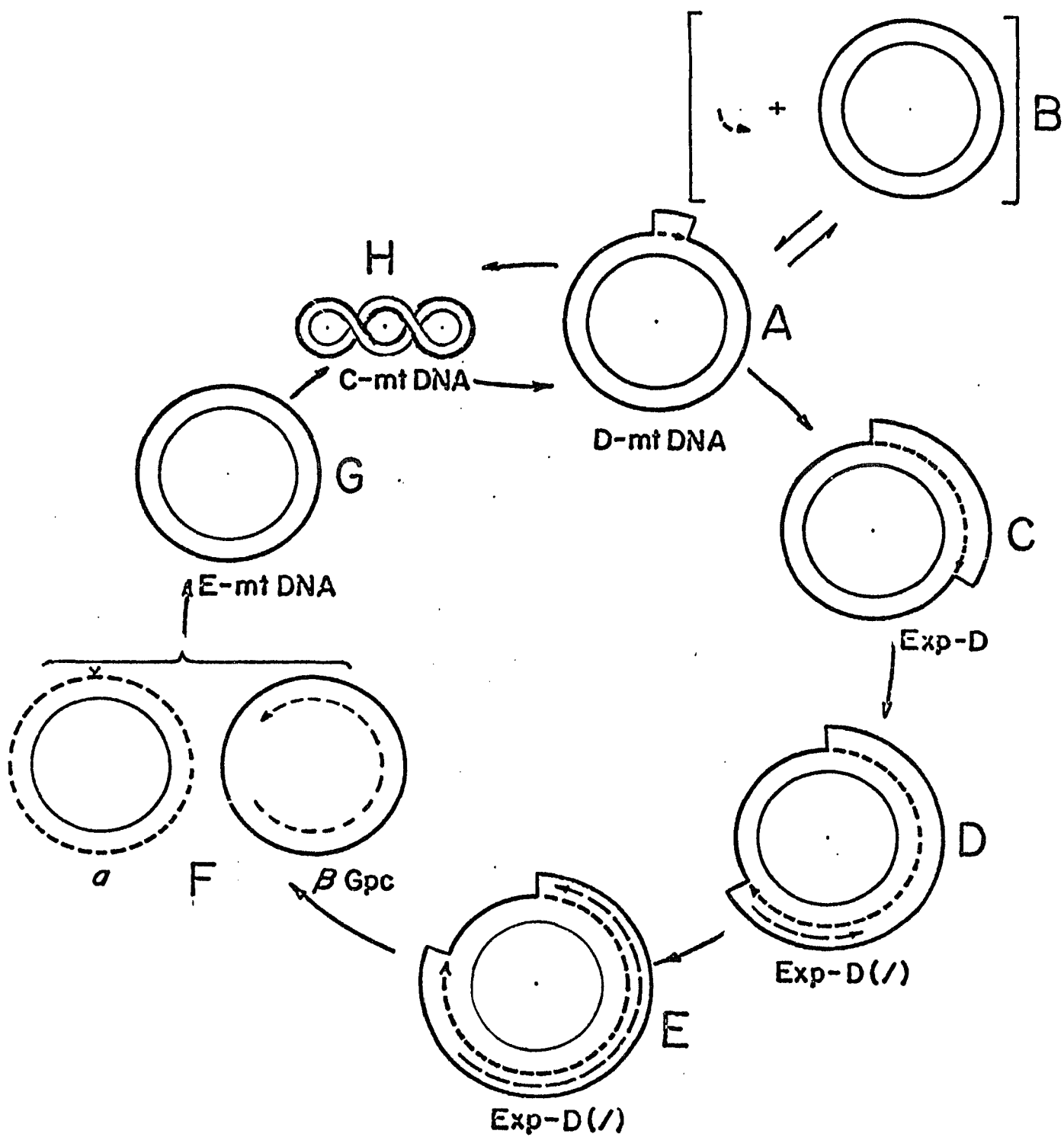
These studies have also established that the direction of leading strand replication (H strand) of mitochondrial DNA is in the opposite direction from the closely situated ribosomal cistrons. (See Figure 1A.)

The newly synthesized H strand can be isolated as an intermediate and has a sedimentation rate in alkaline sucrose gradients of 7S. It has been a major tool in the isolation and sequencing of replication origins from DNA of mouse L (Gillum et al., 1979) and HeLa mitochondria (Crews et al., 1979). Such studies have localized the replication origin of the H strand to within 300 bases of the 5' end of the 12S (small) rRNA, in both organisms.

Grossman (1973) reported the presence of alkali and RNase-H labile RNA in mature mitochondrial DNA though the distribution of such sites was not established. The sequence studies of both H and L strand origins from mouse L cell mitochondrial DNA (Gillum et al., 1979; Martens et al., 1979) has shown that 1-10 ribonucleotides are hydrolyzed from the 5' end of these regions. In addition, the DNA sequence of these origins shows a strong homology with known procaryotic DNA replication origins where RNA priming is found (Øxl74, G4). This is the first compelling evidence of a "eucaryote" showing complicity of RNA priming of DNA replication.

Figure 2. Mitochondrial DNA Replication in mouse L cells
(as proposed by Gillums and Clayton 1980).

The replication cycle begins with (A) as the H strand is displaced, and the complementary light strand forms a duplex with a small linear piece of DNA about 450 nucleotides long. This region is termed the D-loop. The 450 nucleotide piece can be lost as shown in (B). Normal replication proceeds with the D-loop expanding (Exp-D) as shown in C. D and E show replication of the other daughter strand using heavy strand as template. F , G , and H show the possible intermediates, and completed products of replication as discussed in the Introduction.



The findings from mouse L cell mitochondrial DNA replication concerning unidirectionality, discontinuity, and the general position and direction of replication in respect to the ribosomal genes have been prototypical and supported by findings with HeLa, rat liver, sea urchin, Xenopus and chick-embryo cells.

Drosophila mitochondrial DNA replication has also been studied yielding somewhat different results. As in mouse L cell mitochondrial DNA, replication is unidirectional, discontinuous and the origin maps near the ribosomal genes. However, the Drosophila mitochondrial DNA origin lies within a highly A-T rich region, comprising 25% of the mitochondria genome (Goddard et al., 1978). The leading strand is the L strand, therefore replication of the leading strand proceeds in the same direction as does the transcription of the ribosomal genes (from small to large) (Wolstenholme et al., 1979). Furthermore, no D loops are observed. Replication is highly asynchronous, i.e., 10-95% of one strand may undergo synthesis before synthesis of the complementary strand is begun. Also, Drosophila mitochondrial DNA synthesis is much more rapid. Mitochondrial DNA synthesis takes less than 15 minutes to complete as compared to the two hours required for mouse L cells to complete synthesis. This rate is found in mitochondria of Drosophila embryos, larvae, adults as well as cells in culture (Rubinstein et al., 1977). It remains to be seen

whether the considerable differences between rat and Drosophila mitochondrial DNA replication are related to the most obvious biochemical difference between these mitochondrial DNAs, the presence of an extremely large A-T rich region in Drosophila.

Mitochondrial DNA replication has also been studied but with less success, in yeast. The main difficulty lies in isolating mt DNA circles, with which to identify origins, and replication intermediates. It would be of great interest to find replication origins particular in suppressive petite yeast strains. These are deletion mutants of mitochondrial DNA which can selectively amplify that part of the mitochondrial genome that has not been deleted. Putative origins have been identified (Zamoroczy et al., 1979) but only on the basis of homology of sequences that suppressive yeasts share, not yet on the basis of any biochemical evidence.

Mitochondrial Transcription

The primary transcripts:

RNA-DNA Hybridization studies have provided much information about the location of genes as well as the nature of the transcriptional process in mitochondria. Data obtained from the hybridization of mitochondrial DNA with in vivo transcripts of rRNA, tRNA and messenger RNAs are the subject of this section.

Mitochondrial specific tRNAs have been isolated and mapped by hybridization to mitochondrial DNA from Xenopus (Ohi et al., 1978) and HeLa (Angerer et al., 1976). Both H and L strands code for tRNA (see Figure 1). The small number of tRNAs represent a bare minimum required from the genetic code, and is much below the 32 tRNAs required to satisfy the wobble hypothesis.

Saturation hybridization experiments with 16S and 12S mitochondrial rRNA show hybridization exclusively to the H strand (Dawid, 1972). These two cistrons comprise ~15% of the mitochondrial DNA in higher metazoans. There is no evidence of a 5S mitochondrial rRNA cistron. The stability and size characteristics of mitochondrial rRNAs allows ready separation from nuclear contaminants. As such they are often used as indices of mitochondrial gene expression (Chamberlain et al. 1972, Dawid 1972).

Is there a common precursor for the mitochondrial rRNAs? In Xenopus and mouse L some transcripts have been found which include both cistrons. Their scarcity might be expected as a result of rapid processing. (Rasti et al., 1979; Battey et al., 1978). In HeLa, there is a report of a fast-sedimenting mitochondrial RNA species which decreases in proportion as the time of labelling increases. Concomitant to this disappearance is the accumulation of 16S and 12S rRNAs suggesting a precursor-product relationship

(Aloni et al., 1972b). However, caution should be applied in interpreting these results. The most classic demonstration of a large precursor (32S RNA) generating the 2 ribosomal rRNAs observed in Neurospora (Kuriyama and Luck, 1973) was recently shown to be an artifact of RNA aggregation (Lambowitz et al., 1979). In yeast, no such common precursor has been found as might be expected since the ribosomal RNA cistrons are 28 kilobases apart (Sanders et al., 1977).

Hybridization of I^{125} labeled rRNA to specific DNA fragments can be monitored. Such experiments have been used to show that the 12S ribosomal RNA cistron is located within 300 bases of the A-T region of Drosophila mitochondrial DNA (Klukas and Dawid, 1976), and established the 5' 3' transcriptional direction from 12S to 16S rRNA cistron. This polarity has been found as well in Xenopus, HeLa and mouse L mitochondrial DNAs.

The isolation of mitochondrial messenger RNAs is somewhat more difficult in that they appear to be less abundant, have a rapid turnover rate and are heterogeneous in size. This makes a clear distinction from possible nuclear contaminants less likely than with the more stable and discrete sized rRNA and tRNAs. Mitochondria mRNAs have been isolated from cells treated with nuclear RNA synthesis inhibitors such as camptothecin, high concentrations of actinomycin D (Hirsch et al., 1974, Ojala

et al., 1974) or from mitochondrial polysomes (Attardi et al., 1979).

A third approach for detecting mitochondrial mRNAs has been to hybridize bulk total mitochondrial RNA to cloned, labelled fragments of mitochondrial DNA. The mixture is then subjected to S_1 nuclease which digests any free or unhybridized DNA. The resulting RNA-DNA hybrid is then analyzed by alkaline agarose electrophoresis which sizes the protected DNA segment which had hybridized. This technique (Berk & Sharp, 1977) allows the subsequent mapping of transcripts (Battey et al., 1978).

Using these approaches it was demonstrated that at least part of the messenger fraction is post-transcriptionally polyadenylated. The size of the poly A addition is generally 55-90 bases are compared to the 150-250 bases added to nuclear coded cytoplasmic mRNA. The addition of this poly A is rapid and the work of a mitochondrial-specific poly A polymerase (Jacob et al., 1974). The existence of a sizable population of nonpolyadenylated RNA of heterogenous size has been found in HeLa, yeast, and a number of other organisms, but its relationship to poly A-containing transcripts has not yet been fully characterized (Avadhani et al., 1975).

An abundant class of poly A containing transcripts from Xenopus, Mouse-L cell line and Drosophila mitochondria

has been fractionated on denaturing gels resolving 8-11 such transcripts of discrete size (Rasti et al., 1979, Battey et al., 1978, Spradling et al., 1977). Using hybridization techniques like R-looping and Berk-Sharp mapping, it has been demonstrated that these RNAs are transcribed from contiguous sections of the H-strand. The number of stable poly-A containing mitochondrial RNAs corresponds to the number of polypeptides coded for by the mitochondrial genome. In Xenopus and mouse-L a class of longer, less abundant transcripts has been detected that are coded for by regions coding for several of the more abundant RNA species. High resolution analysis of polyadenylated RNAs from HeLa mitochondria (Attardi et al., 1979) resolves 18 species, 17 which hybridize to the H-strand. The total length of these 17 transcripts amounts to 1.7x the size of the H strand. These observations are suggestive that there are long primary transcripts which are rapidly processed. The existence of large precursor transcripts has been found in yeast as well (Grivell et al., 1979; Morimoto et al., 1979), while the presence and significance of polyadenylation of yeast mitochondrial mRNAs is unclear (Jakovcic et al., 1979, Moorman et al., 1978).

Symmetric transcription

Transcription of both H and L strands of mitochondrial DNA was observed in pulse-labelling experiments by Aloni and Attardi (1971). Large RNA molecules transcribed in vivo from HeLa mitochondrial DNA hybridized extensively to both the H and L strands, and the relative extent was dependent upon the labelling time. RNA labelled for short periods of time, hybridized to both strands of mitochondrial DNA. Longer labelling periods showed preferential hybridization of the labeled RNA with the H strand. Experiments of this nature indicated that both strands of mitochondrial DNA are transcribed completely, which suggested that the L strand directed transcripts were rapidly degraded or processed. This was confirmed using kinetic studies (Attardi et al., 1979) which showed that the L strand coded RNAs had a half life between 5-10 minutes while the H-strand RNAs had a half life of between 60-120 minutes.

In addition, RNA could be isolated after short-pulse labelling which would form self-complementary, double-stranded, RNase resistant structures. Also allowing the RNA to self anneal previous to saturation hybridization experiments with mitochondrial DNA, would lower the amount of RNA that would be available to hybridize. This reduction would be a function of the amount of symmetric

transcription that had generated self complementary RNA. Using such an assay, Hendler et al., 1976 demonstrated that symmetrical transcription occurs in only 3-5% of the yeast mitochondrial genome. However, these measurements were done with mitochondrial RNAs extracted from cells labelled for long periods of time--and which were in all likelihood processed, and therefore missing the full extent of L transcription. Thus far, DNA sequence studies of fragments containing cloned tRNA genes in yeast (Martin et al., 1979) and Neurospora (Raj Bhandary et al., 1979) have indicated little evidence of any L-strand transcription in fungi. However, a recent report on a sequenced tRNA indicates that it is transcribed from the L strand in the opposite direction as nearby H-strand localized valine tRNA (Li and Tzagaloff 1979). This is the first report of an L strand specific transcript in fungi, though evidence indicates that 6-7 tRNAs are coded for by the L strand in both HeLa and Xenopus. The full extent of symmetric transcription observed by Attardi's group has not yet been corroborated in other organisms.

Clearly, the importance of RNA processing in mitochondria has tempered and complicated the analysis of in vivo transcription. Given the present data, it is not hard to imagine a rather inefficient mitochondrial system involving full transcription of both strands from one or

more promoters with major control at the level of "proof-reading" processing. Further understanding of mitochondrial transcription may eventually come from in vitro transcription analysis. In vitro dissection of the controls of mitochondrial gene expression may well involve more than the RNA polymerase-DNA interaction, and may require reconstruction of the mitochondrial DNA environment. The next section will briefly discuss the preliminary findings of a mitochondrial 'chromosome', and studies with transcriptional complexes from mitochondria.

Mitochondrial DNA-Protein Environment

Structural Studies

How is the mitochondrial DNA organized in relation to proteins, RNA and the mitochondrial membrane?

Early electron microscope studies by Nass (1969) showed that osmotic rupture of mitochondria produced highly folded DNA attached to membraneous structures. A number of workers have since isolated protein-DNA complexes using gentle lysis of mitochondria by non-ionic detergents and salt. These complexes are characterized by rapid sedimentation rate and subsequent biochemical and electron microscope visualization. Albring and Attardi (1977) described a protein-DNA complex having the appearance of a large membraneous patch similar to that described by Nass. These structures are found associated, one per

circle of mitochondrial DNA. This membrane fragment is reduced in size if rebanded in CsCl, and is proteinase-K sensitive but stable to sarkosyl, SDS and 7M CsCl. In addition, the DNA complexed with this membrane fragment gives a pattern different from purified DNA when digested with restriction enzymes. The specific DNA fragment known to contain the origin of mitochondrial DNA synthesis is displaced. It is concluded that the protection of this region of DNA from digestion suggests a highly specific binding of DNA to the membrane at this site. In vitro binding of purified closed circle P³² labeled mitochondrial DNA to membrane fragments shows no such specific association of DNA to membrane. In another study, Von Tuyle et al., (1979) isolated a similar DNA-protein complex upon sedimentation of detergent-lysed mitochondria through metrizamide. Electron microscope analysis showed a "rosette" structure containing highly folded DNA. The proteins of this complex appear to be in stable association with the DNA even in high salt, sarkosyl and SDS. SDS electrophoresis reveals a subset of these complexed proteins that co-migrate with proteins from the inner membrane, and a subset that doesn't.

A complex having a "nucleosome" like organization has also been isolated from Xenopus laevis oocytes' mitochondria (Pinon et al., 1978) using the same technique

of gentle lysis and rapid sedimentation through sucrose. This complex is visualized by electron microscopy as a relaxed circular molecule consisting of 43 globular particles interconnected by a thin filament. This complex is disrupted by SDS, sarkosyl or CsCl. An analysis of the proteins involved in this complex was not reported. Other studies have described the presence of a basic histone-like protein which appears to be associated with mitochondrial DNA. In one such study using mitochondria from Physarum, Kuroiwa et al. (1976) was able to isolate "nucleoid" structures using a combination of Triton X-100 (1%) and NP-40 (0.01%) to remove cristae and surrounding membranes. These nucleoids are digested by DNase but not RNase. The structure contains a basic protein of around 35,000 as its major component. A similar basic protein isolated by DNA affinity chromatography of yeast mitochondria extracts has been partially characterized (Caron et al., 1979).

These studies all suggest a number of problems. Artifacts might occur as a function of internal cross-linking which some workers think is a rule for mitochondrial preparations (Minssen et al., 1973), as well as non-specific binding and aggregation. The possibility of cytoplasmic and even nuclear proteins contaminating such preparations is considerable, and is not even considered in the "nucleosomal" or "nucleoid" studies mentioned. The problem of

contamination might be overcome with a more routine analysis of the DNA and protein involved using techniques of restriction and SDS electrophoresis. These problems though considerable do not erase the challenge presented to the widely held picture of mitochondrial DNA as "naked". The possibility that such complexes have function in either replication or transcription should be further examined.

Functional Studies: Are there functional units analogous to nuclear chromosomes which upon isolation could be useful probes of mitochondrial transcriptional events?

Studies with bacteria show that "transcriptional complexes" or chromosomes can be isolated, using gentle lysis, which have a highly ordered structure (Worcel et al., 1972). Such structures contain highly folded DNA, nascent RNA, membrane proteins and an RNA polymerase capable of elongating RNA chains which have been initiated in vivo. I shall briefly review evidence that similar complexes can be isolated from eucaryotic organelles.

One such transcriptional complex has been isolated from the chloroplasts of Euglena (Hallick et al., 1976). Lysis of chloroplasts with non-ionic detergent (1% Triton X) and salt (0.15M ammonium sulfate) yields an RNA polymerase-DNA complex found in the excluded fractions of gel filtration column, and subsequently purified by zone sedimentation in glycerol. The transcriptional activity of this complex is

entirely directed by endogenous DNA template. The DNA component of the complex is uncontaminated as seen by its restriction fragment pattern, and its buoyant density in CsCl, while the RNA polymerase shares all the properties of the chloroplast enzyme. Most interesting is that this complex, unlike the bacterial complex, is capable of initiating new RNA chains in vitro as determined by the incorporation of ribonucleotide triphosphates labelled with gamma P³², as well as having elongation function. Recent studies (Hallick et al., 1979) with this complex show that it selectively transcribes the rRNA genes. The lack of inhibition by heparin of either RNA initiation or elongation suggests that the chloroplast RNA polymerase may not be released from the DNA, but allowed to reinitiate further along the same strand.

Levens and coworkers (1979) have isolated a similar complex from yeast mitochondria using detergent lysis and gel filtration approach. When fractions from the excluded volume of a Sepharose 4B column are incubated with all four ribonucleotides and Mg⁺⁺, and RNA is synthesized that has the same hybridization pattern to mitochondrial DNA restriction fragments, as do in vivo transcripts. The pattern was similar to Hallick's result in that both in vivo and in vitro transcripts hybridized extensively to restriction enzyme fragments of mitochondrial DNA containing the

small and large rRNA cistrons. Little hybridization was observed to DNA fragments mapping between the rRNA cistrons, a region of mitochondrial DNA some 27 kilobases in length. This hybridization pattern does not change whether RNA is labelled in vitro for 1.5 or 20 minutes--a possible indication of multiple promoters on the mitochondrial DNA. Unfortunately, this mitochondrial transcription complex can not be used to study initiation events since initiation as gauged by the incorporation of gamma P³² labeled rNTPs does not occur. Therefore this RNA synthesis represents the elongation of in vivo initiated chains. This result is similar to the bacterial complex (Stonington et al., 1971) which could not initiate in vitro due to the demonstrable loss of sigma factor from the RNA polymerase associated with such complexes. The possibility that in vivo RNA processing may have occurred in such complexes makes it difficult to conclude as Levens does that mitochondrial transcription occurs asymmetrically from multiple promoters.

Another large difference between the results with mitochondrial and chloroplast complex is that a soluble mitochondrial RNA polymerase with the same reaction characteristics as the DNA complex-bound enzyme is found as a second peak in the included volume of the Sepharose 4B column. These results suggest that the isolation of components involved in mitochondrial transcription, and

perhaps those specifying initiation or symmetrical transcription are not entirely recoverable with such mitochondrial transcription complexes, at least under these conditions. Such components may be more labile, differently dissociable like sigma factors, or differently solubilized.

The isolation of a 'transcriptional complex' from HeLa mitochondria has been reported (Aloni et al., 1972; Carre et al., 1978). However, this complex is not functional in vitro as no polymerase activity is recovered, but is really a "transcriptional intermediate." Using CsCl-EB gradients, DNA-RNA hybrids are recovered which band at a higher density than closed circular mitochondrial DNA. Such hybrids have the appearance upon electron microscopy of many nascent RNA chains attached along the length of mitochondrial DNA. More such RNA chains were found associated with relaxed than with twisted mitochondrial DNA circles. The nascent RNA from such complexes were labelled in vivo with short pulses of H^3 -uridine, extracted and hybridized to increasing amounts of the separated strands of mitochondrial DNA, with the result that both strands appear to be transcribed to the same extent. The location of these nascent transcripts on the HeLa mitochondrial genome demonstrate that most of the transcripts coded by the L strand map directly to the right of the H-strand

origin (o) of replication with decreasing frequency in a clock-wise direction. Similarly, H-coded transcripts localize to the left of this origin (o) with decreasing frequency in a counter-clockwise direction (Attardi et al., 1979).

This result suggests that a major initiation site for RNA synthesis lies in the region close to the origin of DNA replication (o). A small cluster of L-strand transcripts is localized in the region of the L strand origin suggesting a possible second initiation site for RNA synthesis.

These studies show that a variety of information can come from the use of transcription complexes. Some of the problems with such an approach are found in Attardi's work. RNA aggregation and non-specific binding of nascent RNA to DNA can occur, and complicate interpretation. Also, some associations of longer transcripts might be less stable, and harder to recover using this technique. Lastly, the use of SDS and CsCl, as well as a pronase treatment routinely employed to increase the yield of RNA/DNA hybrids (Carre et al., 1978) limit this technique by precluding the possibility of studying the proteins that might be associated with particular stages of initiation or termination of transcription in such complexes.

In Vitro Transcription

Ultimately the in vitro transcriptional analysis will be used to delineate the regulatory sequences and to reconstruct the events surrounding the initiation and termination of mitochondrial transcription. Attempts at using purified components have used isolated homologous and heterologous polymerases, a variety of templates as well as crude transcriptional complex and mitochondrial lysates. The use of the latter, i.e., isolated mitochondria and crude extracts is limited in that activities of endogenous DNases, RNases, proteases and processing enzymes complicate interpretation. The results with complexes and enzymes are discussed briefly in reference to the major questions that have been asked thus far concerning a) initiation, b) symmetry, c) fidelity.

a) Initiation: No assignment of particular mitochondrial DNA sequences as "promoter regions" has yet been made. Findings from yeast transcriptional complexes suggest multiple promoters, while the findings of Attardi et al. (1979) indicate that major promoter regions may coincide with the origins of replication. Preliminary studies of binding of purified yeast mitochondrial RNA polymerase to 5' labeled DNA fragments have shown that the polymerase preferentially binds to the particular fragments flanking both the ribosomal cistrons (Levens et al., 1979). The same restriction fragments are preferentially hybridized to by in vitro

transcripts. Promoter identification must await a sequencing of the 5' end of these primary transcripts to see if they correspond in sequence to the sequences flanking the polymerase bound regions. Also, promoter-type binding should be tested under more rigorous conditions (high salt, heparin). Other studies using Xenopus oocyte mitochondrial RNA polymerase with mitochondrial DNA (Wu et al., 1974) showed that between 6-8 different oligonucleotide RNA chains/genome can be initiated with 5' gamma labelled phosphate. Further studies will have to correlate these transcripts with in vivo products, as well as more rigorously identify them by fingerprinting and mapping. This latter study also demonstrated that mitochondrial RNA polymerase initiates its transcripts with ATP or CTP.

b) Symmetry and Strand Preference: Similarly to findings with in vivo studies (Aaij et al., 1970) observations of 30-40% RNase resistance of the primary transcripts have been observed with in vitro studies in rat liver (Saccone et al., 1975), suggesting a good deal of symmetrical transcription. These studies have also shown a marked H strand preference--that is, more of the RNAs made in vitro hybridize with the H-strand. The studies of Wu and Dawid (1974) with a purified mitochondrial polymerase and DNA template from Xenopus show that strand selection may be altered by modifying DNA 2° structure. The H strand is

preferred if either closed or nicked circle Xenopus mitochondrial DNA is the template. If denatured mitochondrial DNA is used, the L strand is preferred. This change in strand preference was not influenced by the degree of purification of the polymerase nor by variation in the reaction conditions like salt, or inclusion of Mn^{++} .

It is interesting to note that while symmetry of transcription of mitochondrial DNA is observed using heterologous E. coli and nuclear polymerases (I-II) the strand preference demonstrated by mitochondrial polymerase for its template is not seen. That is, nuclear polymerase I and II show no strand preference (Saccone et al., 1975) while E. coli prefers to transcribe L-Strand DNA as assayed both in vitro and in minicells (Chang et al., 1975). The alteration of preference as a function of changing DNA 2° structure, is observed with only the mitochondrial RNA polymerase.

c) Fidelity: As genes and transcripts become easier to isolate, better characterization will allow more rigorous assays of fidelity.

Another approach towards assaying fidelity of the in vitro transcriptional events has been to use in vitro synthesized RNAs to direct protein synthesis using E. coli translations systems. These translation products are immuno-precipitated with antibodies to mitochondrially

synthesized proteins such as subunits of cytochrome oxidase. SDS electrophoresis is then performed and compared to SDS profiles of in vivo labelled mitochondrial proteins (Saccone et al., 1975). Such studies have shown some success in that a product with the same size and antigenic properties as the in vivo made subunits are produced by an in vitro system. However, also produced are some non-related lower molecular weight products. There is some claim that this is not necessarily due to non-specific transcription, but a problem inherent in using the heterologous E. coli translation system. Moorman and Grivell (1976) have shown this translation system produces a number of artifactual products when programmed with in vivo made mitochondrial RNA. Certainly the issue of faithful transcription will be greatly resolved by the development of a homologous in vitro translation or coupled transcript-translation system from mitochondria.

The Enzyme of Mitochondrial Transcription:

Mitochondrial RNA Polymerase

Mitochondrial RNA polymerase has been isolated and characterized in a handful of organisms: yeast (Tsai et al., 1971; Eccleshall et al., 1974; Rogall and Wintersberger, 1974; Scragg, 1976a; and Levens et al., 1979), Neurospora crassa (Kuntzel and Schafer, 1971), Xenopus laevis ovaries (Wu and Dawid, 1972), rat liver (Reid and

Parsons, 1971; Mukerjee et al., 1973; Gallerani et al., 1972, 1976), bovine spermatozoa (Hecht et al., 1979).

These studies all employed purified mitochondria, and use a variety of methods for solubilization and purification of the enzyme. Solubilization was generally achieved using non-ionic detergents (NP40, Triton-X, deoxycholate, digitonin), or high salt (0.5 M KCl or $(\text{NH}_4)_2\text{SO}_4$), suggesting attachment to the membrane.

Most workers have used glycerol gradient centrifugation, ammonium sulfate fractionation and ion-exchange chromatography for subsequent purification. Using DEAE Sephadex (Tsai et al., 1971; Eccleshall et al., 1974; Hecht et al., 1979; Mukerjee et al., 1973; Reid and Parsons, 1971), DEAE Cellulose (Scragg, 1976) or phosphocellulose (Wu et al., 1972), these studies resolved a heterogeneous pattern of multiple peaks of activity. A number of workers have concentrated on the most active peak, and demonstrated a more homogeneous enzyme after purification (Wu et al., 1972; Scragg, 1976). The multiplicity of activities has been explained as due to endogenous DNA, different interactions of co-purifying proteins, and "ion effects" though none of these have conclusively been shown to be the case.

Another approach was the use of size fractionation as an early step in purification utilizing glycerol

gradients (Kuntzel et al., 1971). This technique took advantage of this enzyme's aggregation in low salt whereby a heavily aggregated activity sediments faster than the bulk of mitochondrial protein facilitating separation. A second gradient in high salt which favors dissociation of the aggregate into a number of lower molecular weight forms was used as a further purification step. This size separation as a function of ionic strength has been observed as well after ion exchange chromatography of the polymerase activity (Scragg, 1976; Wu et al., 1972). A third mode of purification involves affinity chromatography using DNA cellulose (Eccleshall et al., 1974).

These studies have all found purified RNA polymerase(s) whose activity is dependent on all four ribonucleoside triphosphates, and on a DNA template. Incorporation by these activities have in general been found sensitive to actinomycin D, treatment with DNase and RNase, and KOH hydrolysis.

Some properties have been found which distinguish the mitochondrial enzymes from other RNA polymerase activities. These mitochondrial polymerases have a Mg^{++} optima of between 10-20 mM, and are preferentially stimulated by Mg^{++} rather than Mn^{++} , as opposed to the nuclear polymerases. They are not inhibited by high concentration (100 ug/ml) of alpha-amanitin distinguishing them from nuclear polymerases I and III (Phillips et al. 1973, Roeder 1974a,

Weil et al. 1975). Though they can use a broad range of heterologous DNA templates including native or denatured calf thymus DNA, the mitochondrial polymerase(s) have greater activity on poly dAT or mitochondrial DNA templates. High ionic strength (greater than 0.1 M KCl or 0.1 M $(\text{NH}_4)_2\text{SO}_4$) strongly inhibits these enzymes more than 50%. Other properties these mitochondrial enzymes share is that they are extremely labile, as well as having 10-100 fold lower specific activity than E. coli or nuclear RNA polymerases.

Findings of rifampicin sensitivity with these mitochondrial polymerase activities have been quite varied (see Table 12). The Neurospora (Kuntzel et al., 1976), yeast (Scragg, 1976) and rat liver (Gallerani et al., 1976) enzymes appear to be sensitive to this antibiotic though different concentrations are required for the same degree of inhibition. A dose of 10 ug/ml abolishes nearly all the activity with the Neurospora polymerase, while inhibiting less than 30% of the yeast enzyme. A number of other studies have demonstrated that the Xenopus (Wu et al., 1972) and yeast (Rogall et al., 1974; Tsai et al., 1971) enzymes are resistant to 100 ug/ml of rifampicin. Some claim that the rifampicin sensitivity can be lost during the purification process (Saccone et al., 1975). However, amongst very large mitochondrial RNA polymerases (probably aggregate activities) which have been reported one account

reports rifampicin sensitivity (Scragg, 1976), while others don't (Eccleshall et al., 1974; Goldberg et al., 1973). The majority of studies have demonstrated a low molecular weight RNA polymerase with a single subunit of around 45-65,000 daltons resolved as a single band upon SDS electrophoresis. There exists at present, an interesting correlation amongst these low molecular weight activities, that the rifampicin sensitive enzymes all appear to be in the 60-70,000 dalton class while a number of rifampicin resistant enzymes particularly Xenopus (Wu et al., 1972) and yeast (Levens, 1979) are about 45,000 daltons. It is not clear from examining these studies at what stage a rifampicin-sensitivity might be lost as it was not monitored throughout the purification. Its putative loss appears not to be related to the solubilization method employed, or subsequent ion-exchange chromatography as these are shared by procedures yielding both types of response to this inhibitor.

Lastly, the coding of the mitochondrial RNA polymerase by the nuclear genome, and its transport into the mitochondria has only been slightly detailed in the literature. The evidence of increased amounts of mitochondrial RNA polymerase in the cytosol of cells treated with inhibitors of mitochondrial transcription (ethidium bromide) and translation (chloramphenicol) found by Barath and

Kuntzel (1972) is strongly suggestive of the nuclear-origin and cytoplasmic synthesis of this protein. This has been further borne out by the work of Scragg (1976b) who found decreases in labeled mitochondrial RNA polymerase in yeast cells treated with cycloheximide, not with ethidium bromide or chloramphenicol.

Other work has shown that a number of petite strains which sustain small deletions in mitochondrial DNA and lack mitochondrial protein synthesis, maintain a normal concentration of RNA polymerase (Tsai et al., 1971; Scragg, 1976). Data concerning the mechanism and timing of transport as well as turnover of the mitochondrial RNA polymerase has not yet been found in any system. Future studies might well attack the question of transport to see if cycloheximide or chloramphenicol inhibit the "import" of labeled mitochondrial RNA polymerase into the mitochondria, and also might well examine the timing of such an event.

Objective

This study was undertaken to purify the mitochondrial RNA polymerase from Drosophila melanogaster and to characterize it with respect to size, inhibitor sensitivity, and enzyme activity requirements. This activity could then be compared to nuclear RNA polymerases as well as other reported mitochondrial RNA polymerases. The selection of Drosophila was made on the basis of its well-characterized

mitochondrial DNA, its distinct developmental stages, the relative ease of its growth in large amounts and its sophisticated genetics. All of these could contribute to future studies of the structure and function of the mitochondrial RNA polymerase and its role in the gene expression of mitochondria.

MATERIALS AND METHODS

Materials

High molecular weight calf thymus DNA, Sepharose 6B and 4B, Sephacryl S-200, unlabelled nucleoside triphosphates, dithiothreitol, α -amanitin, rifampicin and digitonin were purchased from Sigma Chemical Co., St. Louis, Mo.

Acrylamide, TEMED, ammonium sulfate and NP40 were purchased from Bio-Rad., Richmond, Cal. [H^3]-UTP with a specific activity of 13 Ci/mm and sucrose were purchased from Schwartz-Mann, Orangeberg, N.Y.

Growth and Handling of Experimental Organism

Adults of Drosophila melanogaster (Oregon R) were grown in large population cages kept at room temperature. The flies were fed daily with media containing corn-starch, molasses, propionic acid, dried yeast and agar. Eggs that were deposited on the media were allowed to continue development into larvae and pupae with fresh yeast added daily as a nutritional supplement. Eggs to be used for the preparation of egg mitochondria were collected separately on sheets of agar and yeast-paste, within 12 hours of deposition. Adults were 5-10 days old at the time of homogenization. Pupal material was routinely kept for several days at 4°C prior to use.

All material was extensively washed with distilled H_2O before use. Eggs were dechorionated by washing with a 2% sodium hypochlorite solution for several minutes, filtered on Miracloth and extensively rinsed to remove the hypochlorite.

Solutions

STE - 0.3 M sucrose, 0.02 M Tris-HCl pH 7.9 and 1 mM EDTA.

TGED contained 15% (w/v) glycerol, 50 mM Tris-HCl pH 7.9, 0.1 mM EDTA and 0.2 mM dithiothreitol (DTT).

Solutions of rifampicin were made in three different ways in water, in ethanol and in H_2O with DMSO. No significant difference between them was obtained in the enzyme response.

All other solutions were made in distilled H_2O .

Column Matrix Preparation

Sepharose and Sephacryl matrices can be poured without preparation.

DEAE Sephadex A-25 and phosphocellulose were prepared by the method of Burgess (1969). DEAE Sephadex is equilibrated first with 0.5M HCl, then with 0.5M NH_4OH , and finally with 0.05 M Tris-HCl pH 7.9. Phosphocellulose is equilibrated first with base, then with acid, and then equilibrated to pH 7.9 with addition of Tris-HCl.

Heparin Sepharose 4B was prepared by the coupling of heparin to cyanogen bromide-activated Sepharose 4B according to Sternbach et al. (1975). Sepharose 4B (100 grams) was extensively washed with distilled water to remove sodium azide and salts and resuspended in ice cold 0.2M carbonate buffer. The temperature of the activation step is critical so all solutions were kept at 4°C by the addition of ice. In a ventilation hood, 15g of cyanogen bromide (CNBr) is added to the stirring ice-cold solution of Sepharose 4B. pH was monitored and kept above 11 by the addition of 8 N NaOH until the activation reaction is complete (10-15 minutes). The CNBr activated Sepharose is then filtered, washed with 4-5 liters of 0.1 M carbonate buffer, and 250 mg of heparin in 0.1 M carbonate buffer is added. This mixture is stirred overnight at 4°C, filtered and washed extensively, resuspended in water, and stored at 4°C for further use.

Columns were poured to the sizes indicated in the figure legends, and were in general, equilibrated to 0.05 M $(\text{NH}_4)_2\text{SO}_4$ in TGE unless otherwise stated.

Polylysine kieselguhr chromatography was prepared by the method of Ayad (1972). A slurry of 5 grams of kieselguhr suspended in 25 ml of 0.4 M NaCl in 0.02 M potassium phosphate pH 6.8 was boiled gently to remove trapped air, after which 0.8 ml of polylysine (10 mg/ml)

was added. The column was poured, equilibrated and the sample bound in 0.4 M NaCl in the same buffer. Washing the column to remove protein is done with 1.7 M NaCl in phosphate buffer, while elution of the 260 nm absorbing species is done with a linear gradient of NaCl, 1.7 M to 2.4 M NaCl in phosphate buffer.

Preparation of Templates

Calf thymus DNA purchased from Sigma Chemical Co. was resuspended in buffered saline-EDTA (0.15 M NaCl, 0.01 M Tris-HCl pH 7.5), and twice phenol extracted. The second phenol extraction was followed by ethanol precipitation, and extensive dialysis against a buffer containing 10 mM Tris and 0.1 mM EDTA. This purified DNA was stored at a concentration of approximately 1.1 mg/ml.

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

Poly dAT was stored at 10 units/ml (0.5 mg/ml). When used as template, 10 ug was added to each reaction.

Assays

Assay for RNA polymerase: The assay was done according to the method of Nishiura et al. (1975). The standard reaction mix was 50ul per assay containing:

20 ug DNA (10 mg ss DNA, 10 mg ds DNA)

50 mM Tris-HCl pH 7.9

10 mM Mg^{++}

0.6 mM nucleotides (ATP, CTP, GTP)

1 mM DTT

1 uCi of H^3 UTP (~10,000 CPM/pmol).

The total reaction volume was 0.1 ml with samples of 5 -50 λ being assayed as indicated. The enzyme reactions were terminated after 20 minutes at 25° C by the addition of 10 volumes of cold TCA (10%) containing 2% sodium pyrophosphate. The TCA treated mixtures were allowed to stand on ice for 20 min, filtered through Whatman GF/A glass fiber filters, and washed with three 5 ml portions of TCA (5%) containing 2% sodium pyrophosphate, and with 95% ethanol. After drying, the radioactivity of the filters was determined in 4.5 ml of a toluene-based scintillation cocktail.

One unit of activity reflects the incorporation of 1 pmol UMP into cold TCA precipitable material in 20 min at 25° C.

Protein concentration

Three methods were alternatively used to determine protein concentration.

Absorbance at 280 nm as well as 280/260 ratios were used along with the Warburg-Christian equation. This method could be used with highly diluted samples (1:20-1:50) to eliminate detergent effects.

The methods of Lowry et al. (1951) and Bradford (1977) were used to measure protein. Both techniques have a similar range of sensitivity and gave similar values when compared, however the Bradford technique is more convenient. Standard curves for both protein assays were made with bovine serum albumin. Both tests are distorted by very high concentration of non-ionic detergents (such as 3% Triton) but less so upon dilution. This becomes a problem late in purification for accurate protein estimation since the protein concentration is not large enough to be measured with a diluted sample. However, inclusion of SDS (1%) seems to nullify the effect of detergent on protein-Coomassie binding in the Bradford technique.

DNA concentration

An absorbance of 20 O.D. units was assured to be equivalent to 1 mg/ml of DNA. Indication of the proportion of nucleic acid in polymerase preparations was arrived at from the A280/260 ratio. As with protein determination, detergent concentration had to be monitored closely for its effect on absorbance at 260 nm.

Salt concentration: $(\text{NH}_4)_2 \text{SO}_4$ and NaCl were measured by conductivity by using a Radiometer (model YS 31-Yellow Springs) Instrument Co., Yellow Springs, Ohio. Samples were diluted 1/100 (50 λ in 5 ml distilled H_2O). Standard curves were prepared with buffers of known ionic strength. All measurements were made at room temperature.

Other enzyme assays:

Cytochrome oxidase

0.25 ml 0.1 M Tris-HCl, pH 7.4

0.25 ml cytochrome c (1mg/ml). Sodium dithionite (a few grains) is added until the solution turns pink, and bubbled until H_2S is not detectable.

0.5 ml H_2O

10-50 λ enzyme

The decrease in absorbance at 550 nm is recorded for 2-3 minutes at 20 second intervals.

Malate dehydrogenase

A solution of assay mixes are made up as follows:

5.3 mg NADH in 12.5 ml of 0.2 M Tris HCl pH 7.6

18 mg oxaloacetate in 15 ml of 0.2 M Tris HCl pH 7.6

22.5 ml of 0.2 M Tris HCl pH 7.6

40 ml/40 assays

The decrease in absorbance at A_{340} is recorded for 2 minutes at 15 second intervals.

Yeast Alcohol dehydrogenase

0.2 ml ethanol (12 ml 95% EtOH/100 ml)

0.3 ml NAD (1.6 mg/ml)

0.5 ml 0.032 M pyrophosphate buffer pH 8.8

50 λ enzyme

Increase in absorbance at 340 nm is measured at 15 second intervals for 2 minutes.

CsCl centrifugation

Mitochondrial samples were lysed in 1% SDS and treated with pancreatic RNase (50 ug/ml) at 37°C for 30 min, followed by incubation with 50 ug/ml of self-digested pronase for 1 hr at 37°C. Samples of 1.5 ml with labelled yeast nuclear and mitochondrial DNA as markers (see below) were transferred to cellulose nitrate ultracentrifuge tubes containing 4.1 ml of CsCl. Density was adjusted to 1.695 gm/cc, with the use of a refractometer, and mineral oil was used to fill the tubes. Samples were centrifuged at 33,000 RPM in a Spinco 50Ti rotor for 40 hours at 19 C. Fractions of 0.2 ml were collected from the bottom of the gradient. Aliquots (50ml) of each fraction were assayed for TCA precipitable radioactivity. The remainder of each fraction was brought to 1 ml with distilled H₂O, and absorbance at 260 nm was read.

Preparation of labelled yeast DNAs: Labelled nuclear and mitochondrial DNA were prepared according to the method of Rubin et al. (1977). Spheroplasts of cultures labelled with

H^3 adenine were prepared, lysed in Sarkosyl and centrifuged as above. The collected fractions were subjected to KOH hydrolysis to remove any RNA, followed by the assay of TCA precipitable radioactivity of 50 λ aliquots.

Isolation of mitochondria from Drosophila

10-200g of Oregon-R Drosophila (eggs, larvae, pupae or adults) were broken with mortar and pestle in cold STE and filtered through gauze to remove debris. This homogenate (15-20%) was then centrifuged at 1000, 2000, 2500 and 3000 RPM in a GSA rotor to remove nuclear and granular material. The remaining supernatant was then spun twice at 15,000 x g for 20 minutes, and the pellets from these centrifugations pooled in 10-20 ml of fresh buffer (crude mitochondrial pellet).

The crude mitochondrial pellet was dounced gently and layered on either a 30 ml linear sucrose gradient (0.9 M to 1.9 M) or on a step gradient of 1.2 and 1.5M sucrose with a 2 M cushion and spun for two hours at 20,000 RPM in a Sorvall HB-4 rotor. Fractions (1-2ml) were collected from the top by Pasteur pipette, and assayed for MDH, CO and RNA polymerase activities.

The fractions containing all three activities (~1.32-1.42M sucrose) were pooled, diluted 5 fold with STE and repelleted by centrifuging at 20,000 x g. The pellet was washed, made 10 mM with respect to $MgCl_2$ and incubated

for one hour at 5° C with 100 ug/ml pancreatic DNase I. After DNase treatment the suspension is made 10 mM with respect to EDTA, and washed once with 5 mM EDTA in STE buffer and once with STE buffer. The washed pellet is then resuspended in TGE and frozen at -10° C until use (within one week). The volume of TGE is 1 ml/gm of starting material. This is approximately 1-10 mg/ml protein as determined by Lowry test of mitochondrial suspension.

Both pupae and adult flies were used as starting material in the following experiments with no modification in procedure required. The amount of material used to obtain purified mitochondria could be scaled up to 250 grams, above which significant loss in the yield of organelles occurred. The isolation of mitochondria from 10-20 hour embryos involves a gentler mode of breakage, and fewer low speed spins are required.

Solubilization of Mitochondrial RNA polymerase

Isolated mitochondria were thawed and detergent added to either 3% Triton-X or 0.5% NP 40. This suspension was dounce homogenized several times, and then stirred at 4° C for 20 minutes. After centrifugation at 80,000 xg for 90 minutes, the supernatant fraction was used in the following experiments.

Ammonium sulfate fractionation: Most of the preparations used this procedure to concentrate the protein. The supernatant was made 55-60% saturated with respect to $(\text{NH}_4)_2\text{SO}_4$. The precipitate which result from centrifugation at 20,000 xg for 30 minutes was resuspended in TGED containing 0.05 M $(\text{NH}_4)_2\text{SO}_4$ and 0.5% NP -40. In 2-step ammonium sulfate fractionation, a protein precipitate recovered from a 25% ammonium sulfate saturation, containing no RNA polymerase activity, was discarded. The remaining protein containing the RNA polymerase is precipitated at 50-60% ammonium sulfate saturation.

Polyethylene glycol (PEG) precipitation: In a number of experiments this step was used to remove to endogenous mitochondrial DNA. The technique of Alberts et al. (1971) as modified by Greenleaf et al. (1976) was used, and is described in full detail in the section describing the handling of the whole fly extract.

Treatment of solubilized mitochondrial extracts with DNase: Extracts were made 10 mM with respect to MgCl_2 and 50 ug/ml of pancreatic DNase was added, and incubated at 4° C for 30 minutes.

Gel Filtration Chromatography: Samples (5-10 ml) of solubilized mitochondrial extract were loaded on to Sephacryl S-200 columns in either high or low salt as indicated. Preparation I involves low salt gel filtration followed by high

salt gel filtration. The details of column and fraction size, flow rate and assay are included in the relevant figure legends.

Ion-exchange and affinity chromatography: Extracts were loaded onto columns of heparin-Sepharose, DEAE-Sephadex and phosphocellulose as described. Care was taken that the $(\text{NH}_4)_2 \text{SO}_4$ concentration of the samples as measured by conductivity was less than 0.05M for the DEAE Sephadex and phosphocellulose columns. Heparin-Sepharose was variably loaded at 0.05, 0.1 or at 0.175 M as indicated. All columns were equilibrated with the same salt concentration as found in the sample. Both step and gradient elution were used. Conditions of elution, and fraction collection are indicated. Detergent in the elutant does not effect position of peaks, but does effect the size of fractions. Octyl Sepharose columns were pre-equilibrated with 1 M $(\text{NH}_4)_2 \text{SO}_4$ and TGED, with samples applied in high salt, and elution in low salt and detergent as indicated.

Glycerol Gradient Centrifugation

Peaks with RNA polymerase activity from gel filtration columns were concentrated, and 1.5-2 ml samples with 3% glycerol were layered on 10-30% glycerol gradients of 11 ml. High salt gradients and sample contained 0.5 M KCl, while low salt gradients and sample contained 0.5 M KCl. The gradients were centrifuged in the Beckman

SW 41 rotor for 16 hours at 50,000 RPM. Fractions of 15-20 drops were collected from the bottom of the gradient.

SDS gel electrophoresis

SDS gel electrophoresis was performed by the method of Weber and Osborn (1969). The sample concentrated by either dialysis against PEG 20,000 or $(\text{NH}_4)_2\text{SO}_4$ precipitation was extensively dialyzed against sample buffer and boiled for 3 min in the presence of B-mercaptoethanol. Gels of 7.5 and 10% acrylamide were electrophoresed at 1.5-2 mA/gels and stopped when the tracking dye reached the bottom of the gel. Gels were stained with 0.1% Coomassie brilliant blue R-250 in methanol/acetic acid/ H_2O (5:1:5), destained in the methanol/acetic acid/ H_2O solution, and densitometrically scanned at 590 nm with a Gilford spectrophotometer.

Mitochondrial RNA Polymerase preparation from whole fly homogenates.

Two hundred grams of pupae were broken in a Waring blender in TGED containing 0.5% NP-40. The filtered homogenate was made 1.7 M with respect to NaCl, and 10% with respect to PEG, stirred and centrifuged. (All centrifugations in this preparation were for 30 min at 15,000 xg unless otherwise stated.) The DNA-rich pellet

was discarded. To the supernatant, 1/5 volume of saturated ammonium sulfate (pH 7.9) was added. The solution was stirred and centrifuged resulting in 2 phases. The top phase (PEG-rich) was discarded, and to the bottom phase was added an equal volume of saturated ammonium sulfate. The solution was again stirred and centrifuged. The resulting precipitate was resuspended in TGED and the salt concentration of the extract adjusted to 0.05 M $(\text{NH}_4)_2\text{SO}_4$. The enzyme is then adsorbed batchwise to phosphocellulose pre-equilibrated with 0.05 M $(\text{NH}_4)_2\text{SO}_4$ in TGED buffer. The slurry was stirred for 30 min, collected by filtration in a Buchner funnel, and washed extensively with the same buffer. The RNA polymerase activity was eluted from this ion-exchange resin by suspending the resin in about 2 volumes of 0.6 M $(\text{NH}_4)_2\text{SO}_4$ in TGED, stirring for 30 min, and filtering as before, to remove the phosphocellulose from the RNA polymerase. The protein was concentrated by the addition of an equal volume of saturated ammonium sulfate to the filtrate, stirred and the precipitate collected by centrifugation. This precipitate was loaded and chromatographed on a Sephacryl S-200 column in high salt as described in Figure 10. Peak A¹, the low molecular weight RNA polymerase activity resolved by this column was further purified after adjusting the $(\text{NH}_4)_2\text{SO}_4$ concentration to 0.15 M by binding to a 10 ml heparin-Sepharose column, and eluting with a linear gradient of $(\text{NH}_4)_2\text{SO}_4$, 0.15 M-0.6 M.

RESULTS

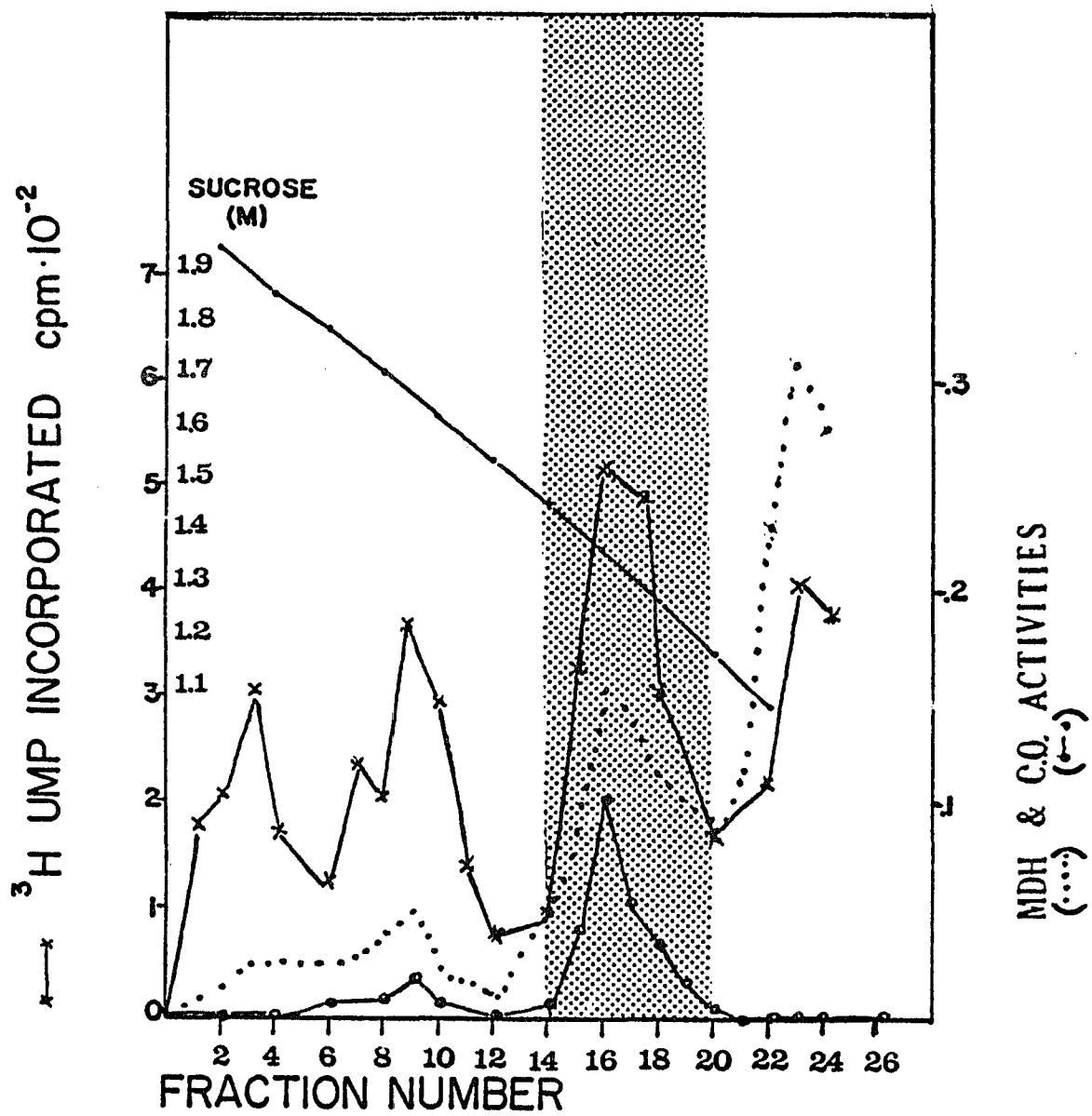
Preparation of mitochondria

The isolation and characterization of mitochondrial proteins necessitates great amounts of purified material. Perhaps because of the limited work done with Drosophila mitochondrial proteins, a technique giving a high yield of pure mitochondria had not yet been developed. The technique used here is a modification of that used by Bultmann and Laird (1973) and Goldring and Peacock (1977) for the isolation of Drosophila mitochondrial DNA.

This approach involves breakage of eggs, adults, and pupae with mortar and pestle in a sucrose buffer, filtration of the homogenate through cheesecloth to remove exoskeleton and cuticular debris, followed by several low speed centrifugations to remove nuclei, cellular debris, and granular material. The supernatant was then subjected to high-speed centrifugation and a pellet of partially purified mitochondria was recovered. This pellet was DNase treated (see Methods), layered on sucrose gradients, and centrifuged at $15000 \times g$ for 120 minutes. Results obtained with a linear-sucrose gradient are shown in Figure 3. Each fraction was assayed for MDH, CO, and RNA polymerase activities. A peak at 1.35 M sucrose of coincident CO, MDH, and RNA polymerase activities contained intact mitochondria as determined by light and electron microscopy. A smaller peak banding at 1.8 M sucrose was also recovered, which may well be mitochondria trapped by the visible eye pigment granules

Figure 3. Fractionation of mitochondria by linear sucrose gradient centrifugation.

A "crude mitochondrial pellet" (see methods) from 50 grams of Adult flies was dounced gently, and layered on a 30 ml 1 M - 1.9 M sucrose gradient, and spun for 2 hours at 20,000 RPM in a Sorvall HB-4 rotor. 1.5 ml fractions are collected from the top by Pasteur pipette, and each fraction assayed for MDH activity (. . . .), cytochrome oxidase (o——o) and RNA polymerase (x——x) as shown. The major peak containing all three activities (fractions 14-20) is shaded in (intact mitochondria). Bottom of the gradient is at left.



banding at this density. Occasional shoulders occur at approximately 1.2 M sucrose which upon microscopic inspection appear to be broken mitochondria. This was further suggested by the fact that the MDH/CO ratio, which has been used as an index of 'intact mitochondria', is consistent from preparation to preparation for the major mitochondrial peak at 1.35 M sucrose but varies for shoulder peaks.

A two step gradient of 1.1-1.5 M sucrose has also proven effective in separating mitochondria from granular debris as well as more soluble proteins. The mitochondria are found at the interface of the two sucrose concentrations.

Quantitation of Mitochondrial recovery

Mitochondrial recovery as a function of different preparative techniques can be monitored by following unit recovery of specific mitochondrial enzymes (Schnaitman et al, 1968) or mitochondrial DNA (Bogenhagen et al., 1974).

Cytochrome oxidase activity was employed for estimates of total recovery of mitochondria primarily because this mitochondrial specific enzyme can be assayed in a crude homogenate as well as after sucrose gradient purification of mitochondria. Malate dehydrogenase (MDH) activity was also monitored during purification. However the recovery of the mitochondrial matrix-localized form of this enzyme can be measured only after sucrose-gradient centrifugation or

TABLE 1
Mitochondrial Recovery

I. Breakage Regimen	C.O. in SUP (units)	C.O. % recov.	After Sucrose-Grdt. Fractionation		DNA (Total) A ₂₆₀ Units PLK
			MDH (units)	C.O. (units)	
Motor Driven					
2 Min	54	73%	--	--	--
1 Min	55	74%	580	18	--
30 Sec	45	60%	--	--	--
Mortar/Pestle	75	100%	1250	40	--
II. Centrifugation Regimen					
No Spin	75	100%	--	--	10.5 (100%)
1000 RPM	68	90%	--	--	--
2000 RPM	50	66%	700	25	--
1000, 2000 RPM	65	85%	--	--	--
1000, 2000 + 2500 . . .	61	81%	1050	35	--
III. Gradient					
Washed Mito Pellet (No Grdt.)	53	70%	--	--	7.1 (68%)
Step	35	46%	--	--	4.2 (40%)
Linear	27	36%	--	--	--

extensive washing of the crude mitochondrial pellet. This is because there is a cytoplasmic non-mitochondrial form of this enzyme which is removed by gradient purification. The fact that the ratio of MDH to CO activity is consistent from preparation to preparation, when the sucrose-gradient purified mitochondria are assayed is an indication of the integrity of the inner mitochondrial membrane. Furthermore, it strongly suggests that the cytoplasmic form of MDH is not present in sucrose-gradient purified mitochondria. These enzyme activities in the purified mitochondria were compared from preparations with the same amount of starting material to determine how mitochondrial recovery might vary as a function of different breakage and centrifugation regimen. These results are shown in Table 1.

As shown in panel 1A, breakage using a hand-powered mortar and pestle was found to lead to better recovery of C.O. activity than the use of motor-driven pestle regardless of the time and speed of homogenization. In testing different low speed centrifugation regimen for their effect on recovery, it is shown in panel B of Table 1 that 10% of the C.O. activity was found in the pellet fraction after centrifugation at 1000 RPM. In a similar experiment, centrifugation at 2000 RPM resulted in a 35% loss of CO activity from the supernatant fraction. Interestingly, centrifugation first at 1000 RPM followed by one at 2000 RPM resulted in only a 15% loss of CO activity, while successfully removing

much of the red granular debris. Additional centrifugations at 2000 and 2500 RPM removed further debris while decreasing the recovery of CO activity only an additional 5% resulting in a net 80% recovery of CO activity in a supernatant fraction.

The subsequent use of a sucrose gradient is essential for further separation of mitochondria from contaminating organelles and soluble supernatant enzymes activities. As shown in the third and fourth columns of Table 1, sucrose gradient purified mitochondria from preparations using different breakage and centrifugation regimens were compared to their MDH and CO activities. A similar proportional increase in total units recovered using mortar-pestle breakage and "graded" centrifugation regimens is found for both CO and MDH activities as shown in the third and fourth columns of table 1. This table also shows that a step gradient gives 70-80% recovery of CO activity when compared to the washed mitochondrial pellet. A linear gradient results in 50-60% recovery.

Another mode of analysis of mitochondrial recovery uses recovery of mitochondrial DNA. Bogenhagen et al. (1974) using highly labelled mitochondrial DNA showed a 72% overall recovery with a "crude" mitochondrial pellet and 26% after step sucrose gradient fractionation. The method I have employed using PLK chromatography shows a 68% recovery with a "crude pellet" and a 38% recovery of mitochondrial DNA

after a step gradient. The purity of mitochondrial DNA as monitored by this technique will be discussed shortly.

Purity of Mitochondria

Since these studies concern a mitochondrial enzyme, it is very important to eliminate the possibility of contamination by bacterial or nuclear RNA polymerases. The elimination of possible contamination was considered at three levels. The first was gross contamination by intact nuclei and/or bacteria in the mitochondrial preparation. The second concerned the possibility that mitochondria trap or bind nuclear or bacterial DNA which in turn might contain DNA bound RNA polymerases. The third level of concern (and perhaps most difficult to control for) was the possible integration of nuclear polymerase into the mitochondria.

To address the problem of gross organelle contamination, sucrose-gradient purified mitochondria were monitored by light microscopy. Such studies utilized fast-green stains, which reliably detect basic histones in isolated nuclei, and showed no intact nuclei. Electron microscope studies showed no intact nuclei or bacterial cells in such preparations. Many intact mitochondria were present in a background of much unidentified fragmented material. To reduce the possibility of bacterial contamination, pupae and adult flies were washed extensively in 0.1% Triton X and water prior to homogenization. A further argument against extensive bacterial contamination comes from the use of

embryos as starting material, as these are effectively made axenic by washing in a 2% solution of sodium hypochlorite which dechorionates the eggs. The embryo mitochondrial RNA polymerase has the same solubilization characteristics and properties as does the mitochondrial RNA polymerase from other developmental stages.

Some studies have found that nuclear DNA frequently binds to isolated mitochondria (Simpson et al., 1967). I have investigated this possibility by the CsCl density gradient profile and PLK chromatography profile of extracts from purified mitochondria.

A DNase treated, sucrose-gradient purified, mitochondrial extract from adults was lysed with SDS, pronase-digested, RNased and centrifuged in CsCl gradients using labelled yeast nuclear and mitochondrial DNAs as markers. These have densities of 1.700 and 1.684 g/cc respectively. As shown in Figure 4 one major UV absorbing peak with a calculated density of 1.68 g/cc is resolved, precisely where Drosophila mitochondrial DNA is expected to band. No peak of UV absorbing material is evident at a density of 1.701, the density of D. melanogaster nuclear DNA (Bultmann et al., 1973). A large amount of UV absorbing material is present at the top of the gradient, and may represent protein and/or lipid-associated material. While this experiment doesn't unequivocally show a single DNA species present in mitochondrial extracts, it does indicate no detectable nuclear

Figure 4. CsCl density gradient profiles of mitochondrial lysates.

A) Profile of ^3H -adenine labeled yeast DNA.

Spheroplasts from cells grown in the presence of 20 uCi/ml of H^3 -adenine were lysed, treated and centrifuged according to the method of Rubin et al. (1977) as described in materials and methods. Fractions of 0.2 ml were collected from the bottom of the gradient (at left). Aliquots of 50 λ were assayed for alkali-stable TCA precipitable counts (—•).

B. Profile of mitochondrial lysate from Drosophila adults using labelled yeast DNA. Purified mitochondria from 50 gm adult flies were lysed with 2% SDS, pronased and RNAsed. To this lysate was added fraction 7 and fraction 14 of yeast DNA (from A). Centrifugation, fractionation and assay for TCA precipitable material (—•) were as described above. In addition, the remainder of each fraction was diluted to 1 ml, and absorbance at 260 nm was monitored (●—●).

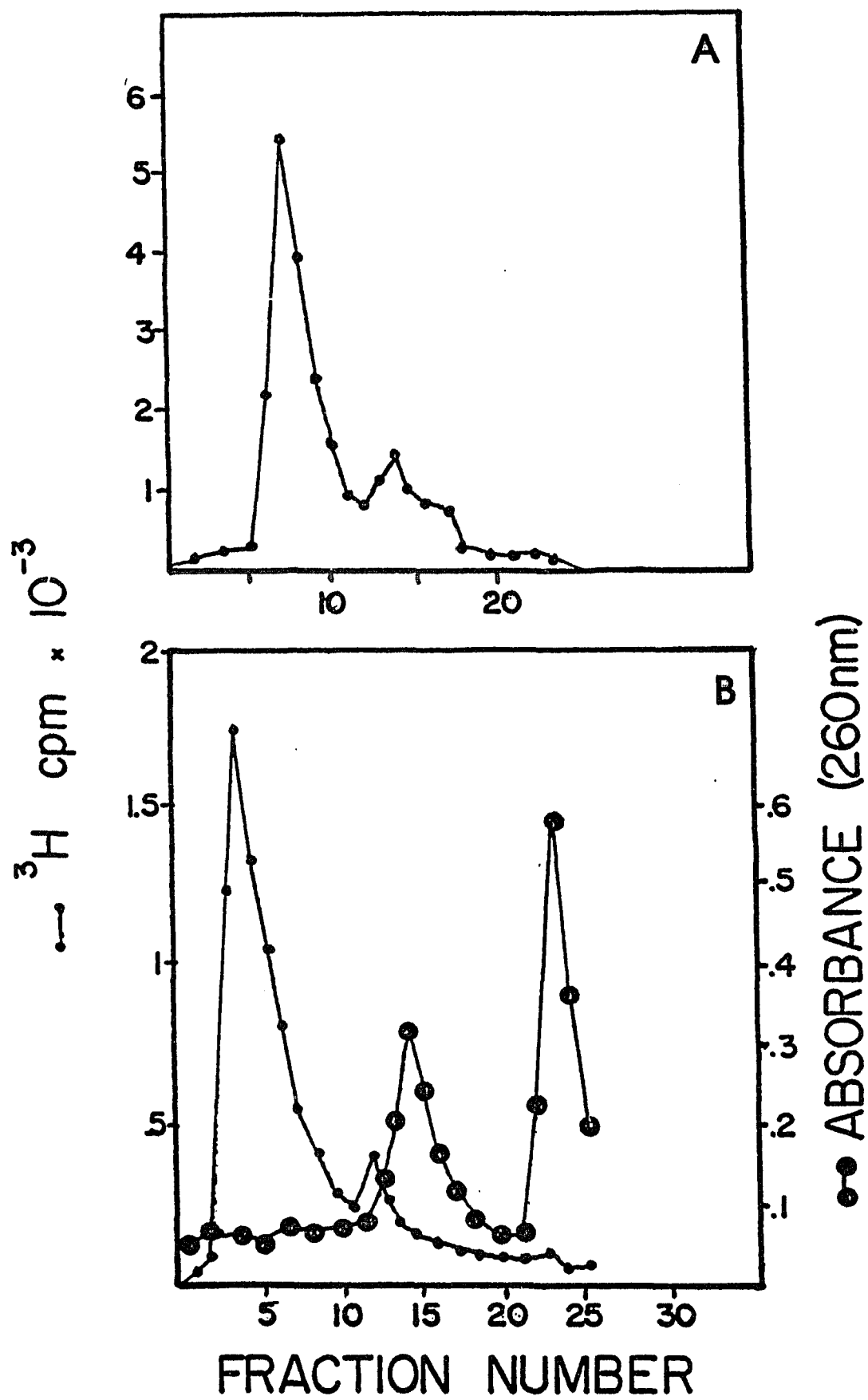
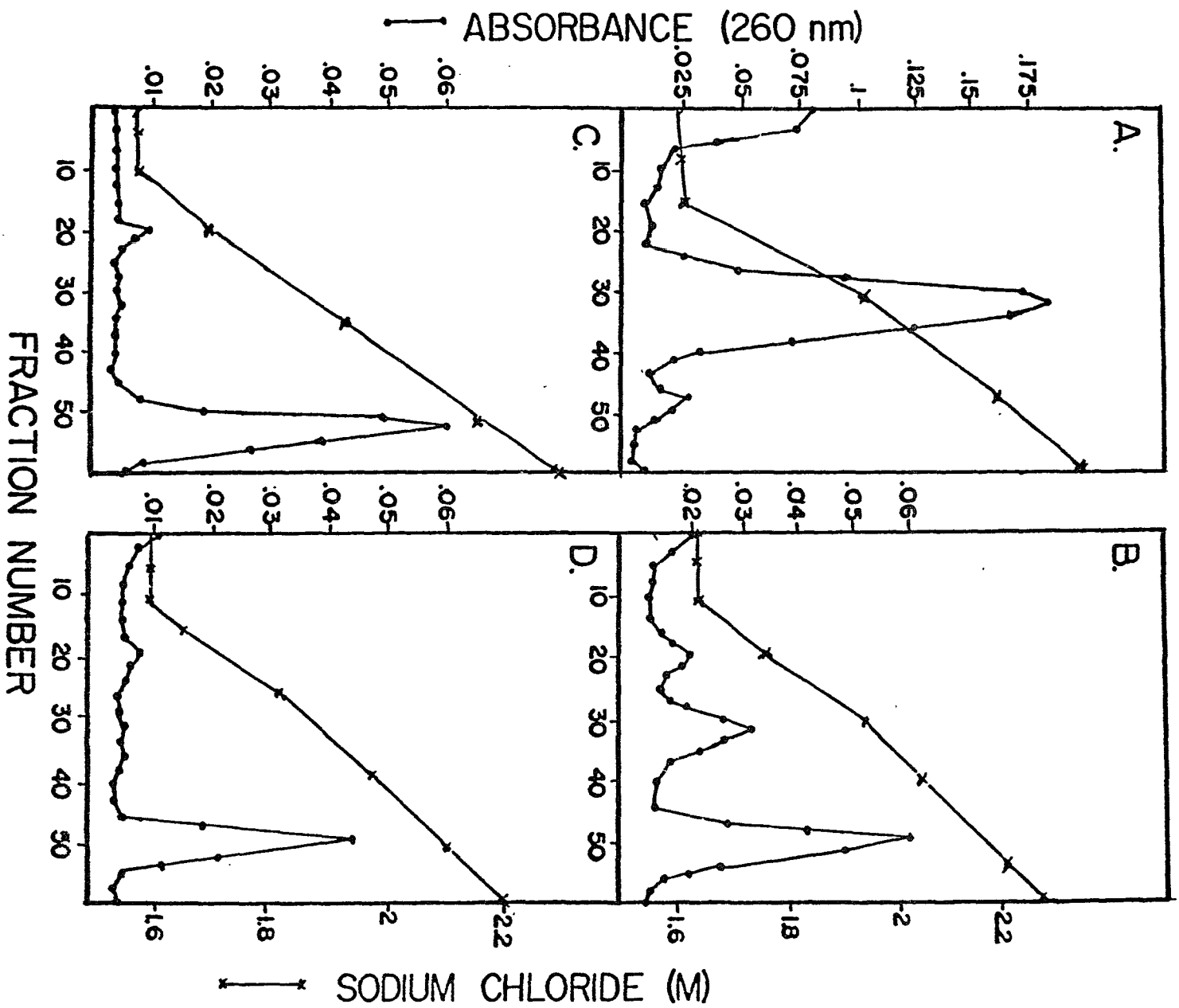


Figure 5. Polylysine Kieselguhr (PLK) chromatography of Crude and mitochondrial extracts.

Extracts are incubated in RNase (50 ug/ml for 25 minutes at 30° C) and then loaded on to columns of polylysine kieselguhr (2 cm x 12 cm). These columns are then washed with 150-200 ml of 1.6 M NaCl in phosphate buffer. Elution is with linear gradients of 1.6-2.4 M NaCl in the same buffer. Fractions of 1 ml are collected, and monitored by absorbance at 260 nm (●—●), and NaCl concentration by refractometer (X—X). Results shown here are from extracts of: A) 5 gms adult flies (sonicated), B) "crude mitochondrial pellet" from 25 gm of adults, C) purified mitochondria from 12 hour collection of eggs (25 gm), D) purified mitochondria from 25 gm of adult flies.



DNA contamination.

Polylysine Kieselguhr (PLK) chromatography has been used successfully to fractionate yeast nuclear from mitochondrial DNA (Blamire et al., 1972) presumably on the bases of differential G-C composition. The results shown in Figure 5 are from the following experiments.

Whole cell extracts were sonicated, RNAsed treated and passed through PLK columns. After washing the column with a 1.6 M buffer to remove protein, a linear NaCl gradient was used to elute the DNA species. The results in Figure 5a show that UV absorbing material is found with a large peak (B) eluting at approximately 1.95 M NaCl, and a smaller peak (C) eluting at 2.15 M NaCl. It should be noted that the 280/260 ratios of both these peaks is approximately 0.5, indicating that most of the UV absorbance is due to nucleic acids, not protein.

The results of PLK chromatography of crude mitochondrial extracts from adult flies resolves as shown in Figure 5b, a larger proportion of the 2.15 M (C) peak with a considerable amount of the 1.9 M (B) species, and a third UV absorbing species eluting at 1.71 M (A). The (C) panel in Figure 5c depicts PLK chromatography of lysed, purified mitochondria from Drosophila eggs collected up to four hours after deposition. These mitochondria were DNAsed, sucrose-gradient purified and osmotically lysed. The lysates were sonicated, RNAsed and then chromatographed on PLK columns

with the following result. A single peak eluting at 2.15 M NaCl (species C) is resolved which contain 90-95% of the total UV absorbance. The (d) panel in Figure 5 depicts the PLK chromatographic profile of purified mitochondrial extracts from adults. Similarly to the results with the egg extracts, 90-95% of the UV absorbance is resolved in the 2.15 M NaCl eluting peak (C). The same result was found using purified mitochondria from pupae. Thus for purified mitochondria, the results show small amounts of UV absorbing material eluting at 1.71 M and 1.95 M NaCl, amounting to less than 5% of total. On the basis of these experiments it appears that species C represents mitochondrial DNA. This species is 5-10% of the total UV absorbance in whole fly preps, is greatly enriched in crude mitochondrial preparations, and is greater than 90% of the total UV absorbance using purified mitochondrial extracts. Species (B) which is present in large amounts in whole cell preps, reduced but present in crude mitochondria, and 95% eliminated with DNase, and sucrose-gradient purified mitochondria is nuclear DNA. It was reasoned that species (A) might either be another form of DNA possibly bacterial or double-stranded RNA. DNase incubation of these extracts appears to eliminate all peaks except the 1.71M species (A) making the suggestion that this peak represents double stranded RNA more probable.

These experiments indicate that some nuclear DNA is present unless the combination of sucrose-gradients and DNase treatment are used. Two cautions remain in interpreting the PLK results. Actual proof that the 2.15M peak is mitochondrial DNA by examining its other properties (CsCL buoyant density, restriction enzyme profile) has been done with yeast, but not Drosophila (Cottrell, unpublished). Secondly, the possibility that bacterial DNA elutes in either of these peaks has not yet been addressed. Drosophila mitochondrial DNA similar to yeast mt DNA has the highest A-T rich genome known, and elutes at high salt concentration as predicted. Nevertheless, further characterization of PLK binding using bacterial DNAs should be done.

The final level of evidence pertaining to possible enzymatic contamination comes from inspection of the activities present in the mitochondrial extract. The possibility of contamination by nuclear or bacterial enzymes and particularly polymerase III which is known to be extranuclearly localized, is made unlikely by the reproducibly distinct properties of the solubilized RNA polymerase activities, as shall be demonstrated.

Assay, Solubilization, Characteristics of Crude RNAP Activity

The test for solubilization of various mitochondrial enzyme activities involved treatment with a solubilizing agent, centrifugation at approximately 15,000 x g for 25

minutes and assaying both supernatant and the resuspended pellet for enzyme activity. It should be noted that a subsequent centrifugation of the supernatant at $140,000 \times g$ for 1 hour was used in all initial experiments with no loss of RNA polymerase or cytochrome oxidase activity from the supernatant and was not routinely employed in later experiments.

Table 2 shows the results of treatments with digitonin, sonication and water-lysis on the solubilization of several mitochondrial enzymes including RNA polymerase, cytochrome oxidase, an inner membrane enzyme, and a matrix-enzyme, MDH. A concentration of 1% digitonin released 74% of MDH but only 20% of cytochrome oxidase, and 16% of the RNA polymerase. Similarly, mild sonication (3×15 seconds) solubilizes 79% of MDH activity and only 12.5% of cytochrome oxidase and 25% of RNA polymerase activity. Osmotic lysis also releases a majority (75%) of the MDH activity, while 15% of cytochrome oxidase and 20-25% of RNA polymerase are solubilized. These results show that the majority of MDH activity is solubilized by treatments which are unable to solubilize a majority of either cytochrome oxidase or RNA polymerase activity. This is consistent with the notion that most of the mitochondrial RNA polymerase activity is bound to the inner membrane.

Figure 6 shows the results of experiments where different concentrations of non-ionic detergents

TABLE 2

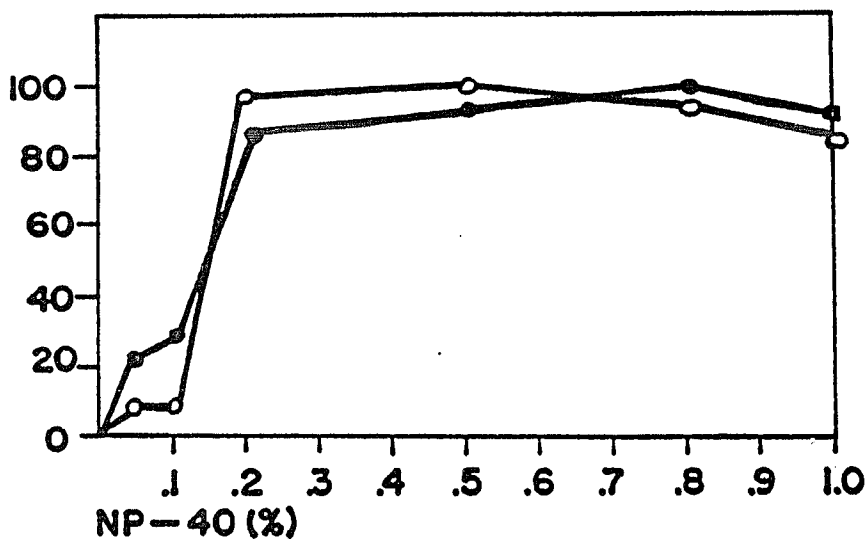
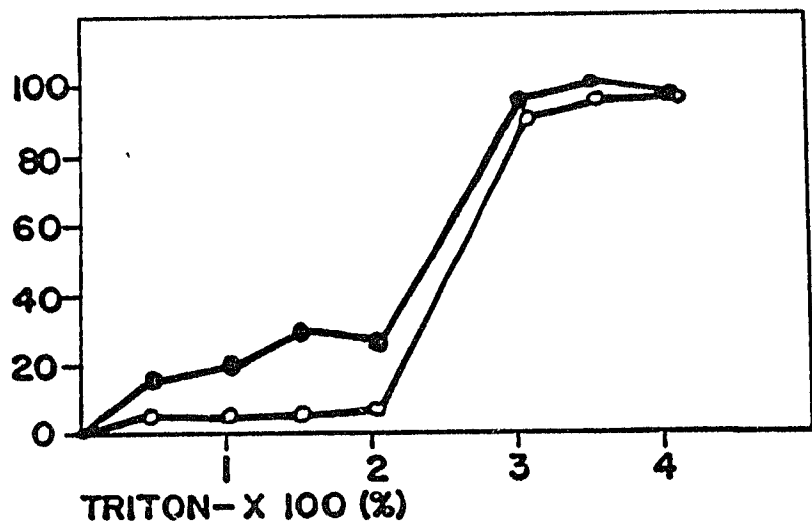
Effects of Sonication, Osmotic Lysis and Digitonin on Solubilization
of Three Mitochondrial Enzymes

	RNA Polymerase (CPM)			Cytochrome Oxidase (Units)			Malate Dehydrogenase (Units)		
	<u>SUP</u>	<u>SUP + Pellet</u>	<u>% Total in SUP</u>	<u>SUP</u>	<u>SUP + Pellet</u>	<u>% Total in Sup</u>	<u>SUP</u>	<u>SUP + Pellet</u>	<u>% Total in Sup</u>
Sonication - 3 x 15 Sec Max Setting	200	1100	25%	0.6	4.8	12.5%	125	150	83%
Osmotic Lysis	300	1350	23%	0.8	5.6	15%	130	165	78%
Digitonin 0.5%	135	1520	10%	0.4	4.6	10%	12	155	10%
Digitonin 1%	270	1600	16%	0.9	4.5	20%	104	140	74%

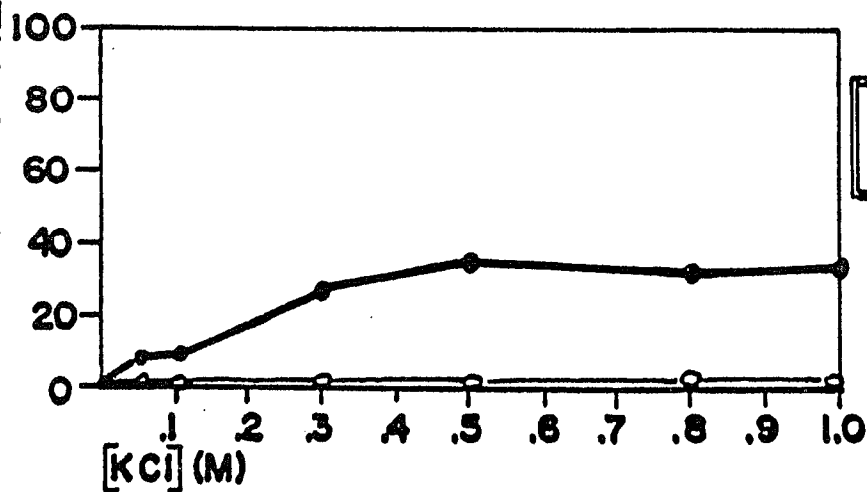
Figure 6. Solubilization of Mitochondrial RNA polymerase.

Aliquots of 0.5ml of mitochondrial suspension containing 2-3 mg/ml protein were treated with the appropriate amount of detergent or salt in a total volume of 1 ml. After incubation for 30 minutes with the solubilizing agent, the mixture was spun at 15,000 x g for 30 minutes. The supernatant and the resuspended pellets of this spin were assayed for RNA polymerase and CO activity as described. The data is shown as % supernatant activity/total activity in both pellet and supernatant. In these experiments, 50 λ of extract was assayed with total activity of pellet and supernatant between 2700-3000 cpm.

ACTIVITY IN SUPERNATANT



% OF TOTAL

RNAP ●—●
C.O. ○—○

(Triton X-100, NP-40) and salt (KCl) were used as solubilizing agents.

Non-ionic detergents solubilized 85-90% of the RNA polymerase activity as well as cytochrome oxidase activity. This co-solubilization is strongly suggestive that both enzymes are bound to the inner membrane.

The sum of the RNA polymerase activities in the pellets and supernatant fractions closely approximates that found in the intact mitochondria. Thus the recovery of polymerase activity in this solubilization assay is nearly quantitative suggesting that there is little activation of RNA polymerase activity at these concentrations of detergents.

One interesting but unexpected observation is that 3% Triton X-100 is required for solubilization of these membrane enzymes where as only 0.2% NP40 is required for the same degree of solubilization. In addition, 20-30% of RNA polymerase is solubilized by 1% Triton X-100, osmotic lysis, sonication and 1% digitonin, as well as high concentrations of KCl (see below). Whether this represents a single class of RNA polymerase molecules which is distinct from the majority of more tightly bound polymerases or not, is unclear. No obvious distinctions in the characteristics of the majority of detergent-solubilized RNA polymerases (see Table 3) from those of the enzyme(s) released by

osmotic lysis, 1% digitonin or 1% Triton-X have been observed.

Another mode of solubilization of mitochondrial RNA polymerase utilizes high salt concentration (Tsai et al., 1971). This technique was substantially less efficient than non-ionic detergents--solubilizing only 30-35% of the activity (see Figure 6). This was true even after a 1 hour exposure to high salt. Cytochrome oxidase was not co-solubilized with this treatment.

Controls were performed to test whether or not solubilization of RNA polymerase activity by high salt resulted in inactivation of the RNA polymerase due to increasing ionic strength. These experiments assayed 10 λ rather than 50 λ aliquots, the reason being that the total salt in the reaction mix would be nearer the enzyme optima. The results were consistent with the other experiments and showed that at high concentration of salt, no more than 30-35% of the total RNA polymerase activity is solubilized. In addition, cytochrome oxidase activity is not markedly reduced by the addition of high salt. Both results indicate that the low recovery of cytochrome oxidase and RNA polymerase activity in supernatant resulting from high salt solubilization of mitochondria is due to the ineffective solubilization of these enzymes, and not inactivation of them by high salt.

Some characteristics of RNA polymerase activity in the pellet and supernatant fractions resulting from these solubilization treatments are shown in Tables 3 and 4. The question of whether the KCl-solubilized activity is a qualitatively different enzyme than the detergent-solubilized activity was addressed by comparing the properties of the RNA polymerase(s) resulting from these different modes of solubilization.

As shown in Table 3, none of the enzymes solubilized were sensitive to alpha-amanitin at concentrations of 50 ug/ml. All activities were stimulated by Mg^{++} more than Mn^{++} as metal cofactor. All activities showed roughly a 25-35% inhibition at 10 ug/ml, and a 40-50% inhibition at 50 ug/ml concentration of rifampicin. These activities show 35-40% inhibition when the reaction mix contained 2 mM Mn^{++} in addition to 10 mM Mg^{++} . Higher concentration of Mn^{++} are more strongly inhibitory using detergent-solubilized activities than with KCl solubilized enzyme. The activity remaining in the pellets (Table 4) were similar in all the above mentioned properties except that the KCl pellet had a greater Mn^{++} inhibition. This Mn^{++} inhibition may be due to a Mn^{++} -activated nuclease that is solubilized more by detergent than by KCl treatment. (The disappearance of this property upon further purification of the polymerase activity using ion-exchange and gel

TABLE 3

Characterization of Solubilized Mitochondrial RNA

Polymerase Activity (Supernatant Activities)

Experimental Conditions	A. KCl (0.5M) Solubilized		B. NP-40 (0.2%) Solubilized		C. NP-40 (0.2%) +KCl (0.5M) Solubilized		D. Triton X-100 (3%) Solubilized	
	Act. (CPM)	Inhib. (%)	Act. (CPM)	Inhib. (%)	Act. (CPM)	Inhib. (%)	Act. (CPM)	Inhib. (%)
Control Rx +10 mM Mg ⁺⁺	320	--	650	--	615	--	675	--
10 ug/ml Rifampicin	210	35	510	25	400	35	465	32
50 ug/ml Rifampicin	190	40	386	44	305	50	365	46
2mM Mn (+10mM Mg ⁺⁺)	225	36	375	46	430	35	480	30
5mM Mn (+ 10mM Mg ⁺⁺)	210	34	225	65	305	50	270	60
50 ug/ml -Amanitin	315	4	650	0	610	2	680	0

TABLE 4

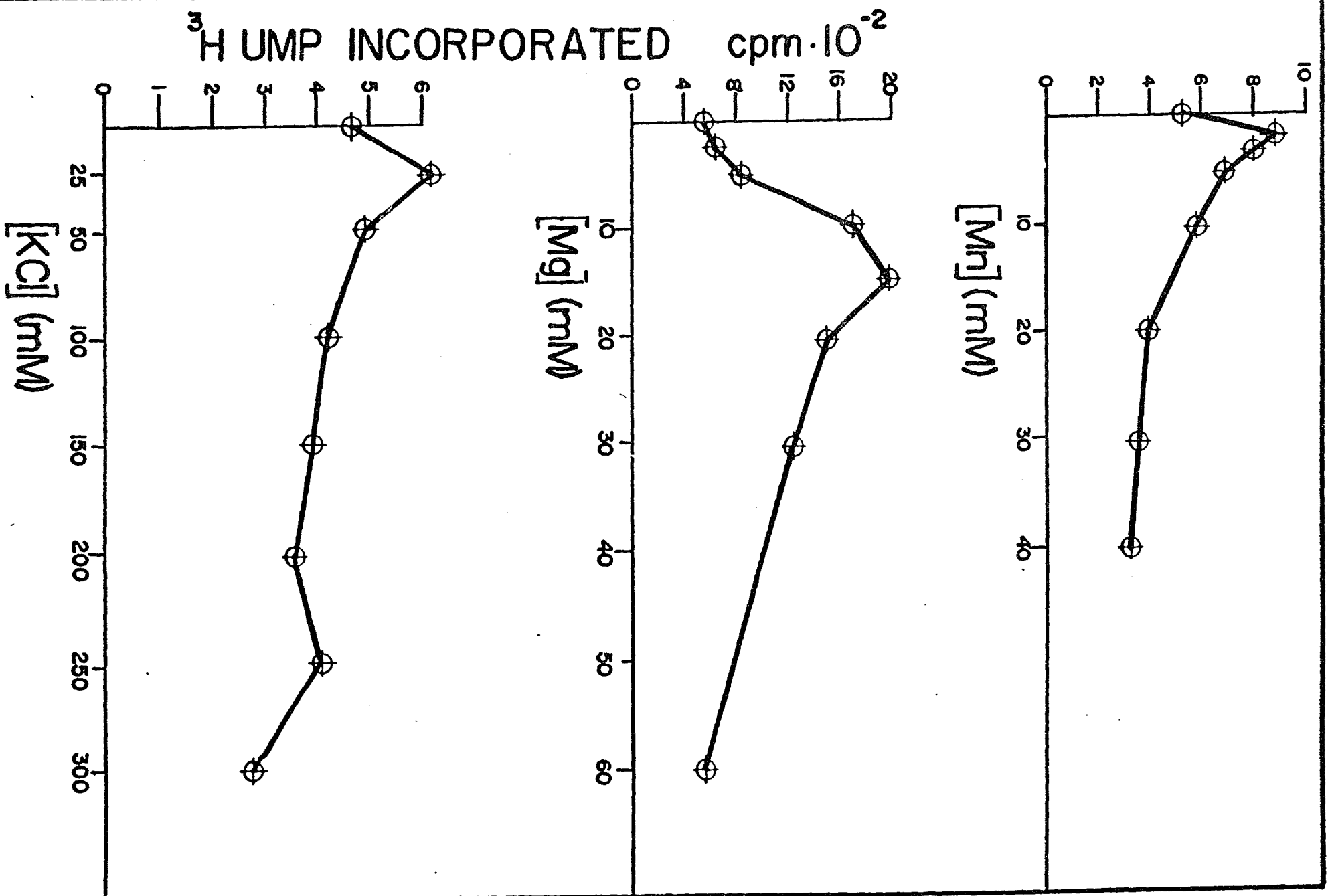
Characterization of Pellet Mitochondrial

RNA Polymerases from Different Solubilization Treatments

	A. KCl (0.5M) Solubilized		C.NP-40 (0.2%) Solubilized		C.NP-40 (0.2%) + KCl (0.5M) Solubilized		D. Triton X-100 (3%) Solubilized	
	Act (CPM)	Inhib (%)	Act (CPM)	Inhib (%)	Act (CPM)	Inhib (%)	Act (CPM)	Inhib (%)
Control Rx	1915	--	230	--	260	--	280	--
10 ug/ml Rifampicin	1400	27	170	27	205	33	225	30
50 ug/ml Rifampicin	1100	43	120	40	150	43	180	40
2 mM Mn ⁺⁺ (+10 mM Mg ⁺⁺)	1375	30	200	15	215	18	240	15
5 mM Mn ⁺⁺ (+10 mM Mg ⁺⁺)	900	53	165	30	190	27	200	30

Figure 7. Ion optima of detergent-solubilized mitochondrial RNA polymerase.

Aliquots of 10 λ of NP40 solubilized mitochondria representing about 50 ug of protein were assayed at each concentration. Each point represents the average of two duplicate reactions.



filtration chromatography is further suggestion that it is separable from other properties of the RNA polymerase.)

The ion optima of the detergent-solubilized enzyme are shown in Figure 7. This activity shows a definite difference from nuclear enzymes in its roughly twofold stimulation by Mg^{++} rather than Mn^{++} . The Mg^{++} optima is between 10-15 mM, the Mn^{++} optima is more narrow at 1 mM. The decrease of polymerase activity with increasing ionic strength (KCl) is interesting in that there is a small but consistent stimulation at high ionic strength (250 mM KCl or 200 mM $(NH_4)_2SO_4$).

The crude detergent-solubilized enzyme appears to be associated with endogenous DNA in that incorporation of H^3 -UTP in the absence of added template is 60-70% of that with exogenously added DNA. DNA-independence is markedly reduced to 10-20% if the solubilized extract is preincubated at 37°C for 30 minutes. To find out if a template-independent nucleotide polymerase like polynucleotide phosphorylase was present in the extract, 20 mM potassiumphosphate, a known inhibitor of that enzyme (Burgess, 1969) was added to the reaction mix, with no resulting effect on the amount of incorporation (see Table 5). The dependence on all four nucleotide triphosphates, and the 90% reduction of TCA-precipitable incorporation by RNase and KOH are strong evidence that an RNA polymerase is responsible for the majority of incorporation.

TABLE 5

Requirements and Characterization of Crude NP40

Solubilized RNA Polymerase Activity

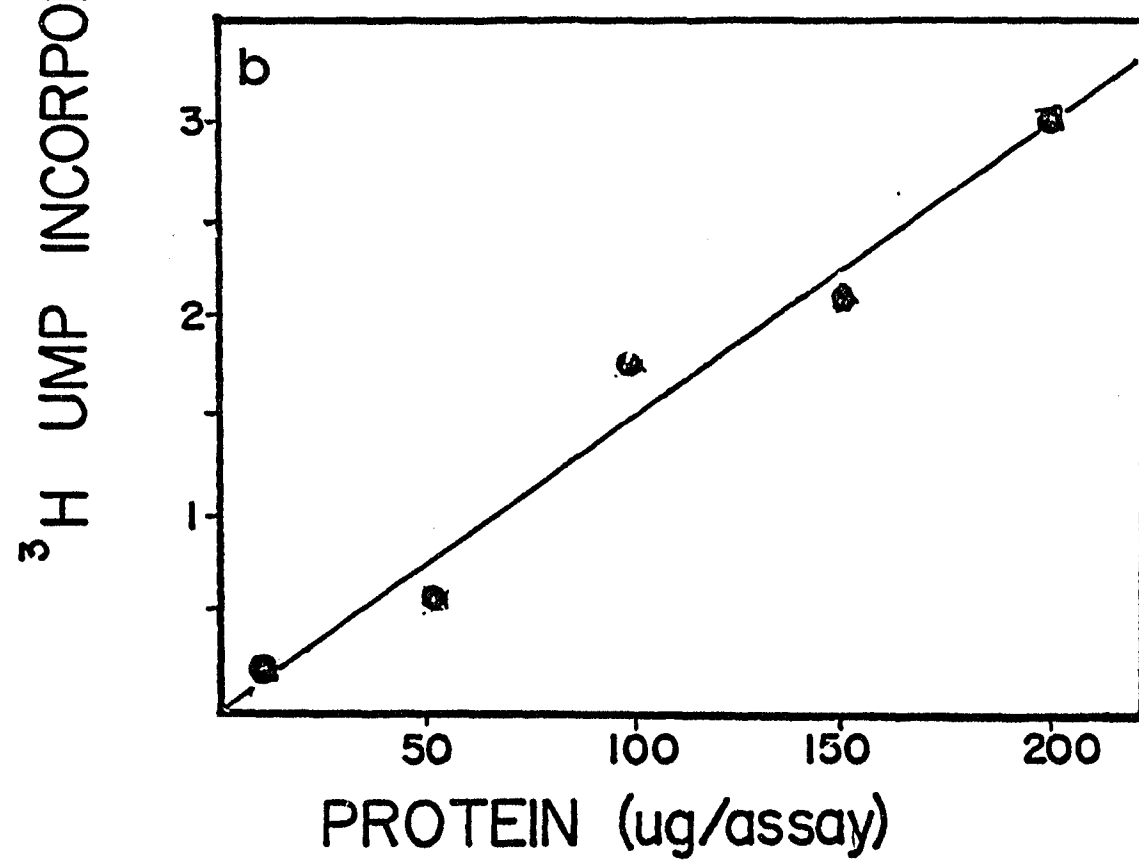
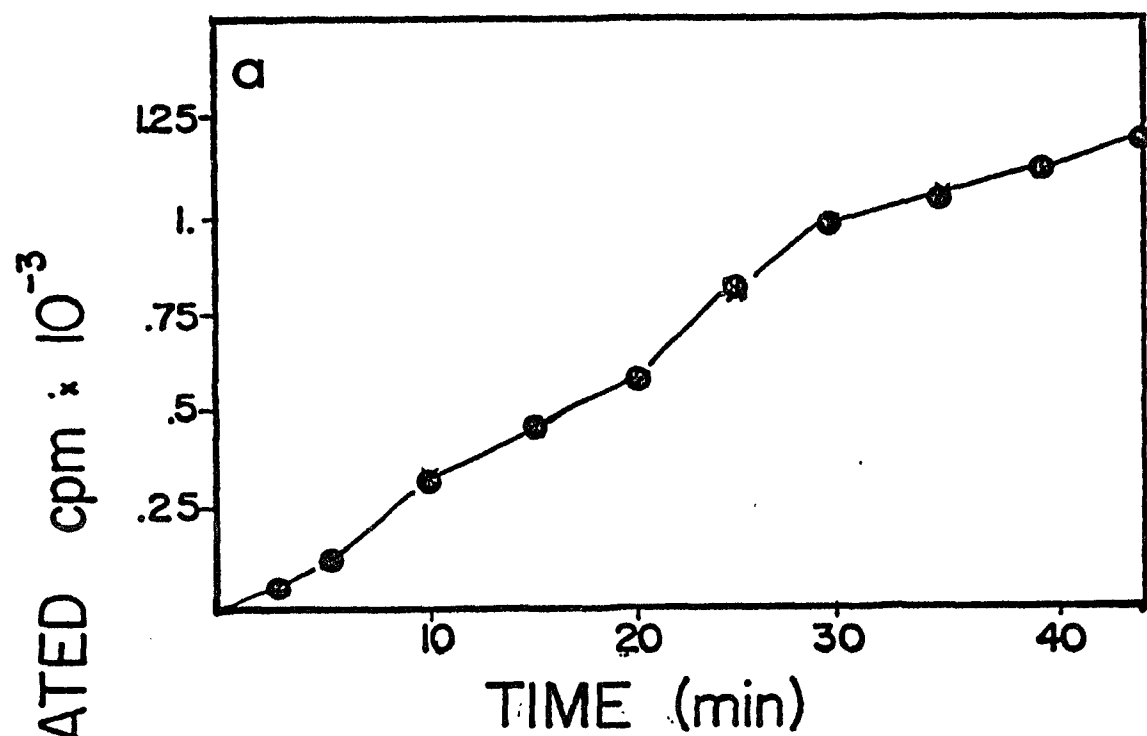
<u>Omission/Addition</u>	<u>CPM</u>	<u>% Control</u>
Std Rx: Control	1570	100
-DNA	940	60
-DNA incubate 37°C for 30 minutes	250	16
-ATP, GTP	155	<10
-GTP	200	13
pH 6,5	720	46
RNAse (50ug/ml)	150	<10
KOH	140	<10
-DTT	1095	70
+ K phosphate (50 mM)	1525	97

Figure 8a. Time course of RNA synthesis by NP40 solubilized mitochondrial RNA polymerase.

Each assay contained 50 ug of protein from detergent-solubilized mitochondria.

Figure 8b. Protein dependence of the activity of NP40 solubilized RNA polymerase.

Each point represents average of duplicate assays.
The standard assay was incubated for 20 minutes.



All assays were conducted in Tris HCl pH 7.9. Incorporation is considerably reduced at lowered pH (6.5). DTT was included in all buffers to stabilize the enzyme. As shown in Figure 8, there is linear incorporation for 35-40 minutes at 25°C using 50 ug of the crude solubilized mitochondrial protein per assay. Enzyme activity increases linearly with increasing protein up to 200 ug/assay.

The KCl solubilized enzyme has a 1.5 fold higher specific activity since the amount of protein solubilized is roughly 5 fold less than with either NP40 or Triton-X 100. The detergent-solubilized enzyme is much more stable allowing further purification. It can be frozen, and assayed several days later with loss of 10-15% activity. The KCl solubilized activity is more labile, 60-80% being lost after 1-2 days storage.

Gel Filtration: Kuntzel and Schafer (1971) showed by glycerol gradient centrifugation that the mitochondrial RNA polymerase differentially aggregated as a function of salt concentration. In addition, experiments described in the introduction showed that certain types of transcriptional complexes could be recovered when detergent lysed mitochondria or chloroplasts were chromatographed on gel filtration columns (Hallick et al., 1977; Levens et al., 1979). Both these approaches suggested that gel filtration chromatography under different salt conditions could be used as an important

preparative and analytical tool in the study of mitochondrial RNA polymerase.

Figures 9 and 10 show the result of experiments in which extracts of detergent solubilized mitochondria were loaded on Sephacryl S-200 columns, and chromatographed in low salt ($0.05 \text{ M} (\text{NH}_4)_2\text{SO}_4$) or in high salt conditions ($0.35 \text{ M} (\text{NH}_4)_2\text{SO}_4$). The elution profile under low salt conditions shows a number of peaks of mitochondrial RNA polymerase activity with a greater proportion of activity found in peaks S_5 and S_4 of high apparent molecular weight. The elution profile under high salt conditions resolves a number of mitochondrial RNA polymerase activities in which peaks S_1 and S_2 of low apparent molecular weight predominate. It is evident then that these columns resolve a number of discrete RNA polymerase activities whose relative proportion appear dependent on ionic strength.

These columns were calibrated by chromatography under the same conditions using proteins of known molecular weight: BSA, cytochrome c and ADH. From this calibration (see Figure 11) the molecular weight of the peaks of mitochondrial RNA polymerase activity were determined to be: S_1 , 60,000, S_2 : 110,000, S_3 160,000, S_4 : 210,000 and S_5 : 250,000 (void volume). The activity of lowest molecular weight (S_1) may well be the monomer or completely dissociated mitochondrial RNA polymerase, since its apparent molecular weight of 60,000 is close to that found by other workers

using SDS gel electrophoresis. The forms of higher apparent molecular weight are sized so as to suggest that they represent multimers of this monomer activity.

In a similar experiment, a KCl solubilized mitochondrial extract was chromatographed on a Sephacryl S-200 column under high salt conditions. The same approximate distribution of peaks was obtained (Figure 12). The pellet activity not solubilized by KCl was further treated with 3% Triton X-100 and chromatographed under high salt conditions on Sephacryl S-200.

Although the elution profile was essentially similar with both the KCl and Triton X-solubilized extracts, there is more activity in peak S_2 and less in peak S_5 in the latter. The significance of this difference is not known. However, the appearance of multiple peaks when the KCl solubilized mitochondrial extract was chromatographed is a strong indication that the size heterogeneity is not due to the presence of detergent.

Some characteristics of peaks S_1 , S_2 and S_5 obtained by gel filtration are shown in Table 6. All the activities are insensitive to α -amanitin up to a concentration of 100 ug/ml, but partially sensitive to rifampicin. Peak S_5 has significant activity in the absence of added DNA and may contain DNA.

Figure 9. Gel filtration chromatography of mitochondrial RNA polymerase activities on Sephacryl S-200 in low salt conditions.

NP 40 solubilized mitochondrial extracts (5-10 ml) were loaded on Sephacryl S-200 columns which had been equilibrated in 0.05 M $(\text{NH}_4)_2\text{SO}_4$ and 0.2% NP40 in TGED. The column (80 cm x 2.5 cm) was then resolved with the same buffer. Flow rate was 30-35 ml/hour. Fraction size was approximately 6 ml. Starting material was 100 grams of pupae. 20 λ of every other fraction was assayed in a total reaction volume of 100 λ . Aliquots of 0.1 ml were diluted 10 fold for absorbance readings at 280 nm. Purified ADH from yeast was chromatographed independently under the same conditions, and eluted as shown.

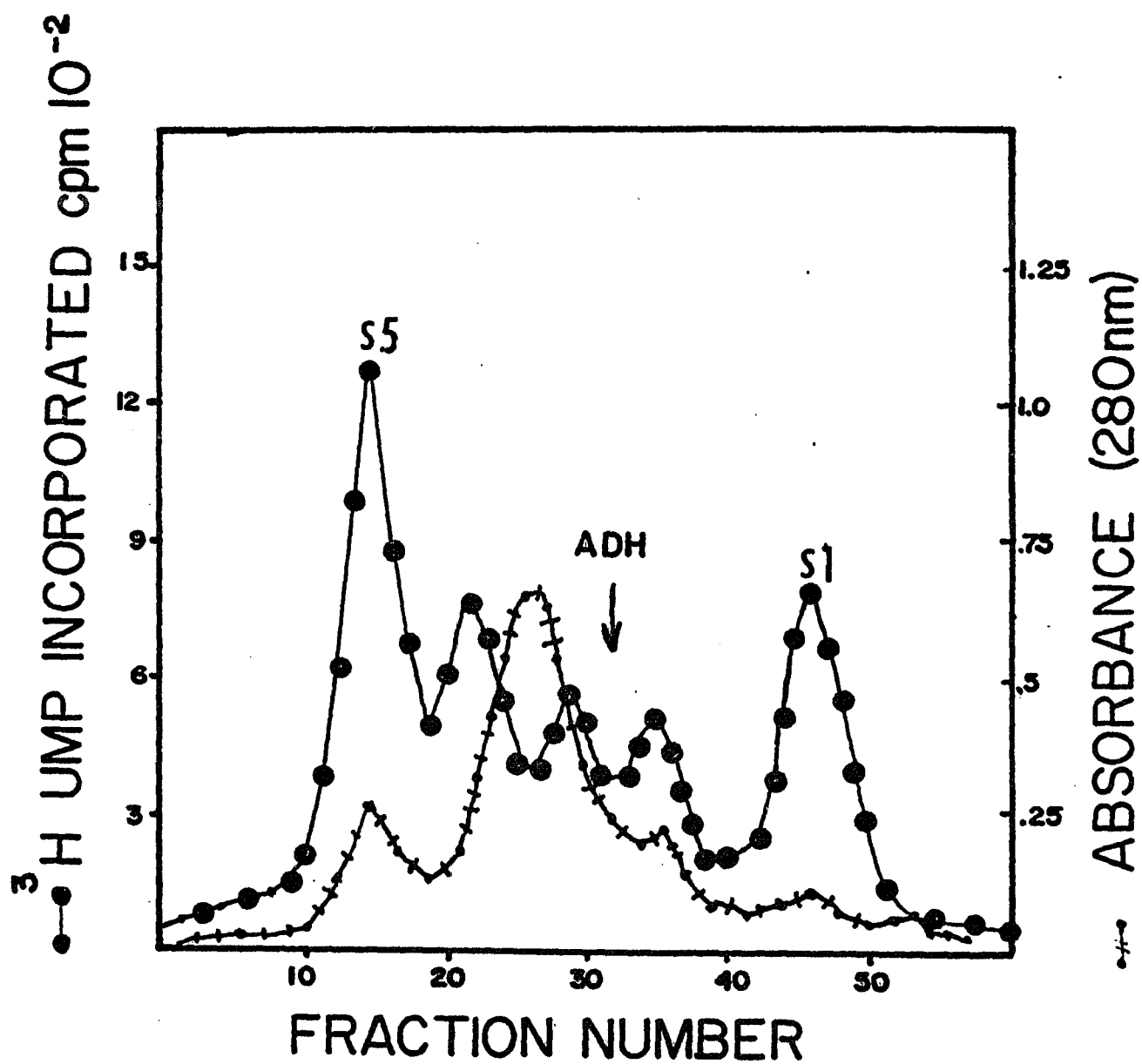


Figure 10. Gel filtration chromatography of mitochondrial extracts of Sephacryl S-200 in high salt conditions.

NP40 solubilized extract was chromatographed on Sephacryl S-200 of the same dimensions as previous. Equilibration and elution buffer was 0.35 M $(\text{NH}_4)_2\text{SO}_4$ in TGED and 0.2% NP40. Fractions 1-20 contained 5 ml, fractions 21-80 were 4 ml. Starting material was 200 grams of pupae. All other conditions were the same as previous.

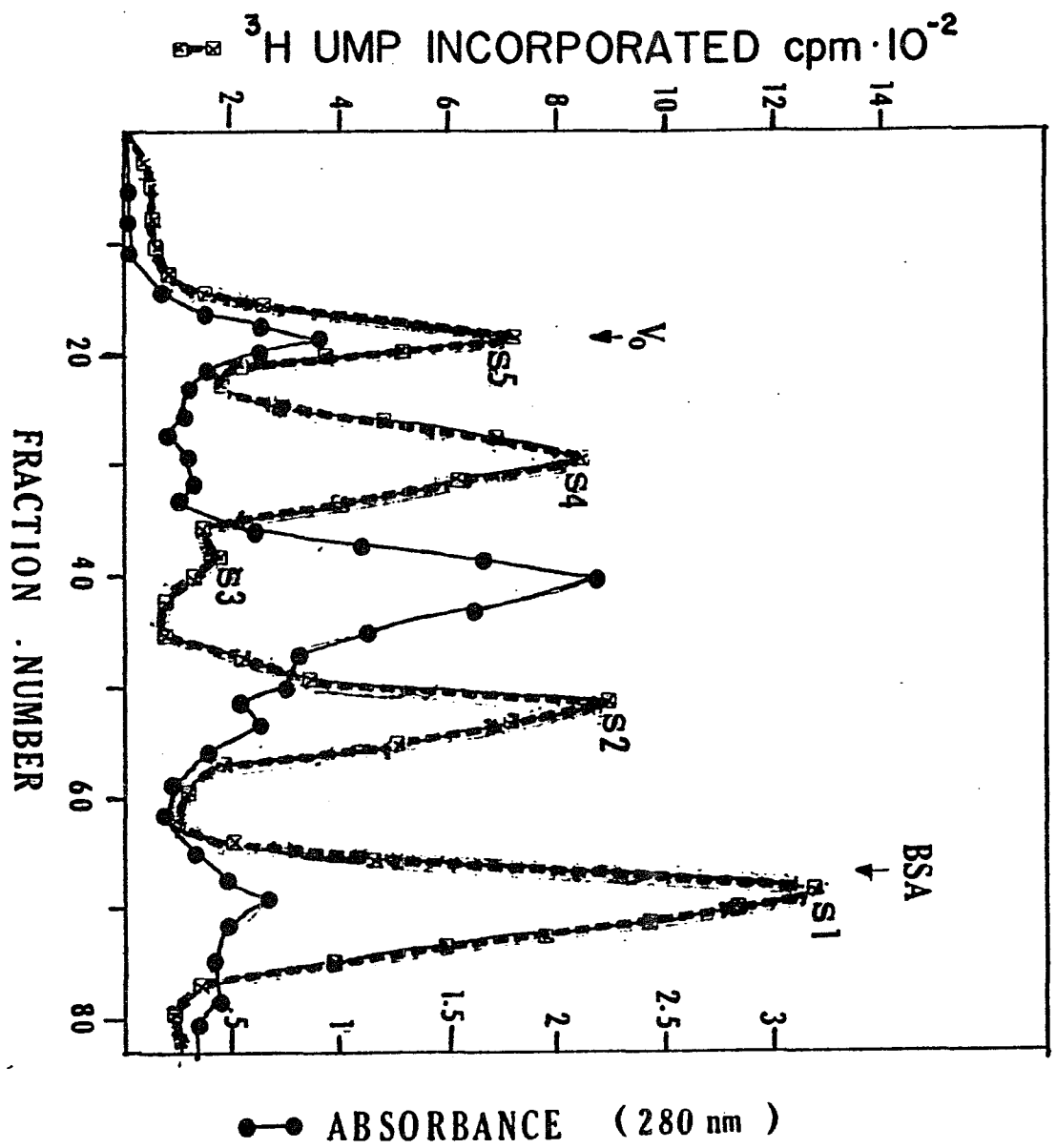


Figure 11. Molecular weight determination of mitochondrial RNA polymerase activities from Sephacryl S-200 gel filtration chromatography.

This determination was calibrated with BSA (67,000 d), yeast alcohol dehydrogenase, ADH (150,000 d) and the exclusion peak, V_0 (250,000 d) in which all markers were chromatographed under the same conditions as the mitochondrial extract. The major peaks of RNA polymerase activities (S_1 - S_5), and mitochondrial protein are shown.

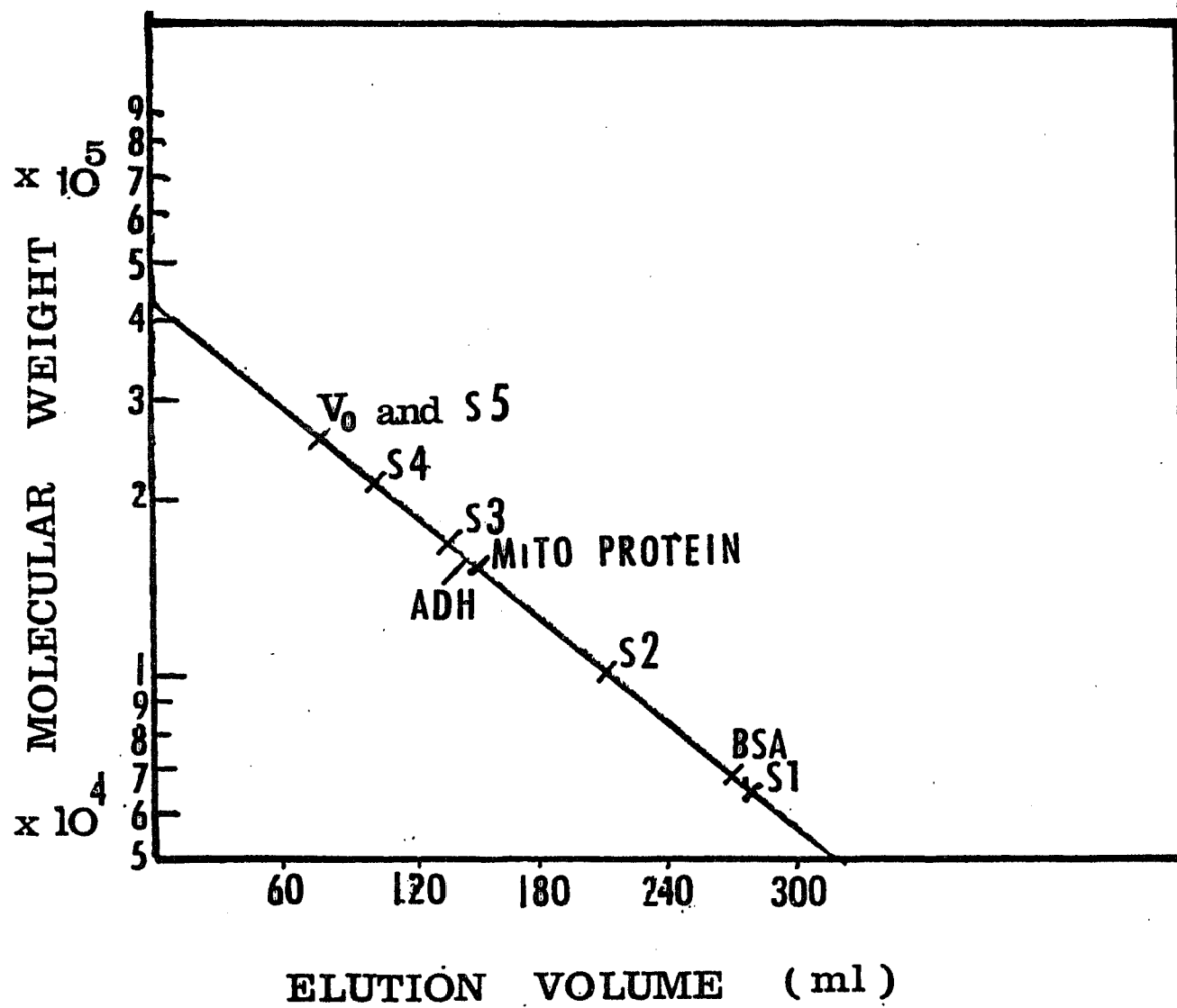


Figure 12a. Gel filtration of KCl solubilized mitochondrial RNA polymerase activities in high salt.

A mitochondrial extract from 200 grams of pupae was solubilized with 0.5M KCl, concentrated by 50% $(\text{NH}_4)_2\text{SO}_4$ precipitation, and loaded onto a Sephacryl S-200 column of the same size as in Fig. 9. This column was equilibrated and resolved with 0.35 M $(\text{NH}_4)_2\text{SO}_4$ in TGED. Cytochrome c was run in parallel experiments as a marker.

Figure 12b. Gel filtration chromatography of Triton X-100 solubilized pellet in high salt.

The non-solubilized pellet from KCl treatment was further treated with 3% Triton x-100, and the solubilized activity loaded on the Sephacryl S-200 column and run in high salt conditions and assayed as previously described.

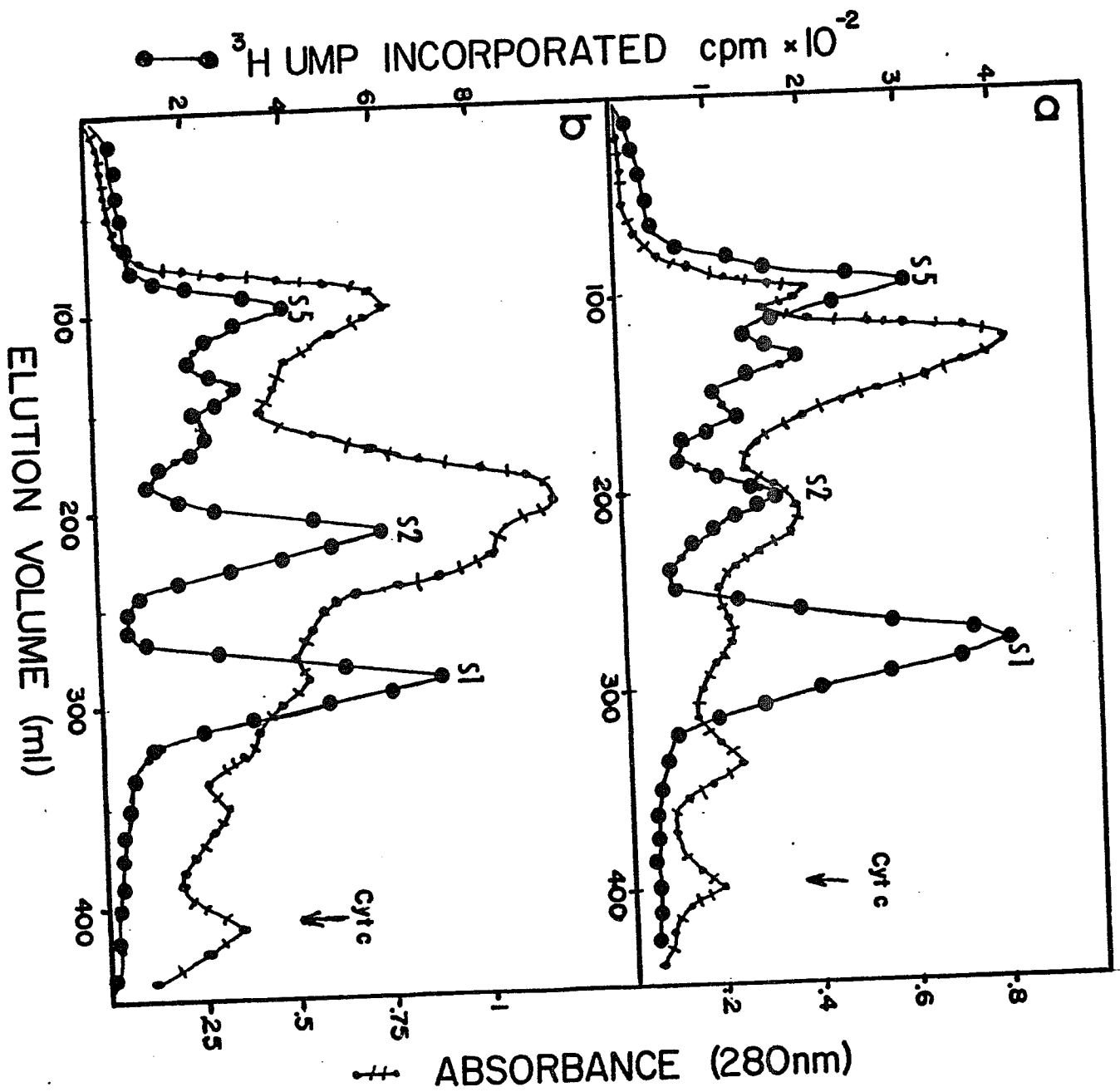


TABLE 6

Properties of Sephacryl resolved
RNA Polymerase Activities.

Expital Conditions	S ₅ 850= CPM		S ₂ 1270= CPM		S ₁ 1620= CPM	
	Control		Control		Control	
	CPM	% Control	CPM	% Control	CPM	% Control
0.15 M Ammonium Sulfate	340	40	575	45	565	35
2mM Mn ⁺⁺ (+10 mM Mg ⁺⁺)	470	55	760	60	1215	75
5mm Mn ⁺⁺ (+10mM Mg ⁺⁺)	340	40	600	47	810	50
100 ug/ml α-Amanitin	845	98	1250	97	1632	100
-DNA	470	55	150	12	130	8
Single- Strand DNA (SS)	977	115	1500	120	2430	150
Double Stranded DNA (DS)	637	75	890	70	972	60
SS/DS DNA	1.53	--	1.71	--	2.5	--
10 ug/ml Rifampicin	595	69	927	73	1250	77
50 ug/ml Rifampicin	425	50	645	51	965	59
100 ug/ml Rifampicin	340	40	560	44	790	49

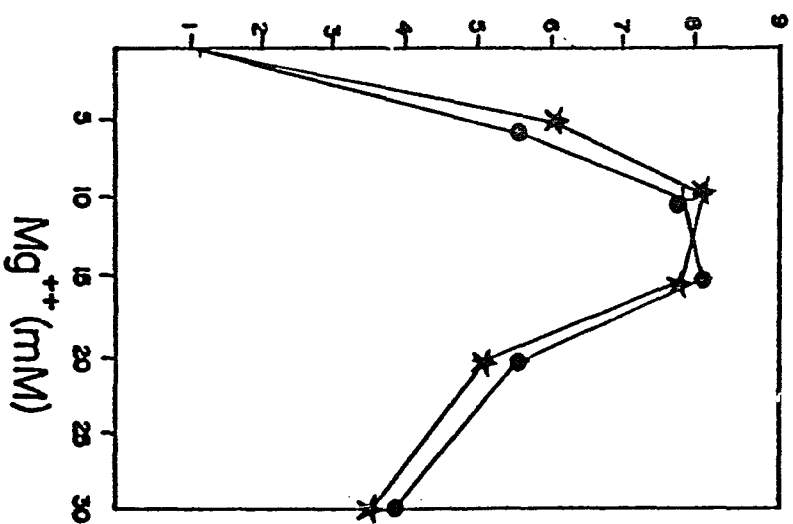
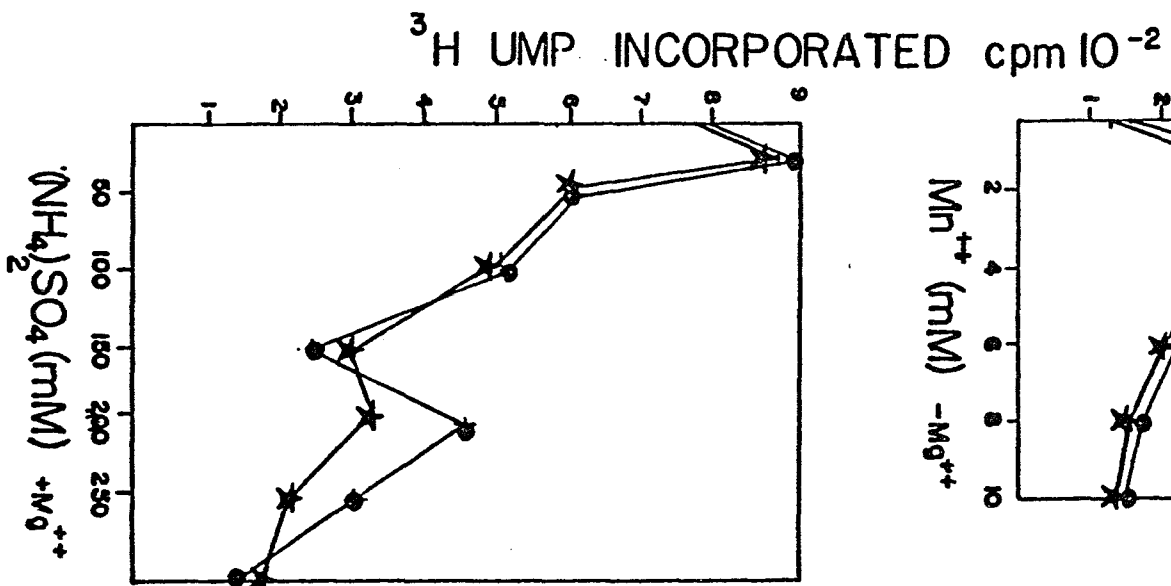
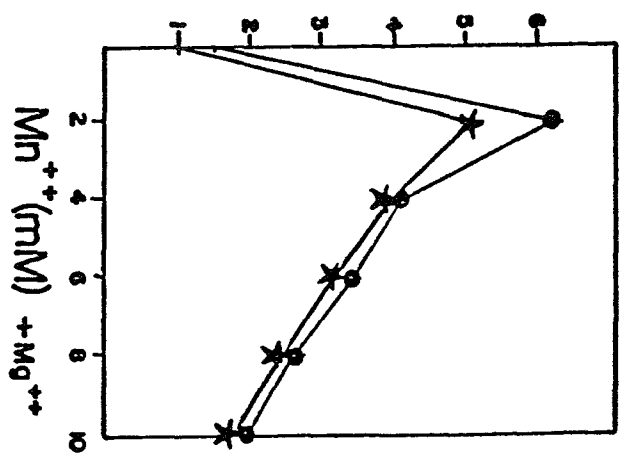
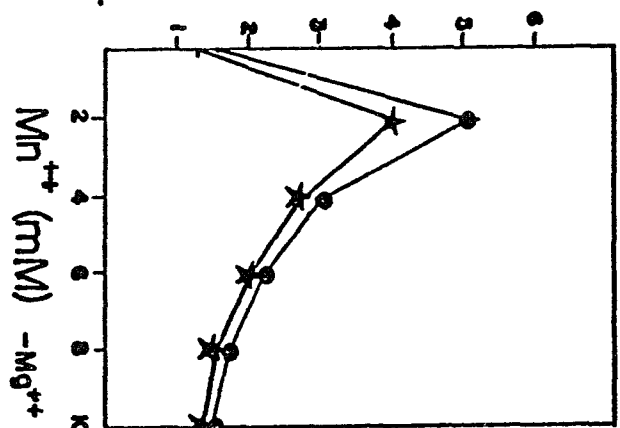
Experiments where PEG precipitation was used to remove DNA from the extracts resulted in a partial loss of higher molecular weight activities but there was no concomittant increase in the other peaks. As far as template specificity is concerned, all the activities are more active with single stranded DNA as template than with double-stranded DNA, though there is a slight increase in double stranded/single stranded activity with higher molecular weight forms. These qualities are very similar to that of the crude solubilized enzyme(s). However, unlike the crude solubilized enzyme, the presence of Mn^{++} does not appear to abolish enzyme activity.

Figure 13 shows the Mg^{++} , Mn^{++} and ammonium sulfate optima of peaks S_1 and S_5 . Both activities have very similar properties with slight but reproducible differences. Both show an optima of 25 mM ammonium sulfate with a slight shoulder at 200 mM. This shoulder is more pronounced with the S_1 activity. There is a broad Mg^{++} optima ranging between 10 and 20 mM. The Mn^{++} optima of 2 mM is considerably sharper. The activity of S_5 is 2 fold greater with Mg^{++} than with Mn^{++} . Fraction S_1 on the other hand has 1.2 fold more activity with Mg^{++} than with Mn^{++} .

In light of the observations of Nass (1969), and Albring et al. (1977) that showed mitochondrial DNA associated with membrane proteins, the resolution of multiple peaks of mitochondrial RNA polymerase may be in part

Figure 13. Ion optima of RNA polymerase activities S_1 and S_5 from Sephacryl S-200 chromatography.

Aliquots of 30 λ of peak $S_1^\bullet$ and 20 λ of peak S_5^* from low salt Sephacryl S-200 gel filtration were assayed in duplicate.



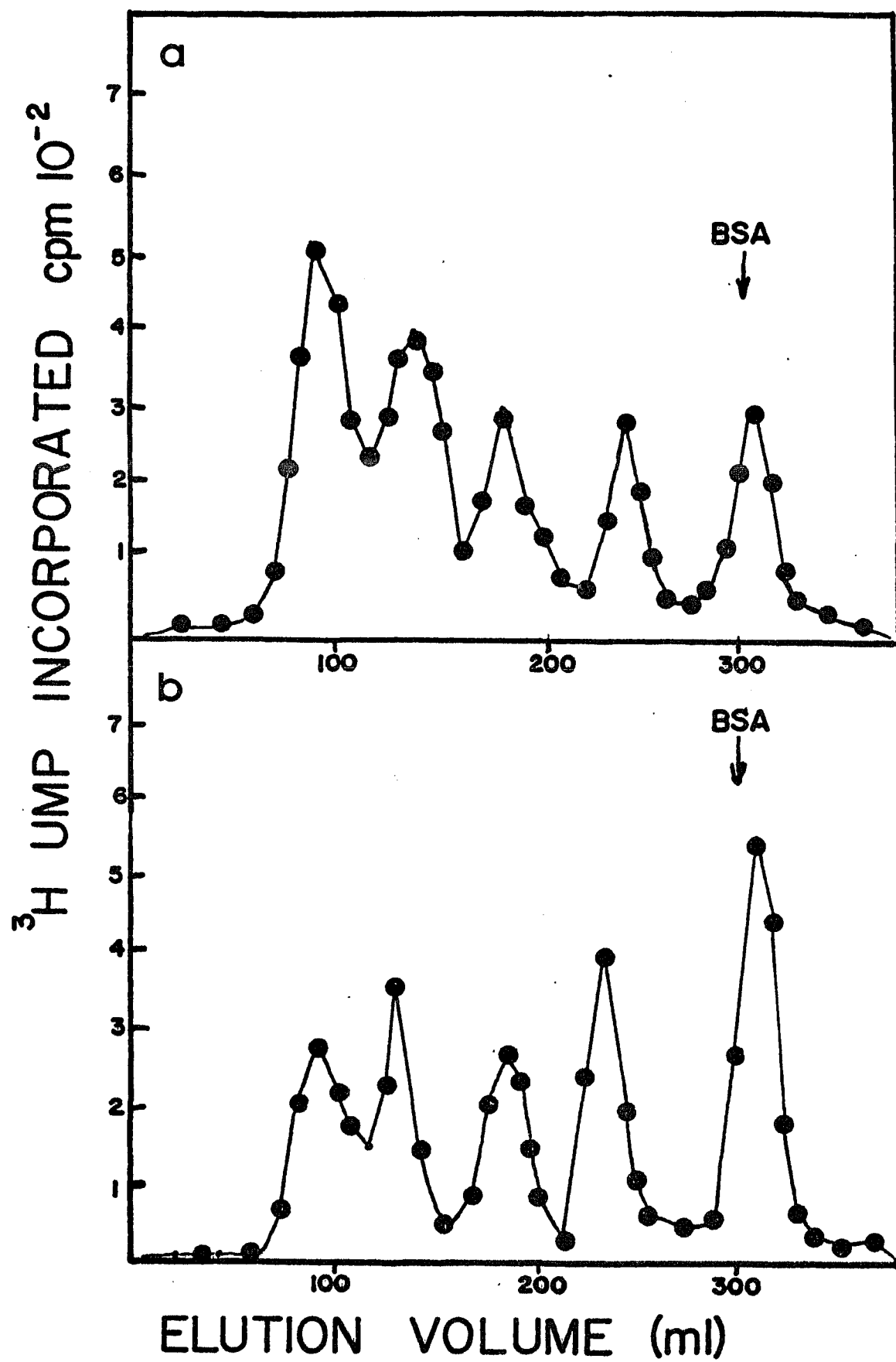
attributed to association of RNA polymerase with mitochondrial DNA and/or various membrane proteins. Certainly both the high concentration of detergent required for solubilization as well as the high degree of DNA-independence of the activity eluting in the void volume are consistent with some membrane and DNA association. Changes of gel filtration profiles in high salt might then be due to increased RNA polymerase dissociation from either DNA or protein factors.

To investigate whether the size heterogeneity might be due to either polymerase bound endogenous mitochondrial DNA or cosolubilized proteins, mitochondrial extracts were subjected to a variety of treatments, and then subjected to Sephacryl S-200 chromatography using high salt (0.35 M $(\text{NH}_4)_2\text{SO}_4$) conditions unless otherwise indicated. These treatments included two-step ammonium sulfate fractionation (see methods) DNase incubation, hi-speed centrifugation, gel filtration in higher salt (1 M ammonium sulfate) and in 2 M urea. (see Figure 14). The results of these experiments show that little change in the heterogenous elution profile of activities is found with these treatments.

The idea that this size heterogeneity is due to differential association of DNA with polymerase molecules is hard to entirely rule out. DNase may have no effect either due to detergent inactivation or polymerase protection. Results using PEG precipitation of the DNA have been ambiguous. Similarly, any number of protein factors could

Figure 14. Gel filtration chromatography of mitochondrial RNA polymerases on Sephacryl S-200 in 2 M urea and a) low salt and b) high salt conditions.

This experiment was performed under the same conditions of chromatography as indicated in Figure 9, except that 2 M urea was included in all the buffers. Aliquots of 20 μ l were assayed. BSA was used as molecular weight marker; its elution profile determined independently under the same conditions.



be co-solubilized along with mitochondrial RNA polymerase as is cytochrome oxidase. These factors would have to remain tightly bound to the polymerase, or be very similar in charge to the polymerase if the same discrete size distribution of activity peaks occurs a) whether different solubilizing agents are used, b) in the presence of ammonium sulfate precipitation, high speed centrifugation, and c) heparin-Sepharose chromatography prior to gel filtration.

Another possibility is that various sized forms appear as a result of irreversible cross-linking in mitochondrial extracts. However, one might imagine some inactivation of polymerase activity, and/or a more random distribution of activity peaks with such a modified RNA polymerase.

The explanation I presently favor is that aggregation of mitochondrial RNA polymerase is the major cause of this size heterogeneity.

Glycerol Gradient Centrifugation and SDS Gel Electrophoresis:

One way to test the possibility that the polymerase molecule is capable of reversible aggregation was to take the activities of the largest (S_5) and smallest (S_1) apparent molecule weight from gel filtration chromatography and submit them to glycerol gradient centrifugation in both high and low salt buffers. As Figures 15 and 16 indicate, such experiments show that both S_1 and S_5 have essentially the same profile on glycerol gradient centrifugation. For both forms, the appearance of a slow-sedimenting activity (G_L)

Figure 15. Glycerol gradient centrifugation of mitochondrial RNA polymerase in low salt.

S_1 , S_2 and S_5 activities from Sephacryl S-200 were concentrated by dialysis against PEG 20,000. Aliquots (1.5-2 ml) with 3% glycerol were layered on 10-30% glycerol gradients of 11 ml. Both sample and gradient contained 0.05 M KCl. Centrifugation was for 16 hours at 40,000 RPM in a SW 41 rotor. Fractions of 15 drops were collected from the bottom of the gradient (at left) and 20 λ of every fraction was assayed for polymerase activity. Marker activity of ADH (6.2 S) and BSA (4.2 S) were run on parallel gradients under the same conditions, and sediment as indicated.

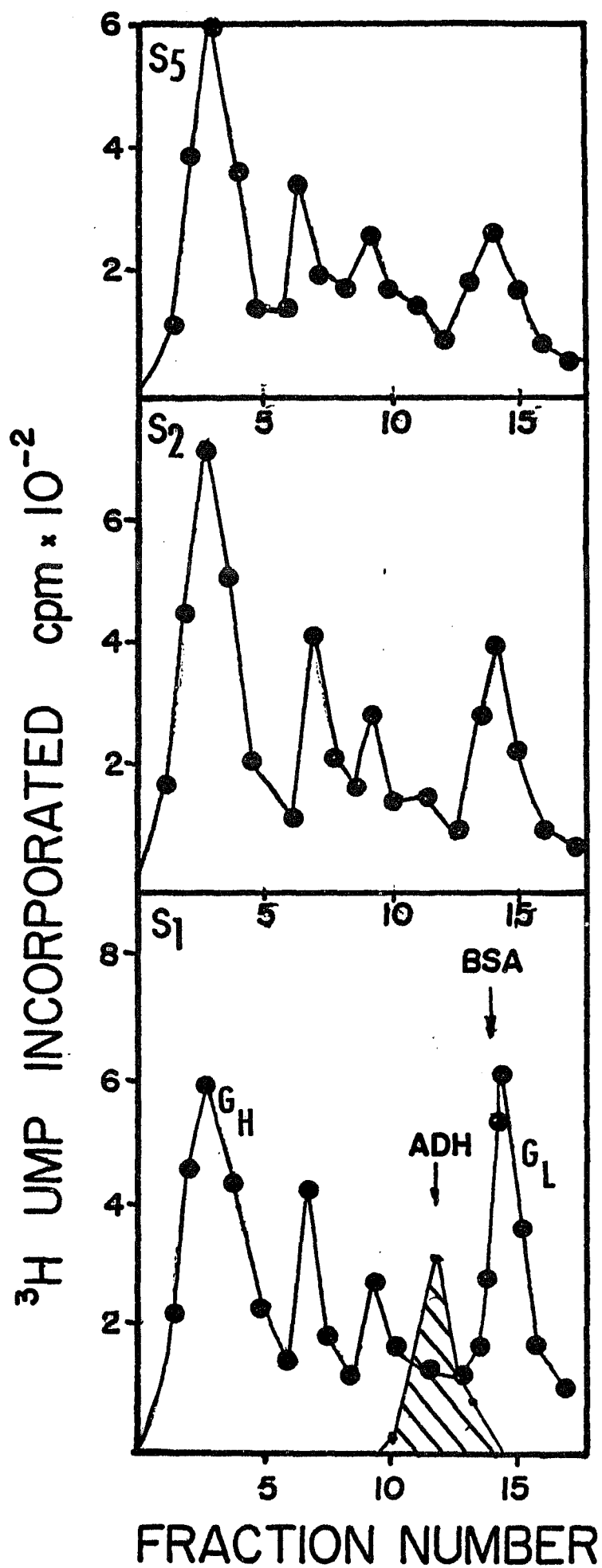


Figure 16. Glycerol gradient centrifugation of mitochondrial RNA polymerase in high salt.

The same activity peaks are layered and centrifuged on 10-30% glycerol gradients as in Figure 14 except that both sample and gradient contain 0.5 M KCl throughout.

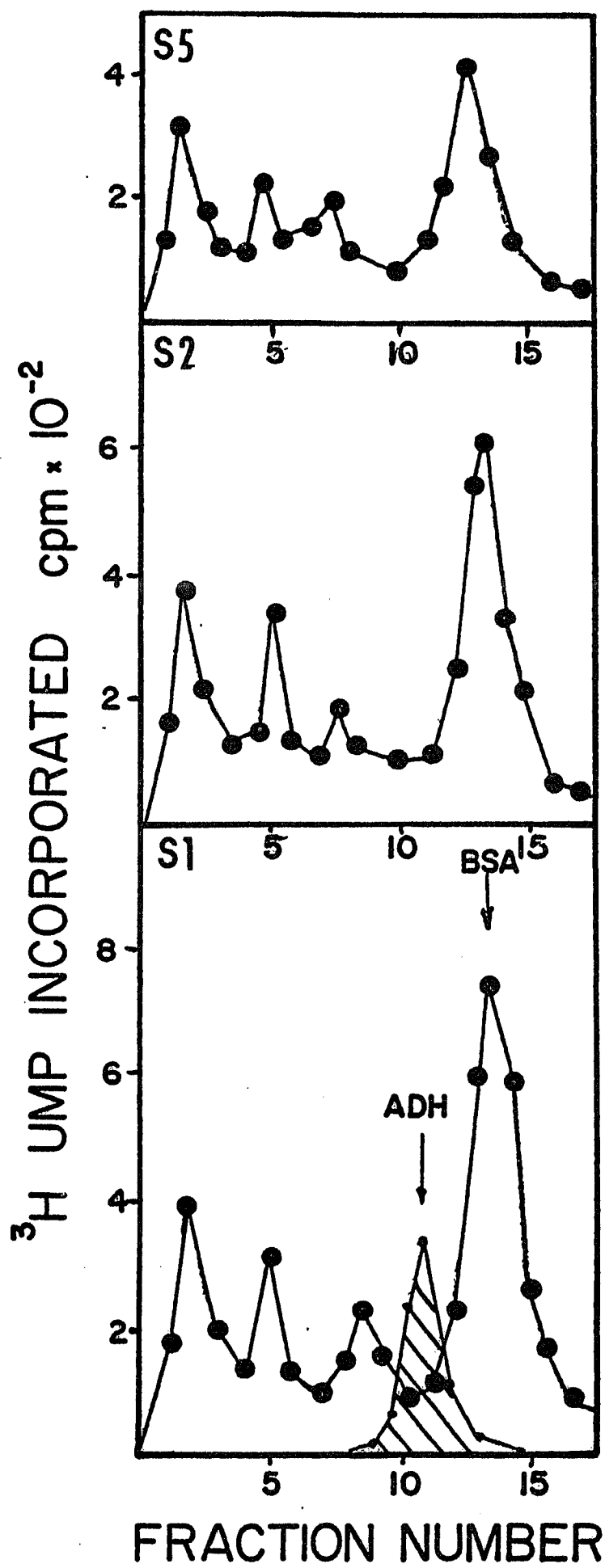
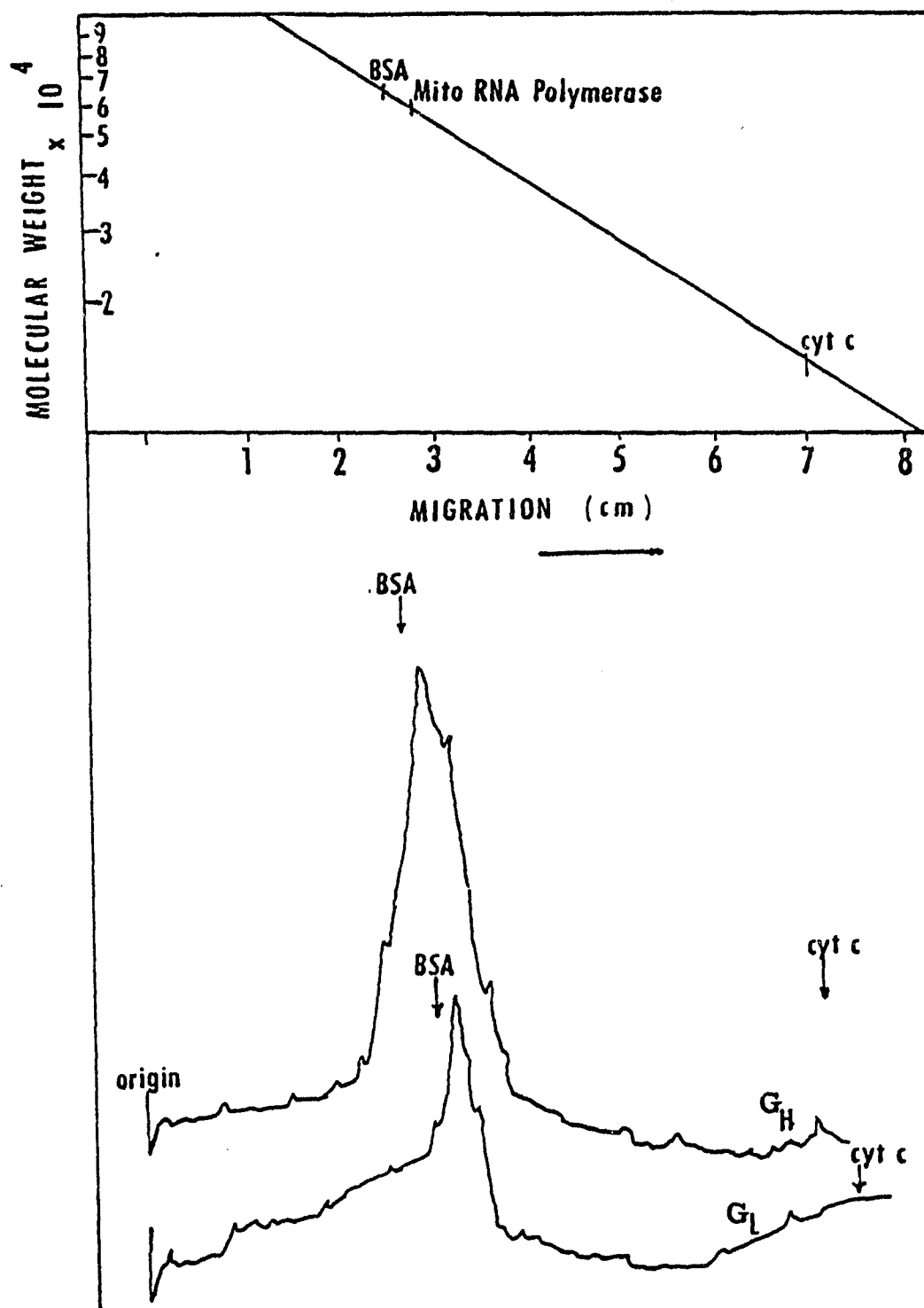


Figure 17. SDS gel electrophoresis of mitochondrial RNA polymerase from glycerol gradient centrifugation.

Peaks of the fastest (G_H) and slowest (G_L) sedimenting polymerase activities from glycerol gradient centrifugation were dialyzed against PEG 20,000 (for concentration), and against sample buffer. These samples containing 20-30 ug protein were then boiled, in the presence of B-mercaptoethanol. SDS gel electrophoresis was performed in the manner and Weber and Osborn (1969) on 10 cm gels of 10% acrylamide. Markers BSA (67,000 d) and cytochrome c (13,000 d) were run on parallel gels. Shown at bottom is the densitometric tracing by Gilford scanner at absorbance of 590 nm, of gels of the G_H and G_L peaks. On top, is the molecular weight determination as calibrated by plotting the migration of the markers (cm) against the log molecular weight.



is favored in high salt conditions yielding a form around 4S, whereas faster sedimenting forms of 30-40 S are favored in low salt. The persistence of all sedimenting forms using either S_5 , S_2 or S_1 at either ionic strength is an indication of reversibility similar to that found by Schafer and Kuntzel (1971). The fact that a fast sedimenting form can be generated under low salt conditions from a low apparent molecular weight activity (S_1) resolved by gel filtration chromatography is more consistent with a self-aggregation phenomena than with an association of protein factors or DNA although these kinds of interactions are not altogether precluded. Further evidence of this contention comes from SDS electrophoresis performed on the fast (G_H) and slowest (G_L) glycerol gradient peaks (see Figure 17). Both show a single major band at approximately 65,000d. The amount of protein applied to these gels was between 5-10 ug. Experiments with more protein may reveal the presence of other protein bands. This technique would not necessarily detect associating protein factors of the same molecular weight.

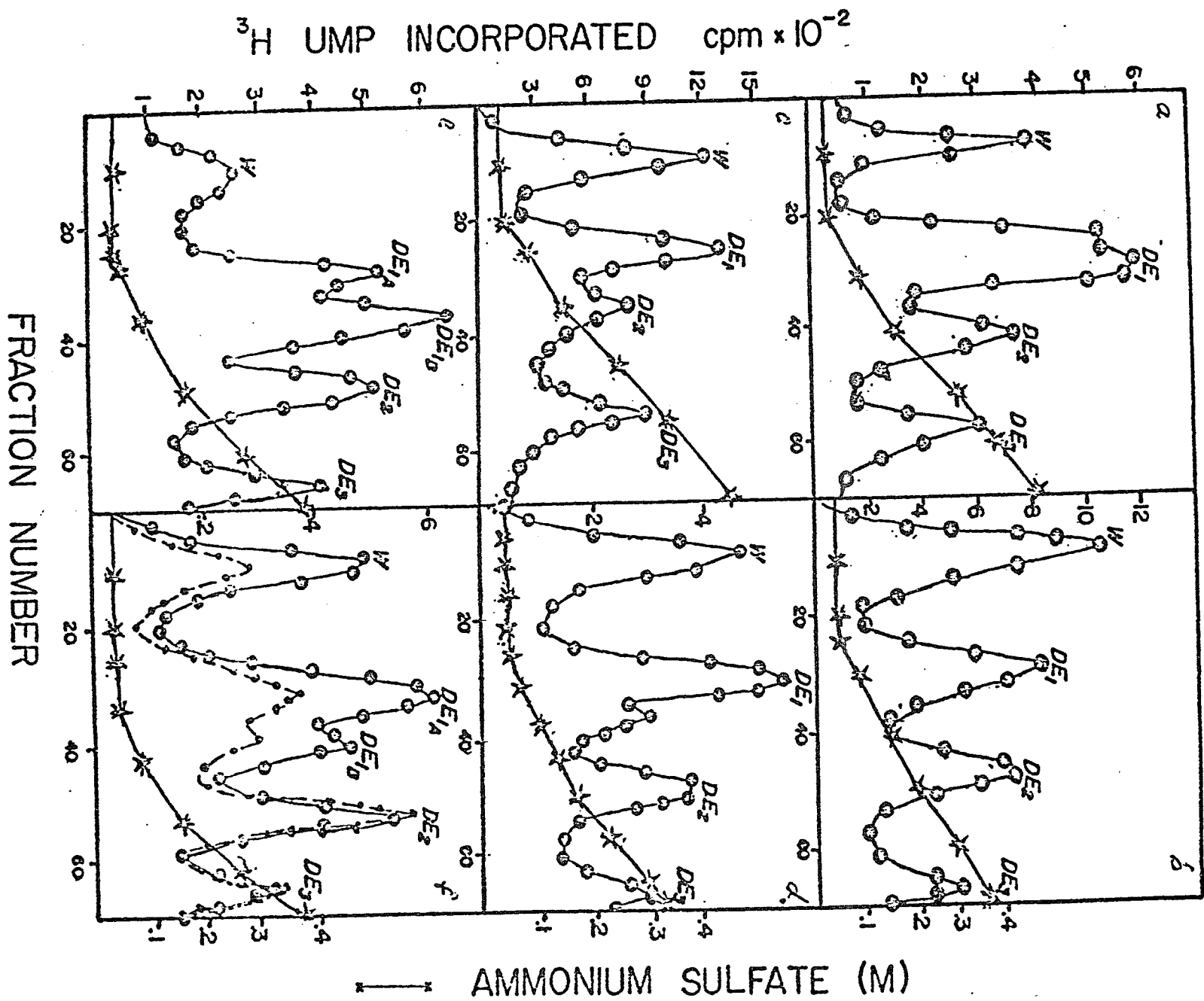
Ion Exchange and Affinity Chromatography

DEAE Sephadex: This kind of chromatography has been quite successfully used in the resolution of multiple eucaryotic nuclear RNA polymerases (Roeder, 1976), and as such, was the first purification method I tried.

Figure 18 shows the result obtained when detergent-lysed mitochondrial extracts from various developmental stages of Drosophila were chromatographed on DEAE Sephadex A-25 columns. Heterogenous elution profiles were found with all developmental stages with distinct peaks eluting at 0.04-0.09M (DE₁), 0.145M (DE₂) and 0.325-0.335M (DE₃). The proportion of activity in these peaks varies from extract to extract. In addition to these peaks, DEAE profiles all resolve a flow-through activity (which in itself is heterodisperse) that doesn't bind to the DEAE-Sephadex matrix. This flow-through (DEW) activity persists whether the column is increased in size, or whether the fraction is reapplied repeatedly to the column suggesting that it is not a result of limited matrix capacity. Much of the activity in the DEW is not dependent upon exogenous template as evidenced by the fact that incorporation in the absence of exogenous template is 60% that found when DNA is added to the reaction mix. DNase incubation of the extract prior to chromatography appears to reduce the size of the DEW peak as shown in Figure 18 with some concomittant increases in peaks eluting at higher salt concentrations, particularly DE₂ and DE₃. Furthermore, DNase treatment markedly reduces the amount of incorporation by DEW in the absence of added template to 20% of the incorporation in the presence of added DNA. This data suggests that the binding of

Figure 18. DEAE-Sephadex chromatography of mitochondrial RNA polymerase.

NP40 solubilized mitochondrial extracts (20-100 mg protein) were loaded onto 2 cm x 15 cm columns of DEAE Sephadex equilibrated with 0.05 M $(\text{NH}_4)_2\text{SO}_4$ in TGED. These columns were washed with 3-4 volumes of the same buffer, and developed with a linear gradient of 0.05 M to 0.5 M $(\text{NH}_4)_2\text{SO}_4$ in TGED. Fractions of 2 ml were collected and 20 λ aliquots assayed for RNA polymerase activity (●—●) and 50 λ aliquots assayed for $(\text{NH}_4)_2\text{SO}_4$ concentration (*—*) by conductivity. These mitochondrial extracts were from: a) 25 grams of 12 hour, dechorionated embryos; b) 50 grams of larvae; c) 50 grams of pupae; d) 25 grams of adult flies, e) same as d) except lysed mitochondria were incubated with 50 ug/ml of DNase for 20 minutes at 30°C; f) 50 grams of adults assayed with Mn^{++} (—) and without Mn^{++} (—) in the presence of 10 mM Mg^{++} .



endogenous mitochondrial DNA to the polymerase is at least part of the reason for incomplete binding to the positively-charged DEAE matrix.

The major discernable difference between extracts of different developmental stages lies in the peaks eluting between 0.04-0.1 M $(\text{NH}_4)_2\text{SO}_4$. Eggs and adults contain two peaks eluting earlier than 0.1 M; one at approximately 0.04 M, the other peak at 0.09 M. Mitochondrial extracts from pupae and larvae do not appear to contain the activity eluting at 0.04 M $(\text{NH}_4)_2\text{SO}_4$. Considering that adult females contain eggs, their DE_{.04M} activity may represent an egg specific polymerase or polymerase-bound factor.

Table 7 shows that the DEW and DE₁ peaks have a high degree of DNA independence. That is, incorporation in the absence of added template was 65% for DEW, and 45% for DE₁, of that obtained in the presence of added template. These peaks are preferentially stimulated by Mg^{++} , have a preference of poly dAT as template and are inhibited by rifampicin (50% at 100 ug/ml) and 2mM Mn^{++} (45%).

The later eluting peaks (DE₂ and DE₃) have a similar preference for Mg^{++} more than Mn^{++} as cofactor, and are inhibited by rifampicin, 60% at 100 ug/ml; DE₂ is slightly more inhibited than DE₃. These peaks all appear completely insensitive to the toxin alpha-amanitin, even at a dose of 100 ug/ml. The latter peaks DE₂ and DE₃ do differ from the early peaks in that they are >90% DNA-dependent, and are not

Figure 19. Rechromatography of the DE_2 peak on DEAE-Sephadex.

Isolated mitochondria from 50 grams of adult flies were NP40-solubilized, and applied to a DEAE-Sephadex column as described. The peak which was eluted at 0.145 M $(NH_4)_2SO_4$ (DE_2) was diluted to 0.05 M $(NH_4)_2SO_4$, reapplied to a second column, and eluted with a linear gradient of 0.05 to 0.6 M $(NH_4)_2SO_4$. One ml fractions were collected, and 20 μ aliquots of every other fraction were assayed.

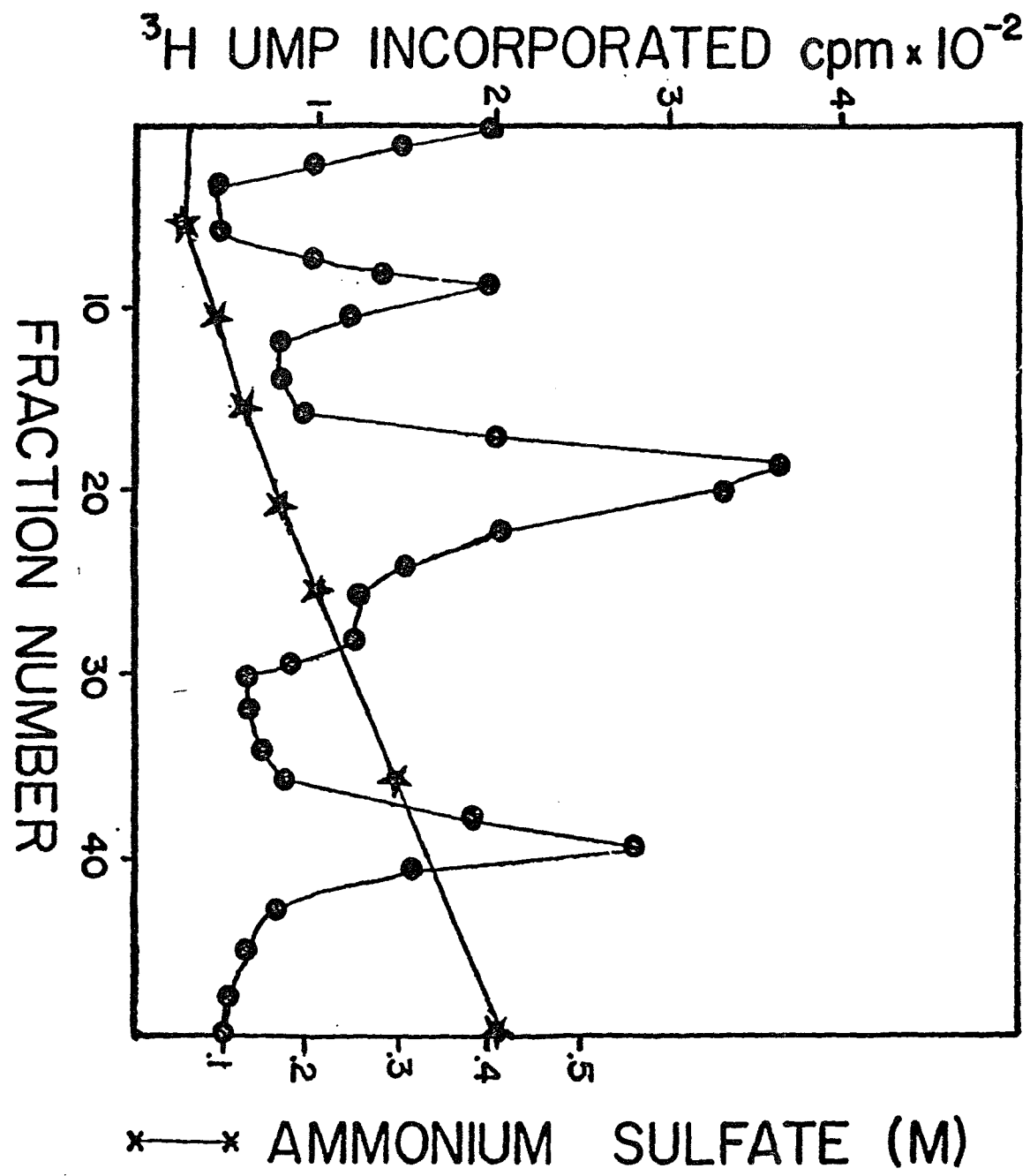


Figure 20. Rechromatography of the DE_3 peak on DEAE-Sephadex.

The 0.33 M $(NH_4)_2SO_4$ peak (DE_3) resolved upon DEAE Sephadex chromatography of the NP40-solubilized mitochondria, was diluted to 0.05 M $(NH_4)_2SO_4$ and rechromatographed on a 1 cm x 6 cm DEAE-Sephadex column. Elution, fractionation and assay were as described in the legend of Fig. 19.

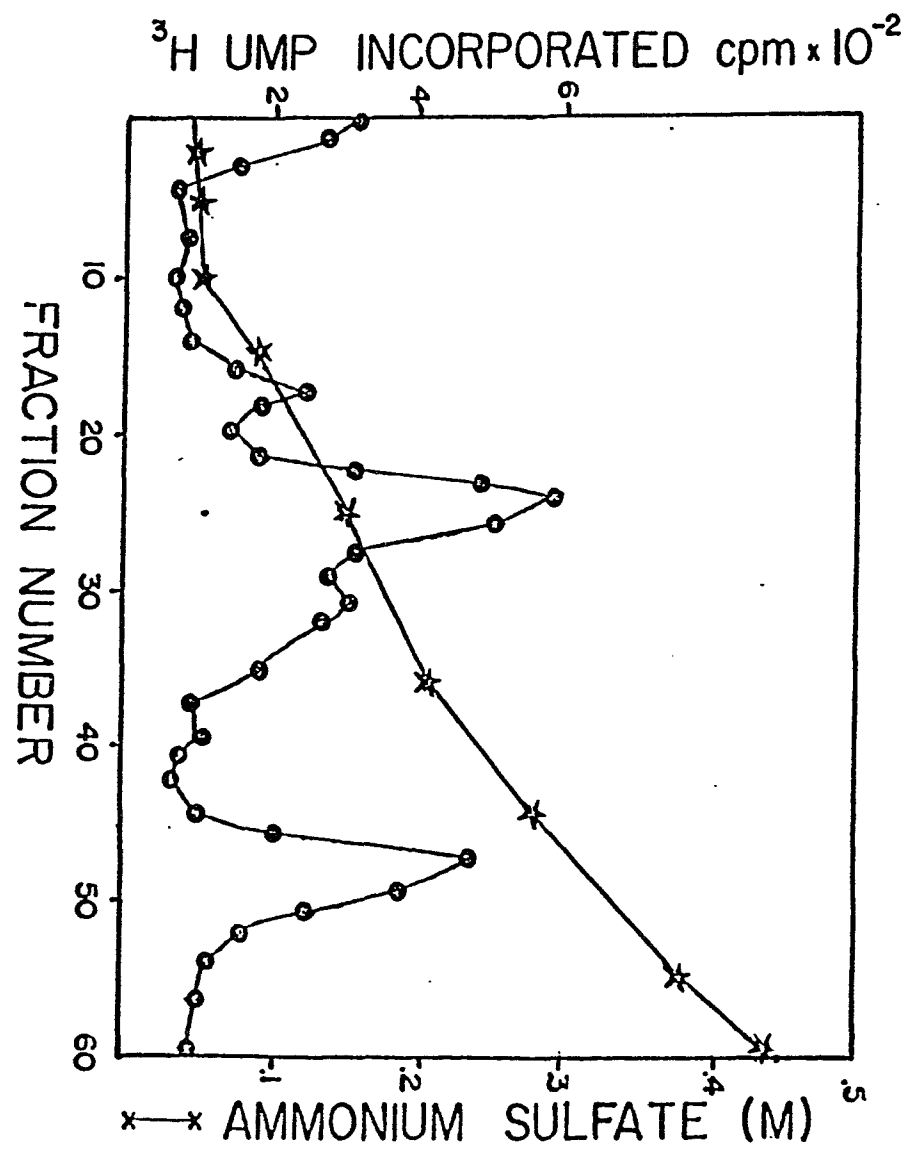


TABLE 7

PROPERTIES OF RNA POLYMERASE ACTIVITIES RESOLVED BY
DEAE-SEPHADEX CHROMATOGRAPHY

	DE _W (wash)	DE ₁ (<0.09M)	DE ₂ (0.145M)	DE ₃ (0.33M)
α -amanitin 100 ug/ml	96	100	105	100
rifampicin 50 ug/ml	50-60	50-60	30-40	50-60
control 10mM Mg ⁺⁺	100	100	100	100
2mM Mn ⁺⁺ (-Mg ⁺⁺)	60	45	53	85
5mM Mn ⁺⁺ (-Mg ⁺⁺)	40	40	50	60
2mM Mn ⁺⁺ + 10 mM Mg ⁺⁺	60	55	70	102
poly dAT	400-500	100-150	400-500	250-300
- DNA	55-60	20	10	5-10
SS DNA	160	120	180	140

Results are expressed as % of control incorporation with 10mM Mg⁺⁺. Assays used 20 λ of the indicated peaks in a total assay volume of 0.1ml. Incorporation for the control assay was 550-600 CMP above background. All results are average of two duplicate assays.

inhibited by 2mM Mn^{++} in the presence of Mg^{++} . In summary, the rifampicin sensitivity, lack of inhibition by alpha-amanitin and preference for Mg^{++} over Mn^{++} distinguish these activities resolved on DEAE from nuclear polymerases I, II and III. In addition, experiments with isolated nuclear enzymes solubilized in high salt show major peaks which elute at 0.11 M and 0.185 M which do not appear in these mitochondrial preparations (Nishiura et al., 1975).

These heterogeneous elution profiles of mitochondrial RNA polymerase activity on DEAE Sephadex were not appreciably altered when the extract was fractionated by ammonium sulfate, or subjected to high speed centrifugation.

Rechromatography of any discrete peak from DEAE-Sephadex resolves the same profile of multiple activities. Figures 19 and 20 show the results of rechromatography of peaks DE_2 and DE_3 on DEAE-Sephadex. One possible explanation for this data involves a model of a rapid, reversible, equilibrium between forms which might result from self-aggregation of polymerase molecules, or association of the polymerase with other proteins.

Phosphocellulose

A second type of ion-exchange resin used was the cation-exchanger, phosphocellulose, which was able to quantitatively bind the positively-charged mitochondrial RNA polymerase. Three predominant activities eluting at 0.1 M

Figure 21. Phosphocellulose chromatography of mitochondrial RNA polymerase.

Isolated mitochondria from 50 grams of adults were lysed with 0.5% NP40, and centrifuged at 15,000 xg for 20 minutes. The supernatant containing between 100-150 mg of mitochondrial protein in 10-20 ml was applied to a phosphocellulose column (2 cm x 25 cm) which had been equilibrated with 0.05M(NH₄)₂SO₄ in TGED. The column was washed with 40-50 ml of 0.05M (NH₄)₂SO₄ and then eluted with 40 ml steps of 0.1 M, 0.2M, 0.5M and 1 M (NH₄)₂SO₄ in TGED. Fractions of 3ml were collected, and 20 μ l aliquots of every other other fraction was assayed for RNA polymerase (●—●) as described in Methods, Absorbance 280 (●—●) and (NH₄)₂SO₄ concentration (x—x). Flow rate was 15-20 ml/hour.

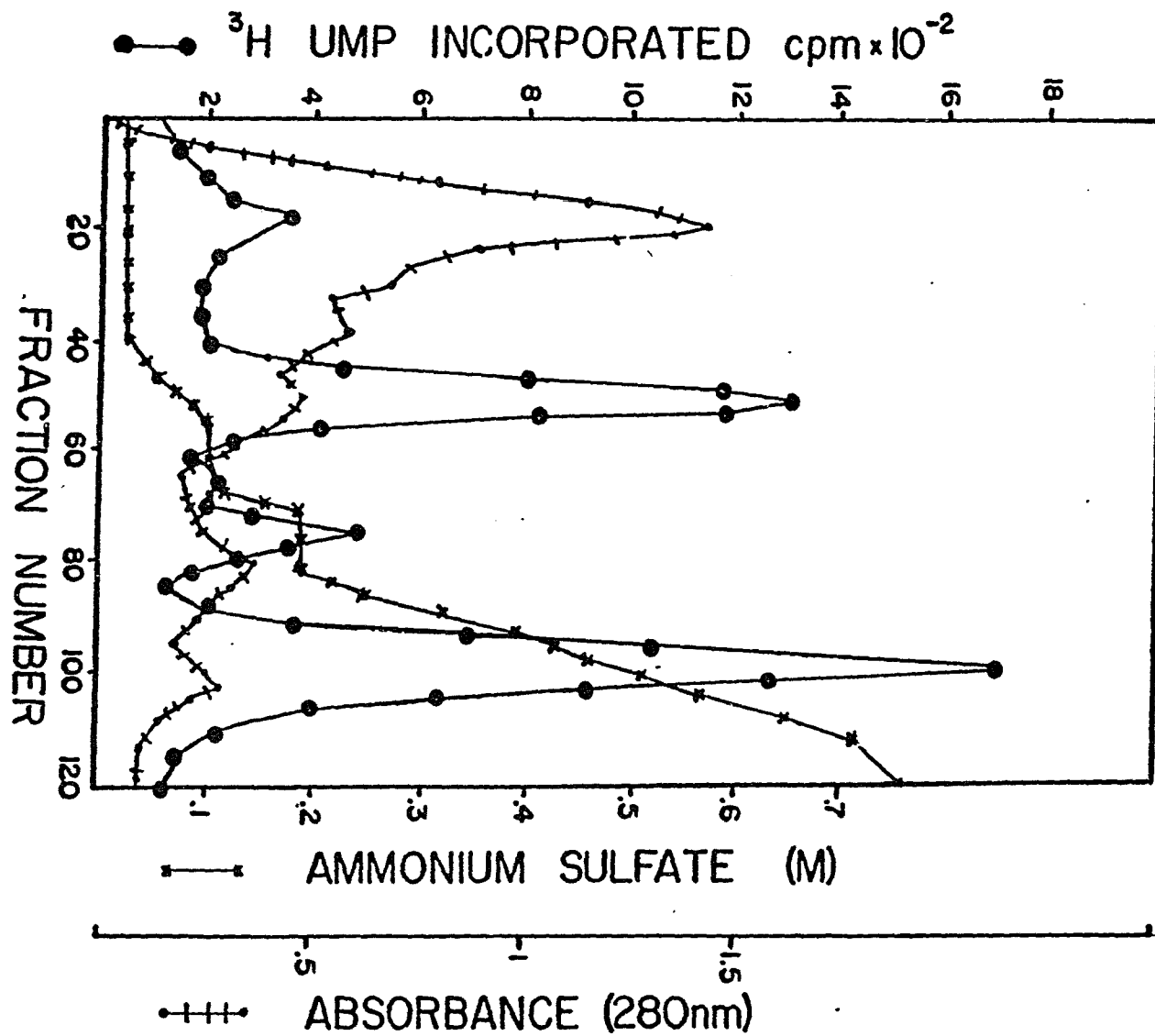


Figure 22. Ion optima of mitochondrial RNA polymerase activities PC_1 and PC_3 from phosphocellulose chromatography.

Peaks PC_1 (0.08-0.1M $(NH_4)_2SO_4$) and PC_3 (0.4-0.45 M $(NH_4)_2SO_4$) from phosphocellulose chromatography were dialyzed 4-5 hours against 2 L of TGED. 25 λ aliquots of PC_1 (★—★) and 15 λ aliquots of PC_3 (●—●) were assayed as indicated in the presence and absence of Mn^{++} and Mg^{++} , and with increasing concentration of $(NH_4)_2SO_4$. All points represent the average of duplicate assays.

^3H UMP INCORPORATED

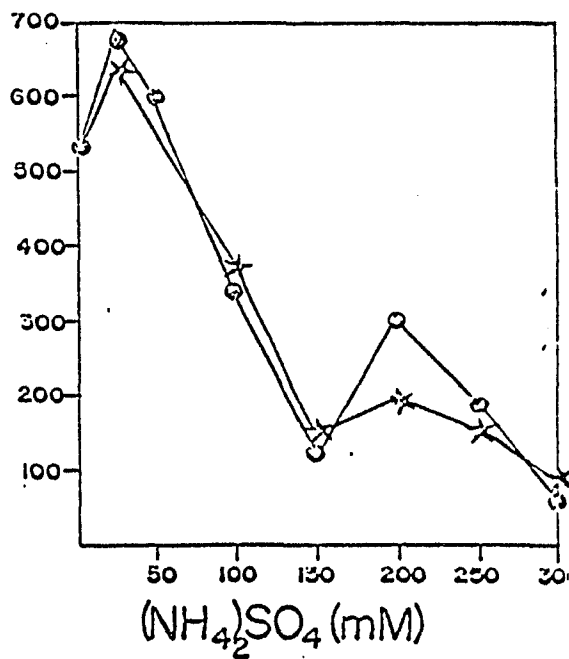
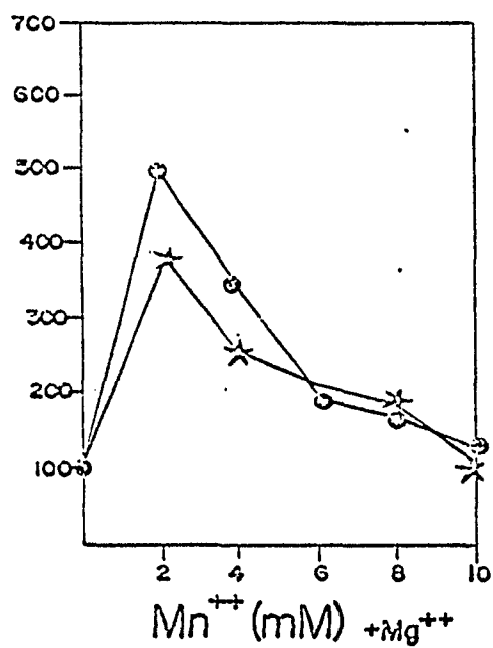
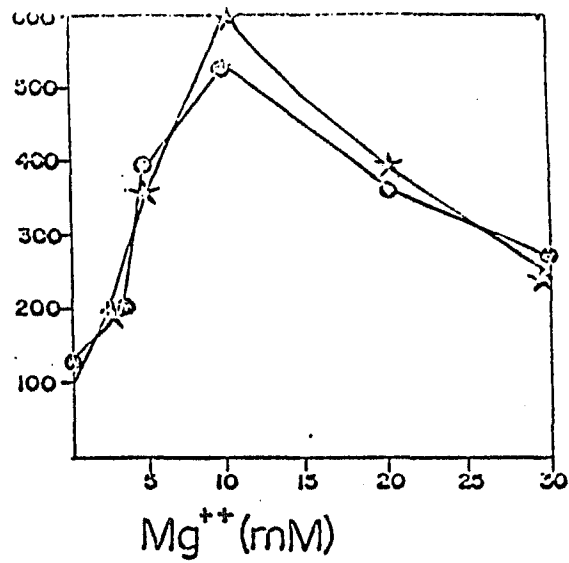
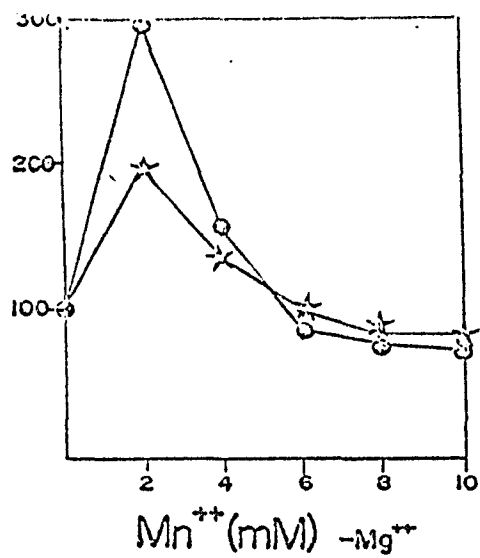


TABLE 8

PROPERTIES OF RNA POLYMERASE ACTIVITIES RESOLVED BY
PHOSPHOCELLULOSE CHROMATOGRAPHY

	PC ₁	PC ₂	PC ₃	Crude mito. extract
	(0.8M)	(0.2M)	(0.4M)	
control reaction (+10mM Mg ⁺⁺)	465	325	490	620
rifampicin 50 ug/ml	250 (55%)	190 (60%)	315 (65%)	380 (60%)
2mM Mn ⁺⁺ -Mg ⁺⁺	230 (50%)	185 (60%)	295 (62%)	320 (55%)
α -amanitin 100 ug/ml	455 (<5%)	330 (0)	500 (0)	610 (<5%)

Results are expressed as CPM incorporated, and as % inhibition of control incorp(in parenthesis). Peaks were desalted by dialysis prior to assay. Aliquots of 20 λ were assayed in duplicate.

(PC₁), 0.2M (PC₂) and 0.4M (PC₃) are resolved by this type of column, as shown in Figure 21.

The properties and optima of the polymerase peaks from phosphocellulose are depicted in Figure 22 and Table 8. All peaks tested were insensitive to alpha-amanitin at 100 ug/ml, and sensitive to rifampicin. The earlier eluting peaks (PC₁ and PC₂) were slightly more sensitive to rifampicin (45% inhibited at 50 ug/ml dose) than later eluting peak (35% inhibited at 50 ug/ml). Both the early and late eluting peaks show similar ion optima, with a broad, greater response to Mg⁺⁺ (10-15 mM) and narrower Mn⁺⁺ optima of 2 mM (see Figure 22). Peaks PC₁ and PC₂ show slightly greater Mn⁺⁺ inhibition when assayed in the presence of 10 mM Mg⁺⁺ than does PC₃; about 31% as compared to 15%. PC₃ also shows increased activity in the presence of Mn⁺⁺ without Mg⁺⁺ in the reaction mix than does PC₁ albeit giving a much lower activity than when assayed in the presence of Mg⁺⁺ alone. When assayed in the presence of Mn⁺⁺ without Mg⁺⁺, PC₃ shows 65-70% of the incorporation found in the presence of Mg⁺⁺. Using Mn⁺⁺, PC₁ shows 50% of the incorporation seen in the presence of Mg⁺⁺. PC₃ also shows a more pronounced biphasic response to increasing (NH₄)₂SO₄ concentration as seen by a 50% greater incorporation at 200 mM ammonium sulfate (see panel D, Figure 22). In these properties, PC₃ is similar to the "monomer" from gel filtration columns, a point we shall return to.

Heparin-Sepharose

Heparin-Sepharose chromatography has been used in RNA polymerase purification because of the high affinity of the polymerase for the polyanion, heparin. Its use generally results in increasing specific activity (Sternbach, 1975) by at least 10-fold. Experiments with mitochondrial extracts chromatographed on this matrix are shown in Figure 23. As shown, greater than 90% of the mitochondrial protein does not bind this matrix which increases the specific activity of the polymerase enzyme which can be bound and eluted from the matrix. The amount of the polymerase activity not bound to heparin-Sepharose is markedly reduced if the column is loaded at higher salt concentration (0.2M). A number of activity peaks are resolved at different salt concentrations, when eluted by a linear gradient of ammonium sulfate concentration. These peaks all are stimulated by Mg^{++} , are rifampicin sensitive and not inhibited by α -amanitin. The activity in the peaks HS_2 and HS_3 are correspondingly increased when the column is loaded at higher concentration (see Figure 24). This result could be due to dissociation of the RNA polymerase from other proteins or from endogenous DNA in high salt conditions, or to a different enzyme conformation at higher salt concentration.

Figure 23. Heparin-Sepharose chromatography of mitochondrial RNA polymerase.

A NP40 solubilized mitochondrial from 40 grams of adult flies was loaded onto a heparin-Sepharose column (2 cm x 20 cm) equilibrated with 0.05 M $(\text{NH}_4)_2\text{SO}_4$ in TGED. The column was washed with 0.05 M $(\text{NH}_4)_2\text{SO}_4$, and eluted with a linear gradient of 0.05 M- 0.7 M $(\text{NH}_4)_2\text{SO}_4$ in TGED. Fractions of 3ml were collected, and 20% of every other fraction was assayed for RNA polymerase activity as described in Methods. Protein was monitored by absorbance at 280 nm as previously described.

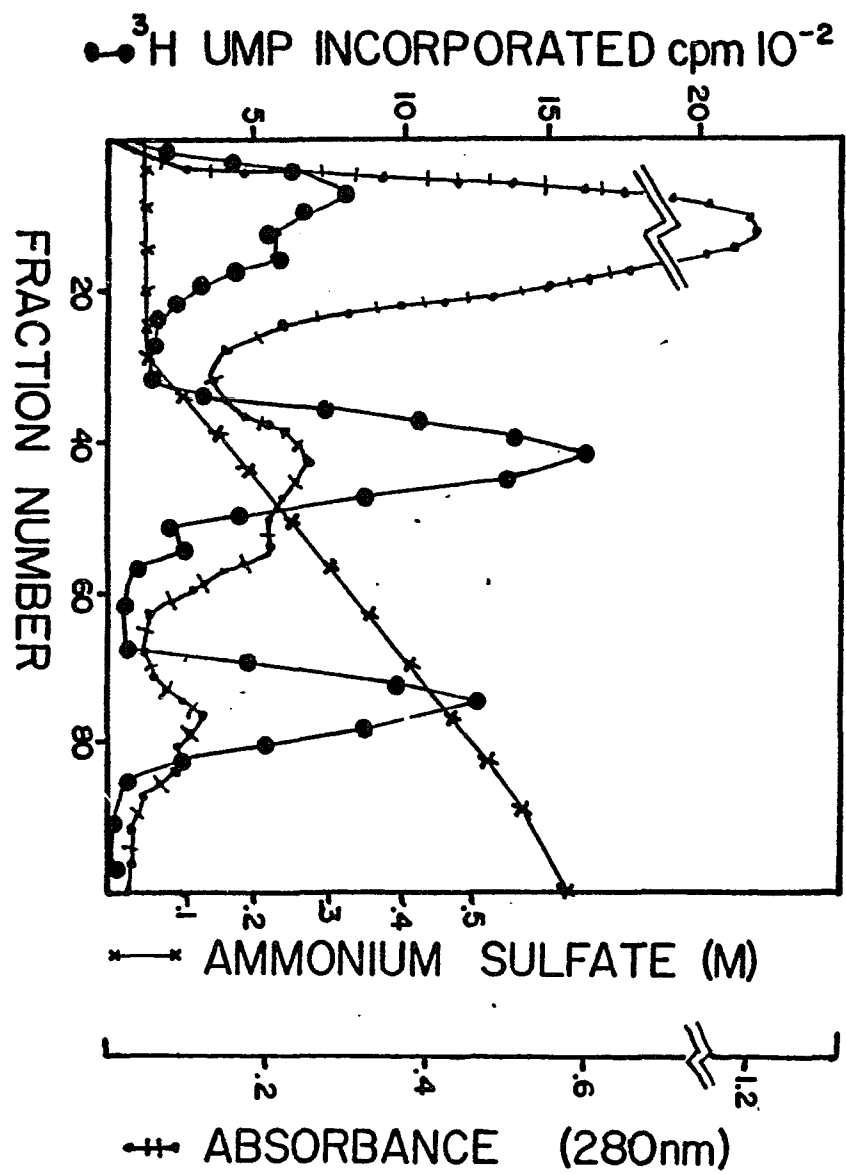
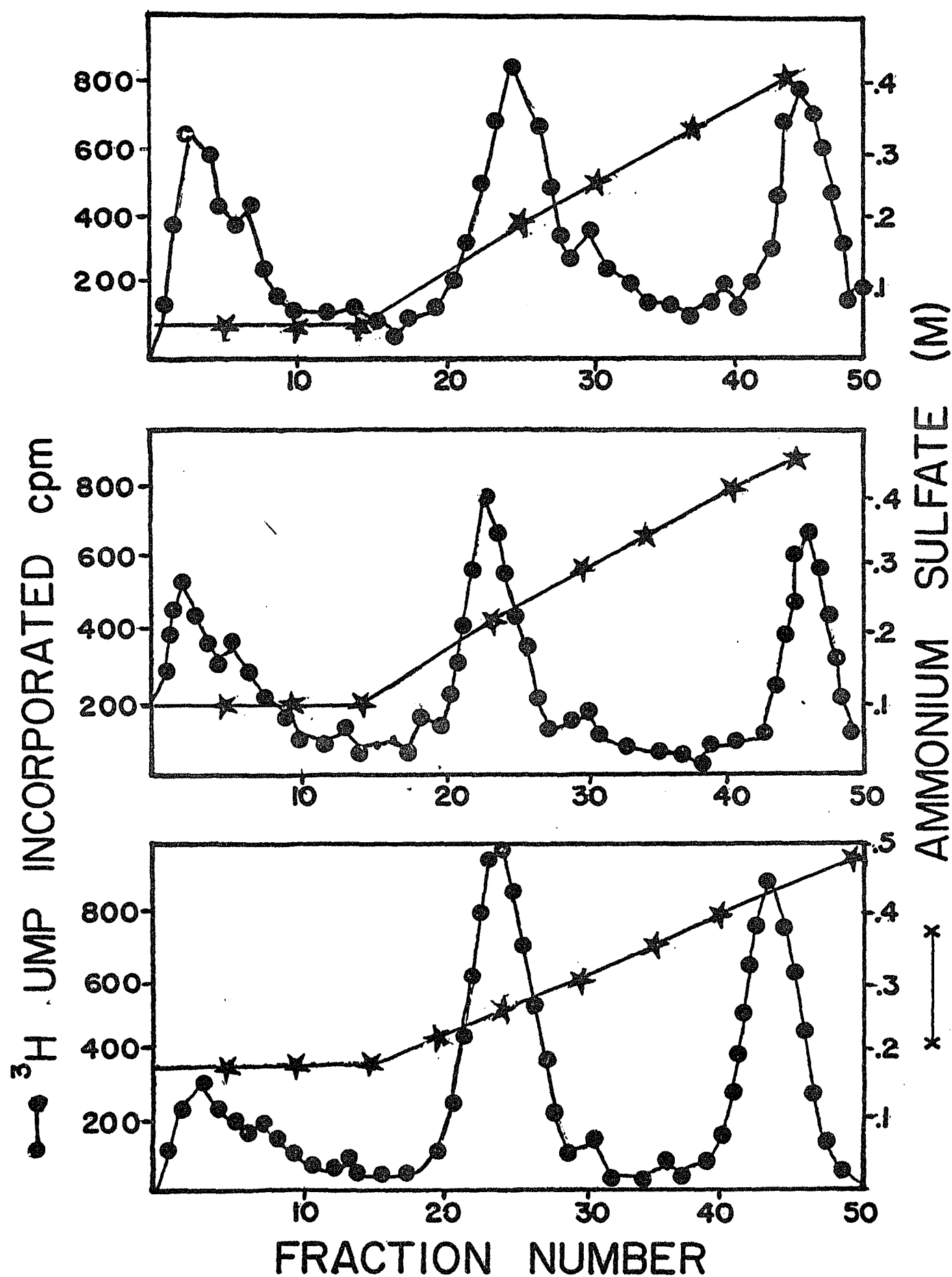


Figure 24. Effect of salt on elution profile of mitochondrial RNA polymerase from Heparin-Sepharose.

An NP40 solubilized mitochondrial extract from 60 grams of Adult flies was split in three parts. These were made 0.05 M $(\text{NH}_4)_2\text{SO}_4$, 0.1 M $(\text{NH}_4)_2\text{SO}_4$ and 0.175 M $(\text{NH}_4)_2\text{SO}_4$ respectively by the addition of 1 M $(\text{NH}_4)_2\text{SO}_4$. The extracts were loaded on to 3 separate heparin-Sepharose columns (1 cm x 15 cm) equilibrated with 0.05, 0.1 and 0.175 M $(\text{NH}_4)_2\text{SO}_4$. Washing, elution and assay conditions were as previously described in Fig. 23.



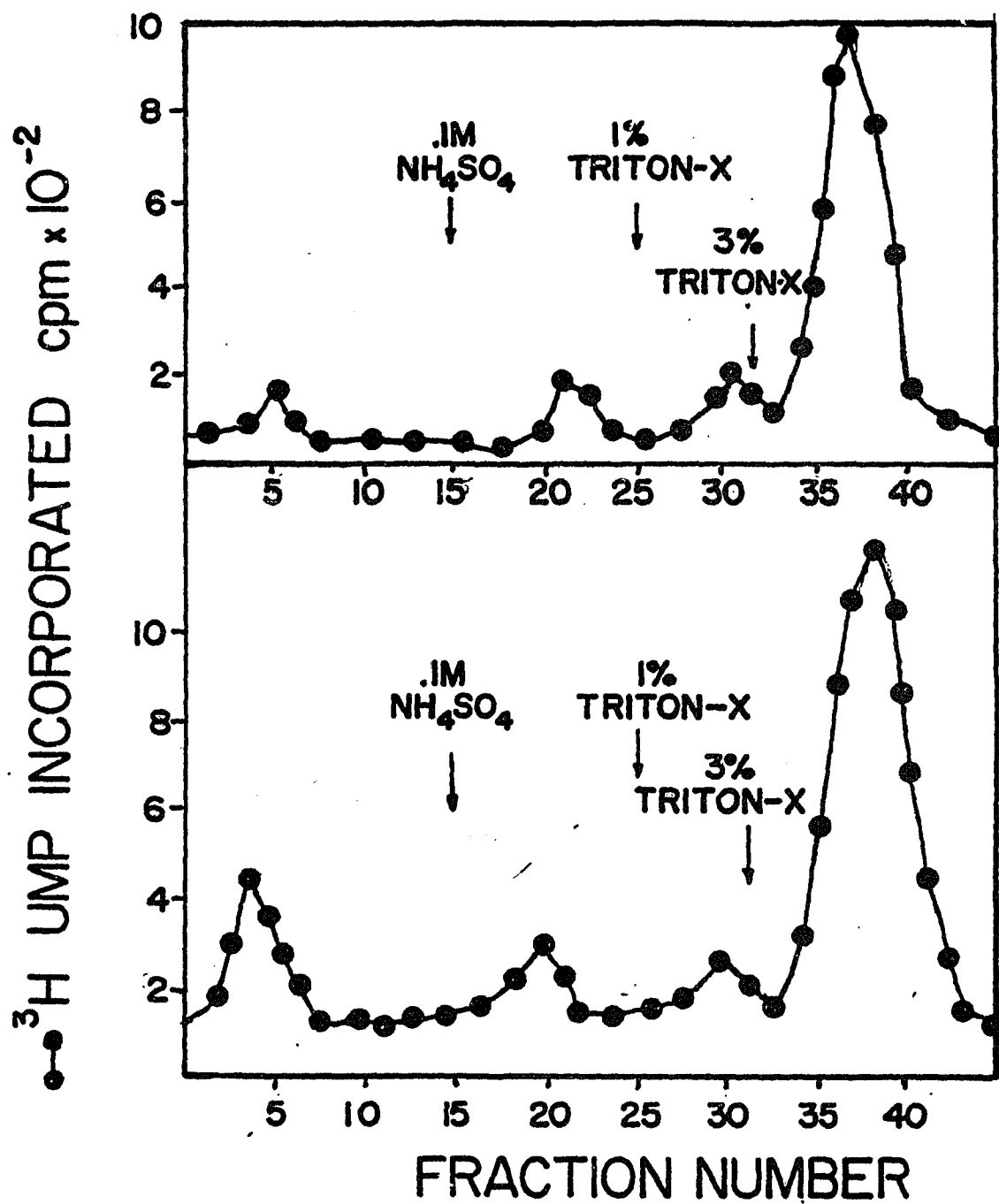
Octyl-Sepharose

Octyl-Sepharose columns were used as well in the purification of mitochondrial RNA polymerase. The fact that the mitochondrial RNA polymerase appears to be associated with the inner membrane and is stabilized by detergent suggests that it may be partially hydrophobic. This technique also serves as a convenient concentration step for polymerase purification as the enzyme binds well to the hydrophobic matrix in high salt, and is eluted homogeneously in 3% Triton X-100. Figure 25 shows the results obtained when the activities of the highest (S_5) and the lowest (S_1) apparent molecular weight obtained from gel filtration on Sephacryl S-200, are chromatographed on octyl-Sepharose columns. 10-20% of either activity is eluted by lowering the salt concentration of the elutant from 0.1 M $(\text{NH}_4)_2\text{SO}_4$. Interestingly, 25% of the "monomer" activity (S_1) doesn't bind this column, whereas less than 5% of the larger activity (S_5) remains unbound. The same proportion of activity remains unbound when larger columns of octyl-Sepharose are used.

Experiments were performed to try to establish the relationship between the different activities resolved by the different kinds of ion-exchange columns. These experiments unexpectedly gave no clear answers to this question. When a discrete peak (PC_1) from phosphocellulose chromatography is applied to DEAE-Sephadex, it resolves with little significant differences the whole gamut of DEAE

Figure 25. Octyl-Sepharose chromatography of mitochondrial RNA polymerase.

NP40 solubilized mitochondrial extracts from 100 grams of pupae were subjected to Sephacryl S-200 gel filtration chromatography in high salt. Peaks S_5 and S_1 were made 1 M $(\text{NH}_4)_2\text{SO}_4$ by the addition of appropriate amounts of saturated, buffered ammonium sulfate, and applied separately to two octyl-Sepharose columns (2 cm x 10 cm) pre-equilibrated with 1 M $(\text{NH}_4)_2\text{SO}_4$ in TGED. The columns were first washed with 1 M $(\text{NH}_4)_2\text{SO}_4$, then with 0.1 M $(\text{NH}_4)_2\text{SO}_4$ in TGED, and then 10 ml steps of 1% and 3% Triton X-100 in 0.1 M $(\text{NH}_4)_2\text{SO}_4$ in TGED. Fractions of 3 ml were collected, and 20 μ l of every other fraction was assayed for RNA polymerase activity. Top, S_5 on octyl-Sepharose. Bottom, S_1 on octyl-Sepharose.



activities as shown in Figure 26. Similarly, if either the DEW or DE₃ are rechromatographed on phosphocellulose, all three forms, i.e., PC₁, PC₂ and PC₃ appear as shown in Figure 27. If different associating factors are responsible for the different charges available for binding, all these peaks must contain all the factors, as all forms are generated upon rechromatography.

Experiments were then performed to see if the heterogeneity seen upon gel filtration chromatography was related to the heterogenous elution profiles on ion-exchange columns. Discrete peaks from ion-exchange columns were chromatographed on gel filtration columns, to see if such peaks represented activities of discrete sizes. Peaks HS₂ and HS₃ were separately chromatographed on Sephacryl S-200, and resolved nearly identical heterogenous profiles (see Figure 28).

Such a result, similar to the resolution of all forms upon rechromatography of discrete DEAE or PC peaks--might be explained by a rapid, reversible association/dissociation equilibrium involving polymerase molecules. However, the involvement of other associating factors is in no way ruled out.

The reverse experiment, that is, rechromatography of gel-filtration activities on ion-exchange matrixes was somewhat more useful in elucidating the relationship between size and charge. Figure 29 depicts the results when the

highest (S_5) and lowest (S_1) apparent molecular weight activities from Sephacryl were chromatographed on heparin-Sepharose columns. The (S_5) peak resolved 45% of activity in peak HS_1 , 40% in HS_2 and 15% in HS_3 . The (S_1) peak resolves a very different distribution of polymerase activity with 10% in peak HS_1 , 80% in HS_2 and 10% in HS_3 .

A similar pattern of distribution of Sephacryl activities upon chromatography on DEAE Sephadex or phosphocellulose matrices was obtained as shown in Figure 30. On DEAE Sephadex, the high apparent molecular weight peak (S_5) resolves 33% activity in the DEW, 10% in DE_1 , 20% in the DE_2 and 36% in DE_3 . The low apparent molecular weight activity (S_1) resolves 10% DEW, 10% DE_1 , 20% DE_2 and 60% in the DE_3 . Similar peak PC_3 is greatly enriched upon re-chromatography of peak S , from Sephacryl on phosphocellulose.

The observation that the "monomer" or lowest apparent molecular weight activity is preferentially resolved into ion exchange peaks DE_3 , PC_3 and HS_2 is further borne out by the qualities that both these particular ion-exchange activities and the monomer activity share. All these activities show a pronounced biphasic salt response, a slight decrease in rifampicin-sensitivity as well as a small but reproducible increase in the ability of Mn^{++} to be used as cofactor compared to other ion-exchange or Sephacryl activities. Conversely, a similar correspondence can be seen between the properties of the earlier eluting

Figure 26. Rechromatography of PC_1 RNA polymerase activity on DEAE-Sephadex.

Peak PC_1 was resolved from phosphocellulose chromatography of a NP40 solubilized extract from 50 grams of adults. This peak was diluted to reach conductivity equivalent to 0.05M $(NH_4)_2SO_4$ with TGED, and then applied to a 2 cm x 15 cm column of DEAE-Sephadex. Fractions of 2 ml were collected, after elution with a linear gradient of 0.05-0.4 M $(NH_4)_2SO_4$. Aliquots of 20 μ were assayed for RNA polymerase activity as previously described.

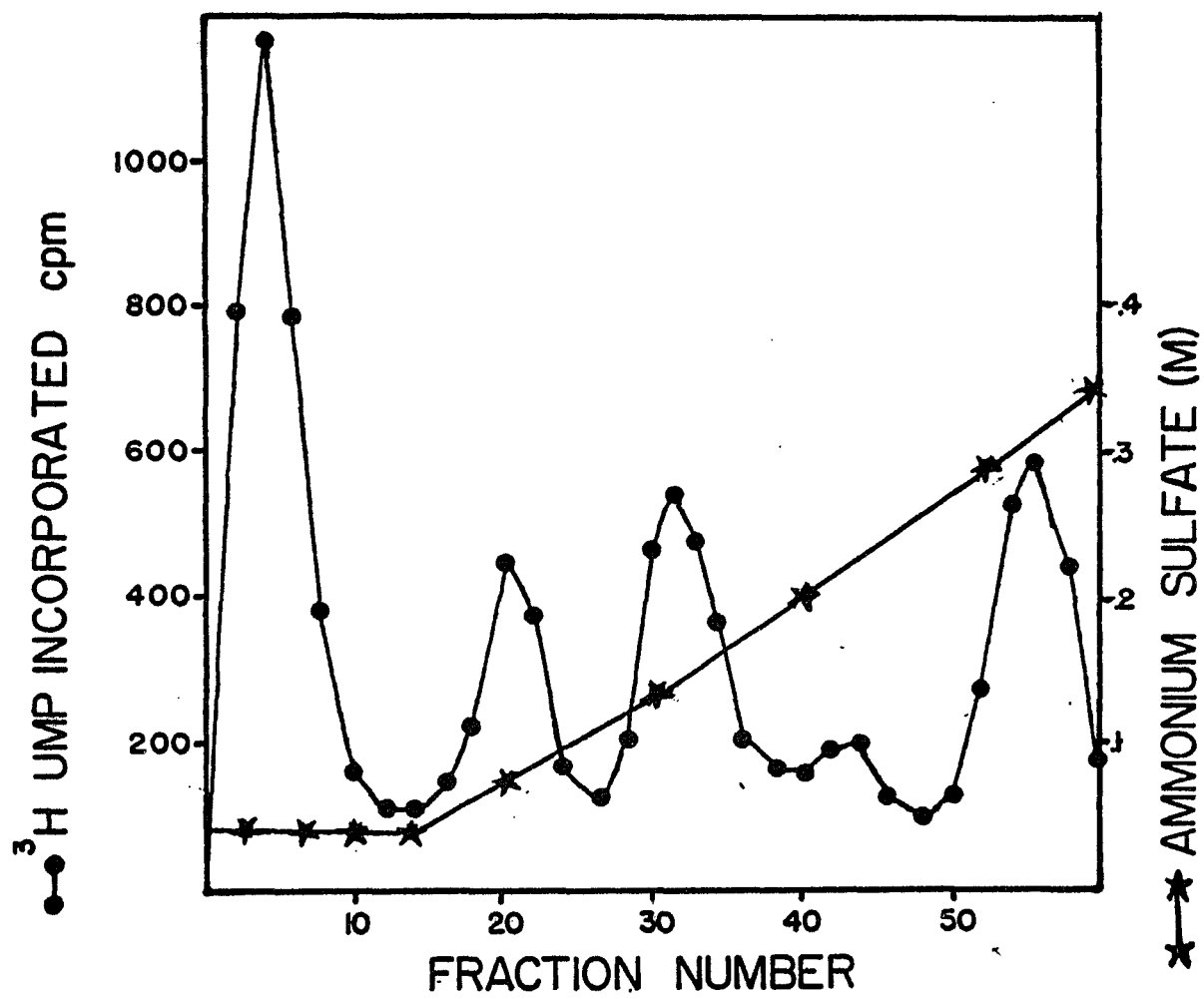


Figure 27. Rechromatography of flow-through RNA polymerase activity from DEAE Sephadex (DEW) on phosphocellulose.

Isolated mitochondria from 40 grams of adults were NP40 solubilized, and loaded on to a DEAE Sephadex column (2 cm x 15 cm) as previously described. The entire flow-through ($\leftarrow$) (50 ml) was then applied to a phosphocellulose column (2 cm x 12 cm) equilibrated with TGED. This column was washed with 25 ml steps of 0.1 M, 0.3 M and 0.5 M $(\text{NH}_4)_2\text{SO}_4$ in TGED. Fractions of 2 ml were collected, and 20 λ aliquots assayed as described earlier.

- a) DEAE-Sephadex profile
- b) phosphocellulose

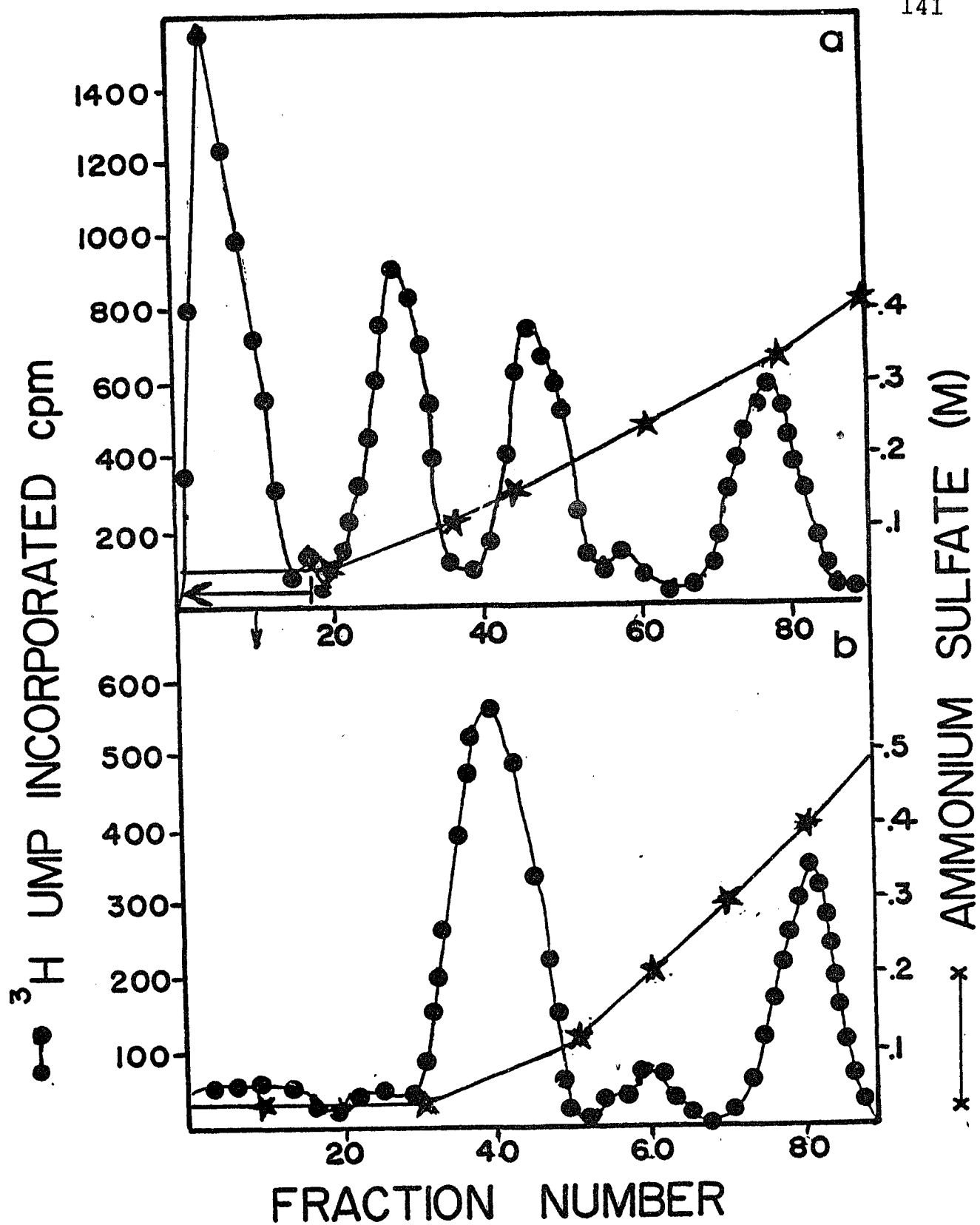


Figure 28. Gel filtration chromatography on Sephacryl S-200 of RNA polymerase peaks HS_2 and HS_3 from Heparin Sepharose chromatography.

NP40 solubilized mitochondria from 100 grams of pupae was made 0.175M $(NH_4)_2SO_4$ and loaded on a heparin-sepharose column equilibrated with 0.175M $(NH_4)_2SO_4$. Washing, elution and assay conditions were as described in Figure 23. Peaks $H-S_2$ (●—●) and $H-S_3$ (○—○) from this column were concentrated, and 3-4 ml of each peak was independently chromatographed on a Sephacryl S-200 column (2 x 80 cm) in high salt as previously described. 3ml fractions were collected, and 20 λ was assayed for polymerase activity. ADH was chromatographed independently under the same conditions.

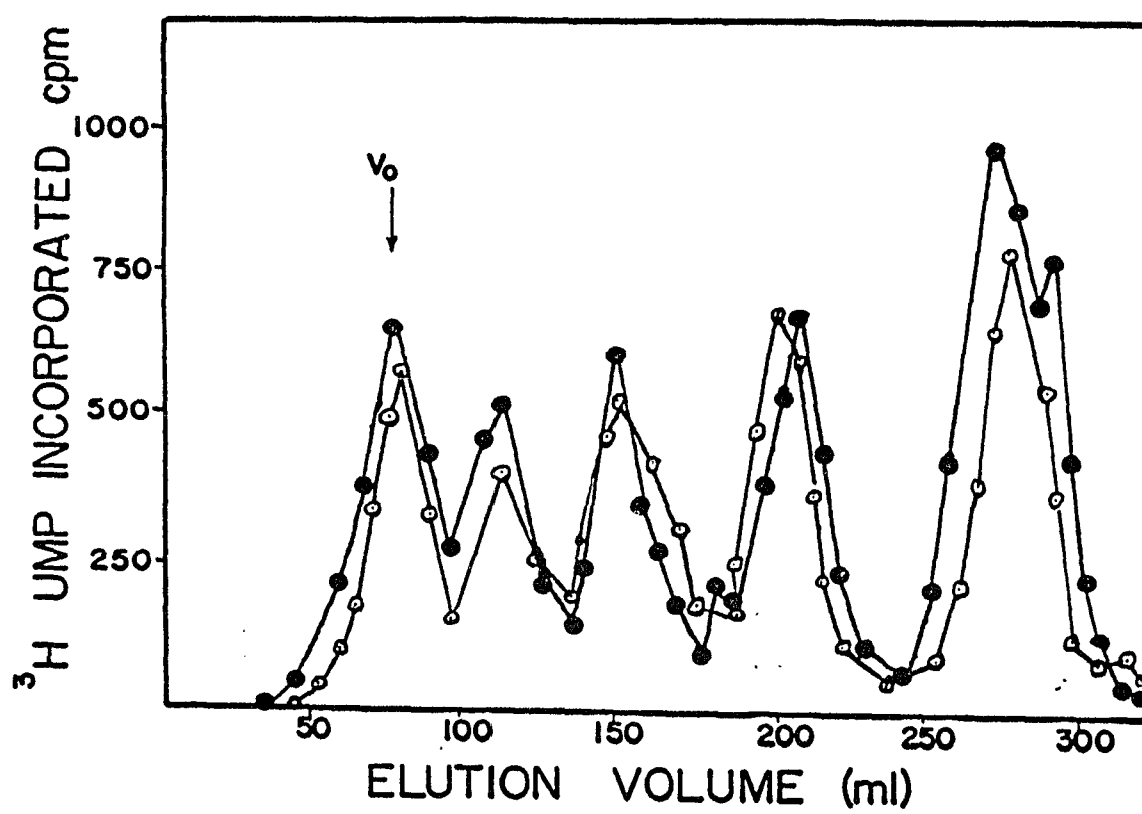


Figure 29. Heparin-Sepharose chromatography of peaks S_1 and S_5 from gel filtration.

A NP40 solubilized mitochondrial extract from 100 grams of pupae was applied to a Sephacryl S-200 column in high salt conditions ($0.35\text{ M }(\text{NH}_4)_2\text{SO}_4$). Peaks S_1 (smallest) and S_5 (largest) were diluted to $0.175\text{ M }(\text{NH}_4)_2\text{SO}_4$ and loaded on two columns of heparin-Sepharose ($1\text{ cm} \times 10\text{ cm}$) equilibrated with $0.175\text{ M }(\text{NH}_4)_2\text{SO}_4$. The columns were washed with the same buffer, and eluted with linear gradients of $0.175 - 0.6\text{ M }(\text{NH}_4)_2\text{SO}_4$ in TGED. Fractions of 4 ml were collected, and 20 λ aliquots assayed from every other fraction for RNA polymerase activity. Top, S_5 chromatographed on heparin Sepharose. Bottom, S_1 chromatographed.

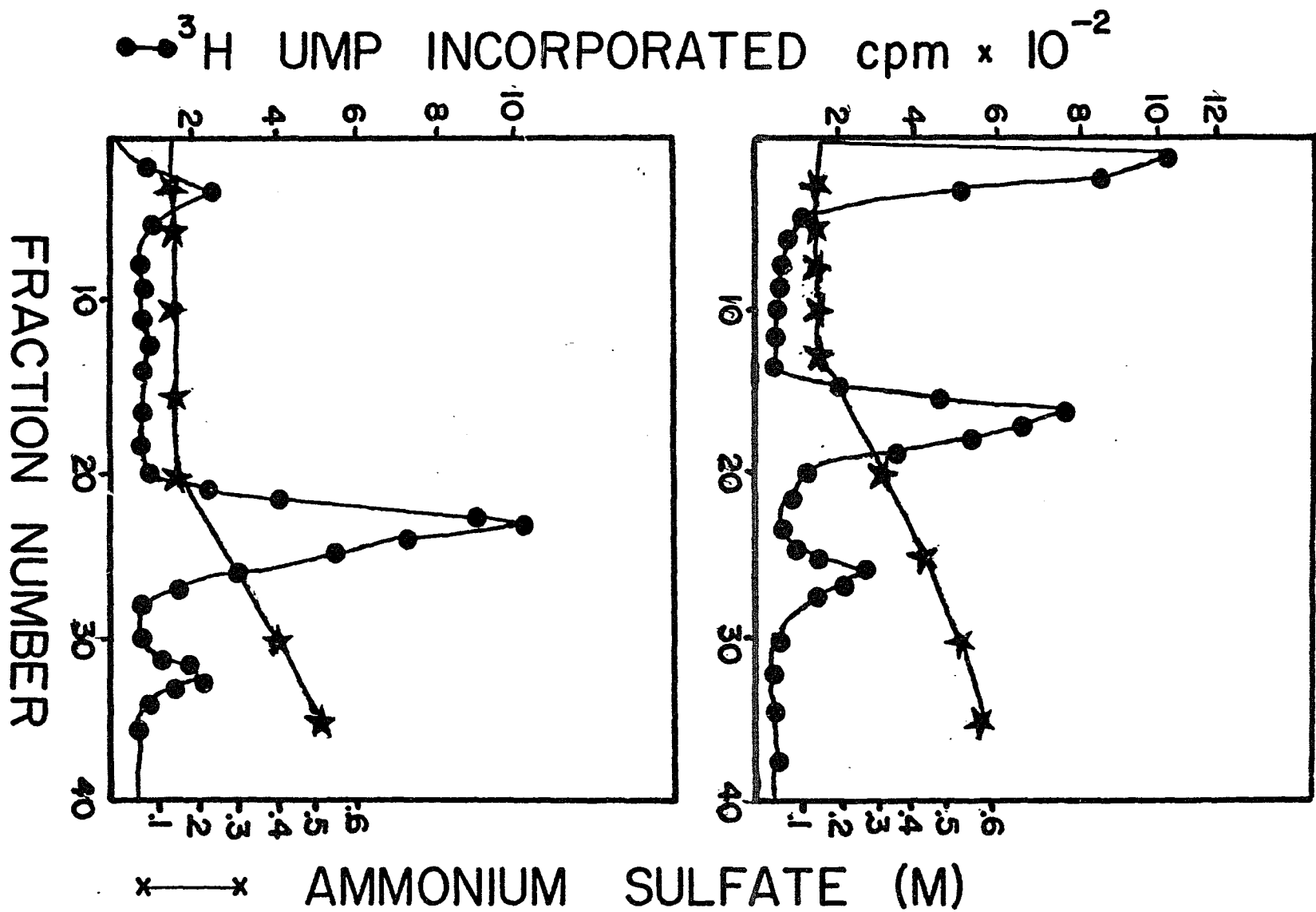
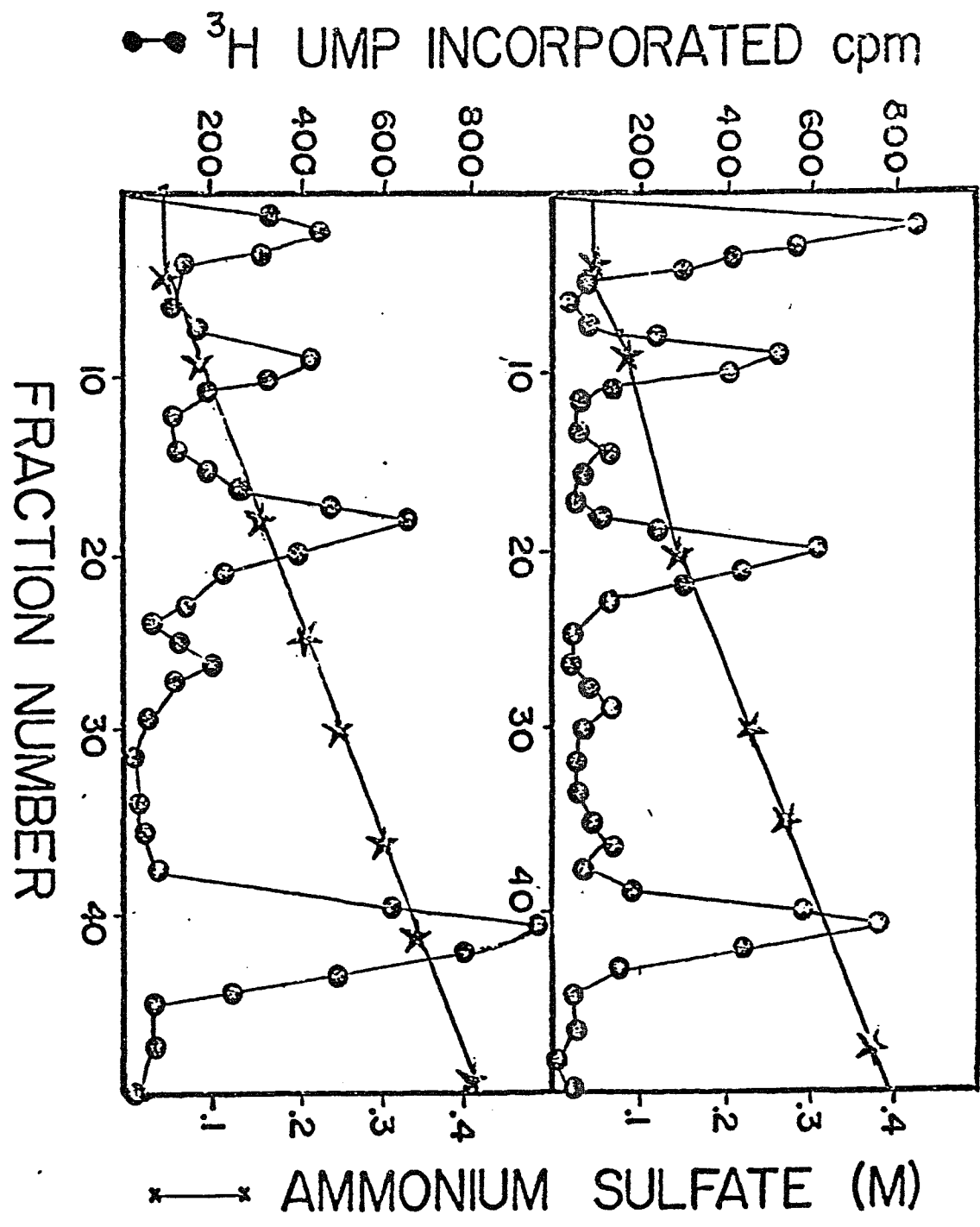


Figure 30. DEAE-Sephadex chromatography of activity peaks S_1 and S_5 from Sephacryl S-200 gel filtration.

A NP40 solubilized mitochondrial extract from 100 grams of pupae was applied to Sephacryl S-200 in high salt. Peaks S_5 and S_1 were diluted to 0.05 M $(\text{NH}_4)_2\text{SO}_4$ and re-applied to two DEAE Sephadex columns (1 cm x 10 cm) equilibrated with 0.05 M $(\text{NH}_4)_2\text{SO}_4$ in TGED. Columns were washed with 30 ml of 0.05 M $(\text{NH}_4)_2\text{SO}_4$ in TGED, and eluted with linear gradients of 0.05-0.5 M $(\text{NH}_4)_2\text{SO}_4$ in TGED. Fractions of 2 ml were collected, and 20 λ aliquots were assayed for RNA polymerase activity. Top, S_5 on DEAE-Sephadex. Bottom, S_1 on DEAE-Sephadex.



species from DEAE Sephadex and PC, and the high molecular weight species from Sephacryl chromatography.

This data might be explained by the suggestion that the polymerase size influences its charge properties. This "size" effect might be due to self associating aggregation of the polymerase, or associating protein factors. The evidence of reversibility on gel filtration, glycerol gradients and rechromatography could satisfy either possibility. The finding that the higher apparent molecular weight form binds well to octyl-Sepharose is interesting in that large enzyme aggregates may be so folded so as to have very little net charge. This may partially explain why these RNA polymerase molecules do not bind well to DEAE Sephadex, and elute early from phosphocellulose. Smaller aggregates would have a higher charge density while the monomer with the highest charge density would be best able to bind either cation or anion exchange columns. Alternatively the different charge binding and hydrophobicity of higher vs. lower molecular weight forms might be due to differential association of membrane factors with the higher forms.

Chromatographic experiments in which we have tried to push the "equilibrium" entirely in one direction have not yet resolved a single species. Chromatography in either 2M urea, or 1M ammonium sulfate have shown little change in profiles of activity. However, the dissociation of these aggregates appears to be complete in SDS and

mercaptoethanol as demonstrated by the SDS electrophoresis experiments. These resolve a single protein subunit, suggesting that the major force in the large form is self-'aggregation', as well as suggesting that SH bonds might well be a major causal factor of aggregation. Further experiments with denaturants such as SDS and guanidine-HCl as well as sulfhydryl--combining reagents such as p-chloromercuribenzoate (PCMB) might be useful in confirming this conclusion. To resolve a single species on both gel filtration and ion-exchange chromatography, such studies require a large amount of highly purified enzyme. This is because polymerase activity is apparently not measurable in the presence of either SDS or guanidine-HCl (personal observation), and the enzyme's presence would have to be followed by monitoring absorbance at 280 nm.

RNA Polymerase recovery

Two schemes of preparative isolation of mitochondrial RNA polymerase are presented here which give somewhat similar recoveries. Preparation I involves Sephacryl S-200 gel filtration first in low salt which allows the separation of the aggregated polymerase from the majority of mitochondrial proteins, then in high salt, to resolve the low molecular weight polymerase. Subsequent purification using heparin-Sepharose and phosphocellulose chromatography is employed. As shown in table 10, this preparation gives a

TABLE 9

Purification of Mitochondrial RNA Polymerase, Preparation I

<u>Purification Stage</u>	<u>Protein (mg)</u>	<u>Total Activity (units)</u>	<u>Specific Activity (units/mg protein)</u>	<u>Volume (ml)</u>	<u>Yield (%)</u>	<u>Fold Purification</u>
Mitochondrial lysate	780	300	0.43	50	100	--
Gel Filtration in Low Salt pooled peaks S ₁ , S ₂ , S ₅	120	240	2.0	115	80	5.6
Gel Filtration in High Salt S ₁ peak	10	120	12	45	40	28
Heparin-Sepharose	0.3	90	300	23	28	1697
Phosphocellulose	0.05	48	960	8	16	2217

TABLE 10Purification of Mitochondrial RNA Polymerase, Preparation II

<u>Purification Stage</u>	<u>Protein (mg)</u>	<u>Total Activity (units)</u>	<u>Specific Activity (units/mg protein)</u>	<u>Volume (ml)</u>	<u>Yield (%)</u>	<u>Fold Purification</u>
Mitochondrial lysate	550	285	0.55	40	100	--
55% Ammonium Sulfate pellet (after dialysis)	200	260	1.3	45	90	2.3
Phosphocellulose	15	220	14.5	36	75	26
Heparin-Sepharose	0.65	180	277	20	45	503
DEAE-Sephadex	0.2	82	410	12	28	773
Octyl-Sepharose	0.065	56	848	6	18	1538

17% yield of RNA polymerase with a specific activity of 960 units/mg. The yield of protein is 50 ug/200 grams of starting material. This enzyme appears to be pure on the basis of SDS electrophoresis.

Preparation II use ammonium sulfate fractionation, phosphocellulose, heparin-Sepharose and DEAE Sephadex chromatography. The yield is 18%, but the specific activity of the enzyme is somewhat lower; 60-65 ug protein is recovered/200 grams of starting material.

The use of preparation I appears to be the method of choice. More efficient ways of concentrating the enzyme such as centrifugation into glycerol gradients as used by Wu et al. (1972) and Kuntzel et al. (1971) might give better yields of purified enzyme for further experiments.

Crude whole fly preparation of mitochondrial RNA polymerase

Another approach to the problem of poor yield was to use whole fly homogenates. The mitochondrial RNA polymerase was solubilized with detergent, PEG precipitation removed most nuclear DNA, and gel filtration under high salt conditions separated the monomer activity from all other polymerase activities as shown in Figure 31. Phosphocellulose and heparin-Sepharose chromatography were subsequently employed (Figure 22). The activity recovered appears to be same enzyme as purified from purified mitochondria (see figure 33). A single band of 60,000 molecular weight is found upon SDS electrophoresis.

Figure 31. Sephacryl S-200 chromatography of RNA polymerase activity(s) from whole fly-extracts.

Whole fly extracts were prepared by homogenization with subsequent PEG precipitation of DNA, batch chromatography using phosphocellulose, and concentration of the enzyme by $(\text{NH}_4)_2\text{SO}_4$ precipitation as described in Methods. The precipitate was resuspended in 5-10 ml TGED and loaded on a Sephacryl S-200 column in high salt. Fraction size for tubes 1-60 was 3 ml, tubes 61-85 6 ml. Protein was estimated by absorbance at 280 ($\bullet \text{---} \bullet$). Polymerase activity was assayed using 10 λ aliquots as described in Methods ($\star \text{---} \star$).

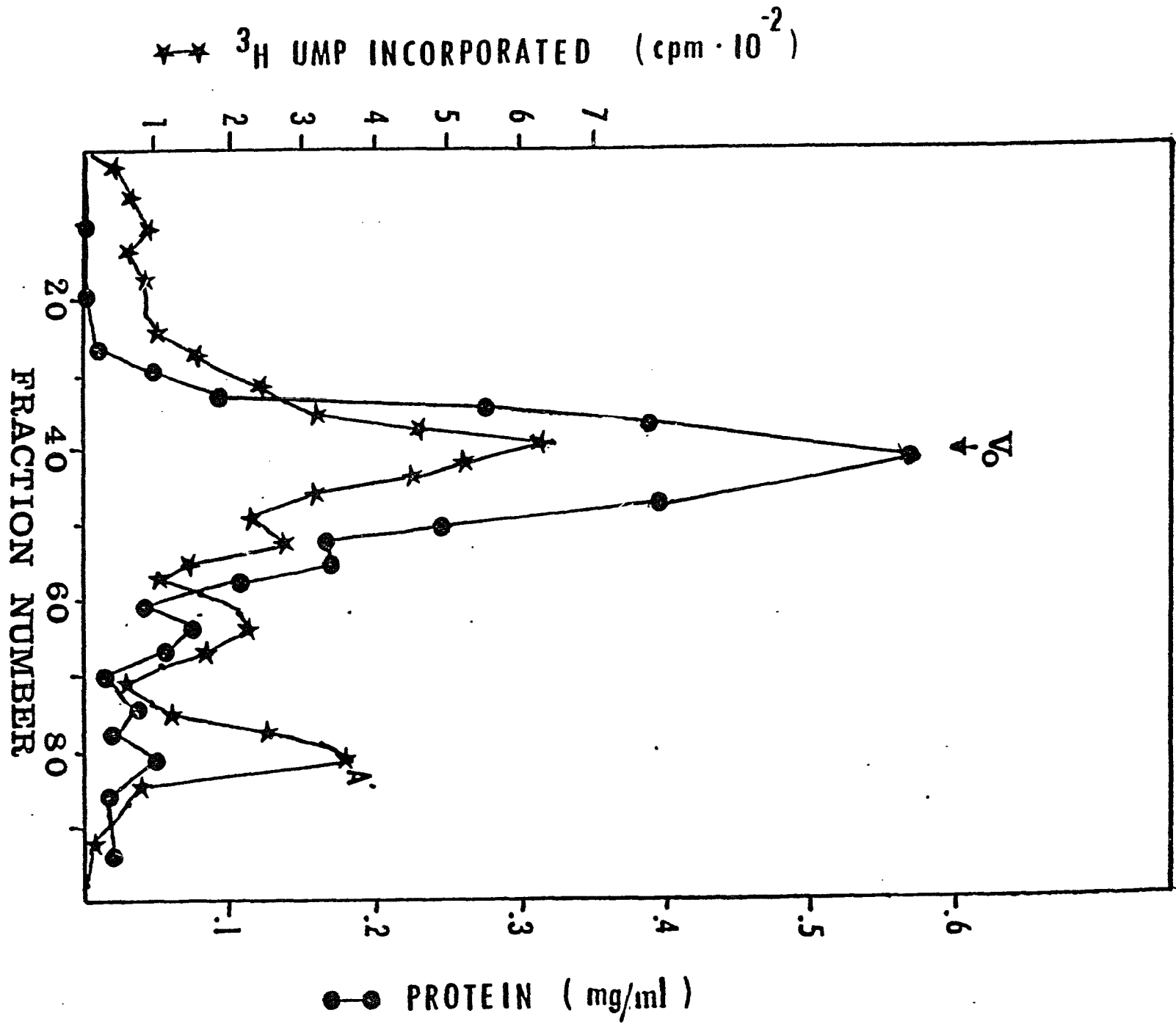


Figure 32. Heparin-Sepharose chromatography of low molecular weight RNA polymerase activity from Sephacryl S-200 filtration of whole fly extract.

The low molecular weight peak from Figure 31 was pooled, diluted 1:1 to make $[(\text{NH}_4)_2\text{SO}_4]$ 0.2M, and applied to a 1 x 10 cm heparin-Sepharose column previously equilibrated with 0.2 M $(\text{NH}_4)_2\text{SO}_4$. Fractions of 4.5 ml were collected, and 20 λ assayed for polymerase activity.

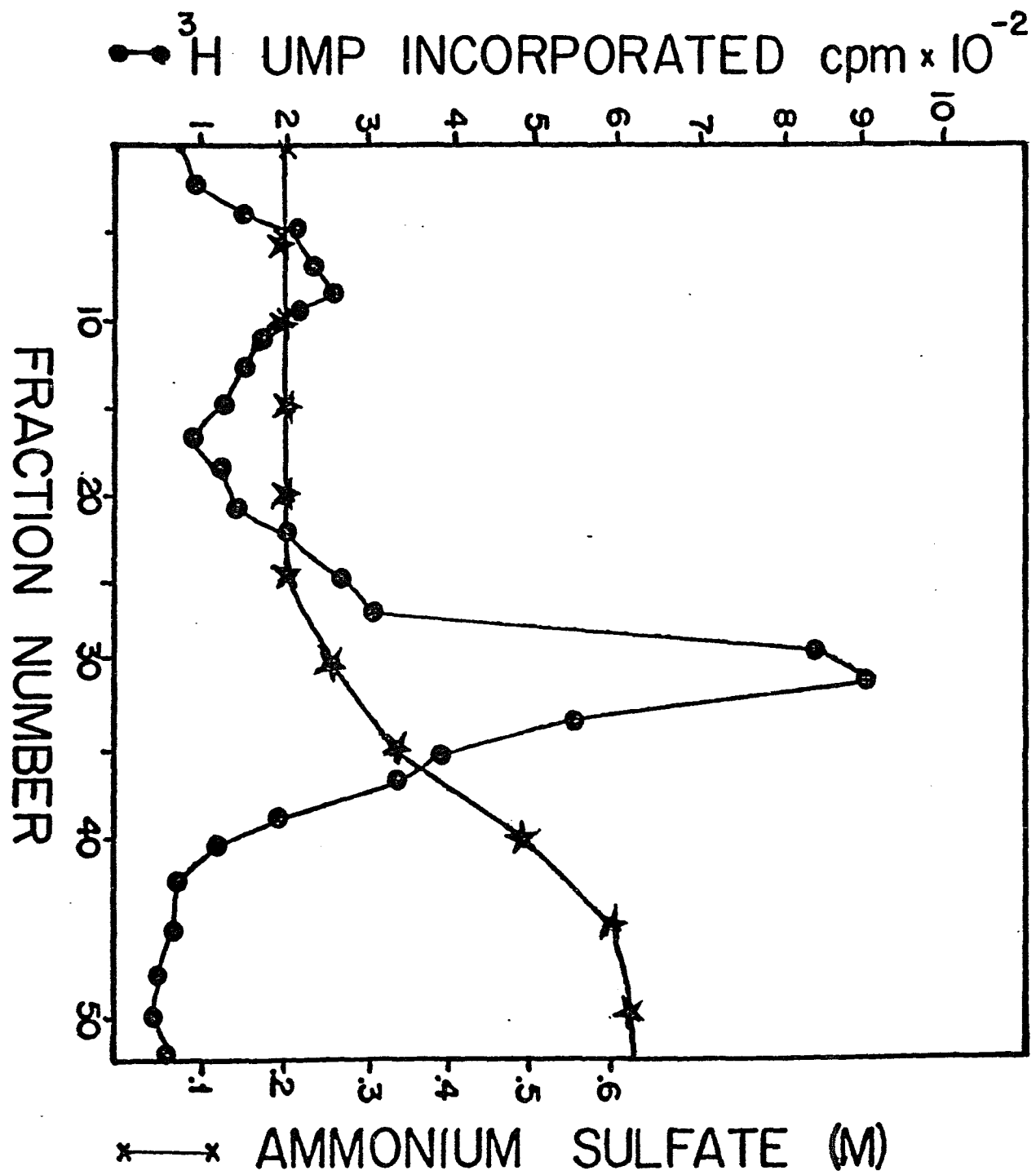


Figure 33. Properties of a purified low molecular weight RNA polymerase from whole fly extract.

Ion optima and response to rifampicin dosage were tested as previously with 10 λ aliquots of the major peak from heparin-Sepharose (Figure 32). All points shown represent an average of duplicate assays.

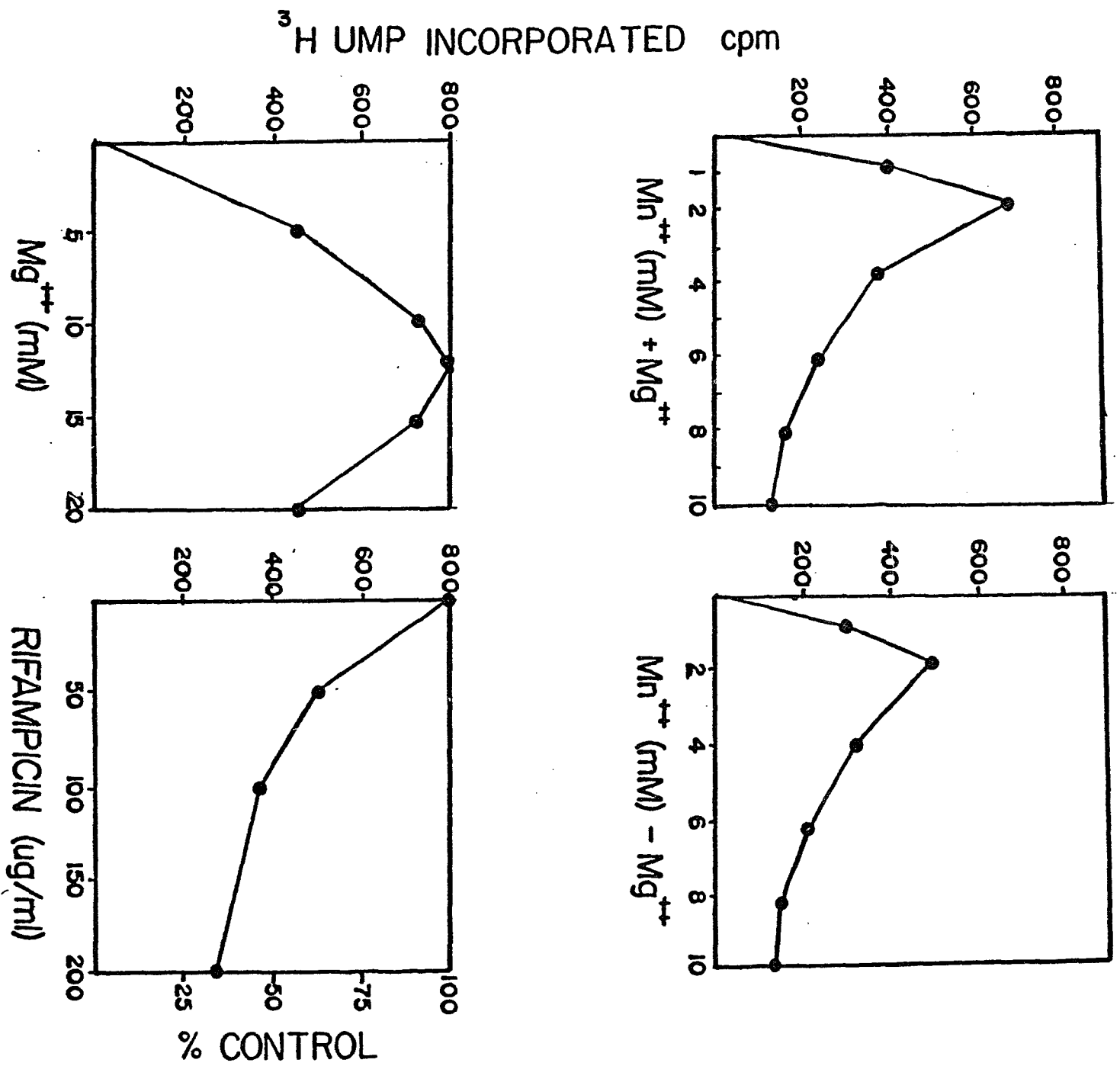


TABLE 11

Purification of Low Molecular Weight RNA Polymerase

Activity from Whole Fly Homogenate, Preparation III

<u>Purification Stage</u>	<u>Protein (mg)</u>	<u>Total Activity (units)</u>	<u>Specific Activity (units/mg protein)</u>	<u>Volume (ml)</u>
Solubilized whole cell lysate	--	--	--	--
Phosphocellulose	20	260	13	25
Sephacryl (high-salt) -peak S ₁	3.5	105	35	45
Heparin-Sepharose	0.15	90	660	24

The time consuming use of PEG precipitation for separating DNA from the polymerase is more than made up by time gained not having to isolate mitochondria. However this step gives quite variable recoveries of enzyme activity. Batch binding to phosphocellulose prior to loading on gel filtration columns seemed to increase the yield of monomer enzymes and remove a good deal of non-enzyme protein. Specific activity was found to be similar to previous estimates using mitochondrial preparations I and II. There is however a greater yield of protein approaching 150 ug, from 200 grams of starting material.

DISCUSSION

The results section presented data concerning the isolation and characterization of several RNA polymerase activities from Drosophila melanogaster mitochondria. The following discussion in summarizing the observed properties and their significance will compare these activities with other RNA polymerases. It is my basic supposition that these isolated activities represent that of a single, true mitochondrial enzyme. Evidence supporting this contention will be discussed. Both the problems in experimental protocols and interpretation as well as suggested future experiments will be presented.

Evidence that this is a distinct class of activity different from nuclear polymerases

Extreme caution was used to eliminate gross organellar or bacterial contamination during the mitochondrial isolation. The mitochondrial preparation was modified to include a number of low speed centrifugations as well as sucrose-gradient fractionation for optimal purity. Microscopic investigation showed no intact nuclei or bacteria in the purified mitochondrial fractions, but some evidence of unidentified membrane fragments which indicated that further controls be employed. The DNA that is associated with such preparations was analyzed on the basis of its

buoyant density in CsCl, as well as its elution from PLK columns. Both criteria show no evidence of a nuclear or bacterial DNA in DNase treated, sucrose gradient fractionated mitochondria.

The properties of the RNA polymerases solubilized from purified mitochondria showed, in both the crude and more purified stages, significant variance with the major properties of nuclear and bacterial RNA polymerases. The lack of any inhibition by alpha amanitin even at a concentration of 100 ug/ml clearly differentiates this activity from nuclear polymerases II and III. Similarly, the mitochondrial activity(s) strong stimulation by Mg^{++} rather than Mn^{++} contrasts with the nuclear polymerases where the reverse is found. Also, the presence of a partial rifampicin sensitivity which is not observed with the nuclear RNA polymerases is further indication that this enzyme is distinct from its nuclear counterparts.

The most obvious difference between the mitochondrial enzyme and the nuclear and bacterial RNA polymerases lies in its small size. Both gel filtration as well as glycerol gradient centrifugation indicate a major activity of approximately 60,000 d. Although this low molecular weight activity is resolved amongst a series of higher molecular weight activities, all these activities share a major subunit of 60,000 daltons resolved by SDS electrophoresis. This is most

certainly different from the large (450-500,000 dalton) and complex (5-12 subunits) RNA polymerase enzymes of the nucleus (Roeder 1976) or of bacteria (Burgess 1969).

The increase in the proportion of activity found in the slowest sedimenting activity peaks (4-6S) from glycerol gradient centrifugation under conditions of high salt has been interpreted by most workers as salt-induced disociation (Scragg, 1976; Wu, et al. 1972; Kuntzel, et al. 1971). Similarly, the increase in higher molecular weight or faster sedimenting forms in low salt conditions is thought to be a form of molecular self-aggregation. The results presented here showing a regular distribution of molecular weight forms with either gel filtration chromatography or glycerol gradient centrifugation where aggregation and dissociation are partially a function of ionic strength

are consistent with this interpretation. This would suggest that the 60,000 dalton activity is the monomer form, which self-aggregates to form larger molecular weight forms. An alternative explanation can not be entirely ruled out that these various higher molecular weight forms are caused by the association of different protein(s) and/or DNA with the 60,000 catalytic subunit preferentially in low salt. The argument that this is a proteolytic artifact of a larger molecule is difficult to maintain in light of the strong similarity in the reaction

characteristics of all the activity peaks, the consistent distribution of the chromatographic patterns, the lack of any significant change if proteolytic inhibitors such as PMSF are used throughout, and lastly, the ability of the low molecular weight activity to reversibly aggregate to form the larger forms.

This enzyme has been further localized in the mitochondria by virtue of its attachment to the inner membrane. This observation had been suggested by most early workers (Scragg, Wu) who found that only upon rigorous solubilization in high concentration of digitonin or deoxycholate would the polymerase activity be solubilized. Similarly, my results demonstrate that non-ionic detergents such as Triton X-100 and NP-40 are the most effective solubilizing agents of RNA polymerase activity, and that the inner membrane enzyme, cytochrome oxidase is co-solubilized with the RNA polymerase activity. The poor yield of RNA polymerase using simple salt extraction, osmotic lysis and mild sonication which do solubilize most of the matrix enzyme MDH suggests further that the majority of mitochondrial RNA polymerase is bound to the inner membrane. The class of more easily solubilized RNA polymerase constituting 20-30% of the total activity may be molecules not bound to the membrane. However they have the same basic catalytic properties as the detergent solubilized enzyme.

Their biological significance (if different) is not yet clear. It is interesting to note that the conditions used to obtain transcription complexes such as have been isolated by Levens and Hallick (see Introduction) would appear to maintain the mitochondrial RNA polymerase in association with the inner mitochondrial membrane.

Further observations and conclusions concerning the heterogeneity of mitochondrial RNA polymerase in size and charge

The heterogeneity obtained upon gel filtration chromatography is observed as well with ion exchange chromatography on either DEAE-Sephadex or phosphocellulose resins. Similar heterogeneity had been found in most early studies and was interpreted as being due to endogenous DNA (Gallerani et al. 1976), salt-effects causing aggregation (Scragg 1976) and as possibly due to associated proteins (Hecht et al. 1979). The results have not eliminated any of these possibilities conclusively. The inter-conversion of size (seen in the glycerol gradient and gel filtration experiments) and charge (seen upon rechromatography) are indicative of some type of equilibrium as a function of ionic strength. The conditions for complete conversion of one form into another have not yet been found.

The data presented here suggests that the different sized RNA polymerase activities may have different net charges. For example, species of high molecular weight are eluted from DEAE Sephadex and phosphocellulose

columns in greater proportion at low salt conditions whereas the activity of the lowest apparent molecular weight elutes from ion exchange columns preferentially at high salt conditions. In addition, the activity of highest apparent molecular weight has similar catalytic properties to the activity that elutes from ion exchange columns at low salt concentration. Likewise, the activity of lowest apparent molecular weight has catalytic properties similar to the activities eluting from ion exchange columns at high salt concentration. The largest aggregate shows lower activity with Mn^{++} than it has with Mg^{++} as cofactor with the Mn/Mg activity ratio of approximately 0.55. Similarly, the Mn/Mg activity ratios of the low salt eluting species from DEAE Sephadex and phosphocellulose are in the 0.5-0.6 range. These peaks also have about the same degree of rifampicin sensitivity, that is 40-50% of the control incorporation in the presence of 100 ug/ml rifampicin. On the other hand, the properties of the "monomer" enzyme activity correlate well with high salt peaks P_3 and DE_3 from ion-exchange in showing greater incorporation in the presence of Mn^{++} (with the Mn/Mg activity ratio between 0.7-0.85), a lower sensitivity to rifampicin (50-60% of the control incorporation in 100 ug/ml rifampicin and a more pronounced biphasic response to ammonium sulfate. Similar differences between activities resolved on DEAE Sephadex had been reported by Reid and Parsons (1971), Tsai et al. 1971,

Levens et al. 1979 and Hecht et al. 1979. In addition, the incorporation with a double stranded DNA template appears to be higher with the larger molecular weight/less charged species as compared to the lower molecular/more charged form.

One possible explanation for the change in catalytic properties as a function of apparent molecular weight might be that high salt induces an alteration in polymerase conformation which in turn affects reaction properties. An aggregated form might have less charges exposed and therefore bind poorly to ion exchange matrix while binding well to a more hydrophobic matrix, such as the inner mitochondrial membrane. A monomer form with its charges fully exposed will bind well to the ion exchange columns, and less well to the hydrophobic mitochondrial membrane. This hypothesis is somewhat borne out by the differential behavior of the monomer as compared to the large aggregate activity in binding the hydrophobic affinity matrix, octyl-Sepharose. Some physiological consequences of this model as well as some suggestions for further testing it are addressed later in the discussion.

A second possibility is that conformational shifts are mediated by a protein(s) which is tightly bound to the polymerase. Such a protein might be tightly bound in low salt resulting in a RNA polymerase with high apparent molecular weight, and low affinity to ion-exchange matrices. Its own hydrophobicity could also dictate

binding to the octyl-Sepharose column. Such a protein would have to be the same size as the 60,000d catalytic subunit or in low concentration since there is no evidence of it upon SDS electrophoresis of the slow sedimenting activity from glycerol gradients. The use of a more sensitive electrophoretic technique such as that developed by Switzer et al. (1980) might be used to detect such a protein. The discovery and isolation of such a factor which may confer initiation function on the mitochondrial polymerase analogously to the sigma factor in bacteria would be an extremely important control element in mitochondrial transcription.

A third and less likely explanation lies in the influence of endogenous mitochondrial DNA on the chromatographic properties of the polymerase activities. It is clear that the largest aggregate from Sephacryl filtration, and the flow-through activities from ion-exchange columns contain endogenous DNA as gauged by their activity in the absence of exogenous DNA. However, DNase treatment, PEG precipitation and $(\text{NH}_4)_2\text{SO}_4$ fractionation--all which remove the majority of DNA from the polymerase do not significantly alter the heterogenous chromatographic profile of gel filtration. In addition, chromatography on gel filtration of distinct peaks of activity from heparin-Sepharose chromatography which in theory should contain no DNA (personal communication, M. Holland) resolves the same

heterogenous profiles as previously found. Lastly, it is interesting to note that Niles et al. (1974) has found that the RNA polymerase of phage T7 of molecular weight 107,000 eluted from phosphocellulose in a heterogenous manner. Furthermore, this heterogeneity remained when any one of the peaks was rechromatographed on phosphocellulose. Although he did not identify the cause of this heterogeneity, this elution profile was not a function of DNA association since protamine sulfate removal of DNA from the polymerase did not eliminate the heterogeneity.

In conclusion, the data supports a model of a single polymerase which aggregates under low ionic strength, which may or may not be under the control of associating proteins. The different forms of polymerase recoverable from ion-exchange and gel filtration columns have similar reaction properties, yet with some differences which may be operative in vivo.

Other characteristics of the mitochondrial polymerase

As far as the general isolation of the enzyme goes, the manner of solubilization whether using nonionic detergent or high salt as solubilizing agent, does not significantly change the nature of the resultant enzyme. Similarly, the preparation of the enzyme from whole fly extracts without preparing purified mitochondria gives similar yields of a low molecular weight activity (about

60,000d) from gel filtration chromatography which shows the same qualities as the activity found with the mitochondrial isolation. This reduces the likelihood that this activity or its properties are an artifact of the mitochondrial isolation or DNase treatment. While findings with ion exchange chromatography and glycerol gradient centrifugation such as ours are well documented in the literature, the use of gel filtration, heparin-Sepharose and octyl-Sepharose chromatography in the purification of the mitochondrial RNA polymerase have not yet been characterized elsewhere. Of interest, however, is the recent report (Nagasawa et al., 1979) concerning the purification of cytochrome oxidase using octyl-Sepharose chromatography from which the oxidase activity is eluted with 3% Triton X-100, the same concentration as we use to not only elute the RNA polymerase activity from such a column, but to solubilize the polymerase as well from the mitochondrial membrane. One wonders whether the polymerase will show the same amount of hydrophobic residues as the oxidase? This degree of hydrophobicity may well be related to the general finding of instability with the mitochondrial polymerase, which was suggested in our studies as well. The presence of detergent appears to increase stability probably by providing a hydrophobic environment for the enzyme. This effect appears to carry through the preparation, since unlike Schafer and Kuntzel who found that

they lost most of the enzyme activity during ammonium sulfate fractionation, our enzyme is relatively stable through this fractionation. This might well be due to the inclusion and coprecipitation of the detergent with the enzyme.

One feature of the mitochondrial enzyme appears to be an ability to utilize a number of different templates. These studies indicate that calf thymus double-stranded and single stranded DNA and poly dAT all support activity of the polymerase. Though most studies have shown there is a preference for mitochondrial DNA, poly dAT and single-stranded DNA in that order, there is general agreement that unlike the small phage polymerases T3 and T7 which are highly specific in their use of templates, the mitochondrial RNA polymerase effectively uses a broad spectrum of templates. (Dunn et al. 1971, Oakley et al. 1979).

The specific activity of our enzyme is similar to that found in a number of studies (see Table 12). Differences in specific activity might well be a function of both the template used, and the conditions used in the assay of the enzyme. The specific activity of the mitochondrial RNA polymerase of most studies including our own are consistently much lower than nuclear or bacterial counterparts. This limits the present use of an in vitro transcription-translation system in that enough RNA might not be made by such a polymerase to make the appropriate translation product.

TABLE 12
Properties of Reported Mitochondrial RNA Polymerases

<u>Source</u>	<u>Rifampicin Sensitivity</u>		<u>Subunit Molecular Weight</u>	<u>Specific Activity</u>	<u>Worker</u>
	<u>Dose</u>	<u>Inhibition</u>			
Neurospora	6 ug/ml	90% inhib.	63,000	4	Kuntzel, Schafer (1971)
Rat Liver	10 ug/ml	35% inhib.	66,000	0.517	Reid, Parsons (1971)
	10 ug/ml	80% inhib.	68,000	0.322	Murkerjee <u>et al.</u> (1973)
	20 ug/ml	70% inhib.	62,000	8.5	Gallerani <u>et al.</u> (1976)
Yeast	10 ug/ml	95% inhib.	63-69,000	1.56	Scragg (1976)
	20 ug/ml	10% inhib.	N.D.	17	Wintersberger (1971)
	40 ug/ml	10% inhib.	N.D.	33	Tsai <u>et al.</u> (1971)
	100 ug/ml	40% inhib.	180,000, 120,000	22	Eccleshall <u>et al.</u> (1974)
	20 ug/ml	10% inhib.	63,000	1-1.3	Rogall <u>et al.</u> (1974)
	400 ug/ml	50% inhib.	45,000	300	Levins <u>et al.</u> (1979)
Xenopus laevis oocytes	50 ug/ml	0 inhib.	47,000	8.63	Wu, Dawid (1972)
Blastocladiel- la emersonii	Sensitive but not quanti- fied		N.D.	N.D.	Horgen <u>et al.</u> (1971)
Bull sperm	30 ug/ml	50% inhib.	N.D.	N.D.	Hecht (1979)

Evidence for precursor polymerases?

The results here have been generally consistent with earlier descriptions of the mitochondrial RNA polymerase as a single, subunit enzyme clearly different from the large multisubunit RNA polymerases of the nucleus and bacteria. However, a number of similarities to the properties of polymerase III are evident with the mitochondrial activity. The biphasic salt optima as well as the use of polydAT as template are particularly like polymerase III which unlike the other nuclear polymerases is substantially localized in the cytoplasm of the cell (Roeder 1976). One possible explanation is that polymerase III is a precursor to the mitochondrial polymerase. The lack of alpha amanitin sensitivity and size difference may indicate that the mitochondrial polymerase represents the catalytic subunit of polymerase III with these qualities lost along with the "extraneous" subunits. This explanation has a number of flaws. How would the rifampicin sensitivity be gained? With all the work done on bacterial and eucaryotic nuclear polymerases, why has no "catalytic subunit" been isolated capable of transcribing DNA without the presence of the other subunits? Another explanation might be that the similarity in catalytic properties may be related to other functional and not necessarily to structural similarities, e.g., both initiate on duplex DNA (in contrast to polymerase I and II). One way to elucidate the relationship between

the mitochondrial and polymerase III would be to probe the antigenic relationship between them.

Another suggestion of a "precursor" relationship or perhaps of a cytoplasmic pool comes from the recent findings of a RNA polymerase from Drosophila (Gross and Beer 1975) with the same degree of rifampicin sensitivity as the mitochondrial enzyme. This polymerase is solubilized by high salt extraction of crude cell homogenates, elutes from DEAE Sephadex at 0.145 M ammonium sulfate, and has a large molecular weight (500,000) as measured by glycerol gradient centrifugation in high salt conditions. Although these authors consider this activity a nuclear enzyme (calling it IB), evidence concerning the subcellular location and subunit characterization are unclear. Though these authors assert that this is a non-mitochondrial enzyme on the basis of its sedimentation, it could be an oligomeric form of the mitochondrial enzyme. Large oligomeric forms of mitochondrial RNA polymerase have also been found extramitochondrially in the work of Barath and Kuntzel (1972) and Scragg (1976). These forms have sedimentation values corresponding to a MW of 500,000 even in high salt but are composed of single subunits (60,000 d) as resolved by SDS electrophoresis. Furthermore, the elution of the activity by 0.145 M $(\text{NH}_4)_2\text{SO}_4$ is similar to peak DE₂ consistently found above which has been correlated with activities of high apparent molecular weight. Although very preliminary, these results may

indicate that an oligomeric form of the mitochondrial RNA polymerase may reside in the cytoplasm, and be the pre-mitochondrially processed form of the mitochondrial enzyme.

What is the physiologically active form of the mitochondrial RNA polymerase?

The question arises, which is the active form of the mitochondrial polymerase, the aggregated or monomer forms, or alternatively, the membrane-bound or the membrane free forms?

It might be stated at the outset of such a discussion, that even studies with the well characterized bacterial RNA polymerase have not unequivocally proven the predominance of the monomer (13S) versus the aggregated dimer (26S) form. Richardson (1966) demonstrated that the 26S form is more apt to be present under physiological salt conditions in vivo. The weight of evidence however with in vitro of polymerase binding to DNA or poly dAT (Krakow, 1971; Smith, 1967) have shown the predominance of the monomeric form in binding DNA. Chamberlin (1974) has corroborated this by showing that the 13S form has higher specific affinity under initiation conditions. Yet other workers (Mangel et al. 1974) suggest that these findings may be a function of the particular templates used in these experiments.

One molecular interaction that might be affected by a monomer aggregate transition is the polymerase binding to the template. Although the evidence is preliminary,

there appears in my studies of the mitochondrial RNA polymerase to be greater activity on the double stranded DNA and poly dAT templates with the highest apparent molecular weight activity peaks than with the monomer enzyme. An interesting model might be proposed in which the oligomeric polymerase is necessary for recognizing palindromic control sequences and secondary structure of DNA at duplex initiation sites. Such a model has precedence in the lac and lambda repressors interaction with DNA (Von Hippel, 1979) as well as restriction enzymes interaction with DNA (Nathans, 1979), all of which appear to be most effective in binding to DNA as oligomers. Increasingly, nucleic acid-protein interaction appears to be controlled at the level of conformational interaction between DNA-protein and protein-protein (Pribnow, 1979). In the context of mitochondrial transcription as discussed in the introduction it was found that the secondary structure of DNA was highly important in determining the strand preference of transcription (Wu, et al. 1972) by a purified mitochondrial polymerase. The possibility that duplex-binding increases significantly with an oligomeric form of RNA polymerase certainly warrants further investigation. Two approaches for further studies are suggested. A) By molecularly cross-linking the oligomers (higher molecular weight activity), and then assaying both the binding and initiation of RNA synthesis on double

stranded and single stranded mitochondrial DNA templates, this hypothesis could be tested. The reverse experiment (B), i.e., reducing irreversibly the oligomers to monomers and assaying binding and initiation functions on different templates, as well as strand preference, might serve as a control. The maintenance of measurable activity under conditions of cross-linking or complete dissociation is the challenge with such experiments. It should be mentioned that these experiments would give the same expected result whether the increased duplex binding by the higher molecular weight form is due to the polymerase self-aggregation, or due to some associated membrane or protein factor.

A second equally speculative function for a monomer aggregate transition might be in the mitochondrial RNA polymerases' involvement in the control of DNA synthesis initiation. The finding that RNA priming is associated with DNA synthesis in the mitochondria (Gillum et al., 1979; Martens et al. 1979), the similarity of the monomer polymerase in size and preference for single-stranded DNA to the procaryotic primases and dnaG protein (Rowen et al. 1978) which are involved in DNA replication--all are suggestive that the mitochondrial RNA polymerase may play some role in the initiation of DNA synthesis. There is also increasing evidence that the site of initiation of both mitochondrial transcription and replication may be the same (Van Etten et al. 1980; Attardi et al. 1979). One possible control mechanism

for regulating which program occurs, i.e., transcription or DNA replication, might be a function of the state of the RNA polymerase, that is, monomer or aggregate. A similar model could be proposed where regulation would be dependent on the association of membrane elements with the mitochondrial RNA polymerase.

Future prospects

A number of experiments are suggested by the observations and hypotheses proposed in these studies.

Structural: At the level of enzyme structure, larger amounts of the purified enzyme than we have at present are necessary for analytical studies. In order to investigate the forces and significance of aggregation, it is suggested that an oligomer monomer transition be attempted using guanidine HCl or sulfhydryl reagents.

A possible precursor product relationship with the nuclear enzymes might be investigated by any cross-reaction of the mitochondrial polymerase to antibodies made to the more plentiful nuclear polymerases. Any homology would be further investigated using peptide maps of the subunits in question.

The question of rifampicin interaction with the mitochondrial RNA polymerase might be clarified by binding studies with labelled rifampicin. This technique could be used to assay changes in rifampicin binding as a function

of polymerase aggregation, as well as a means of quantification of RNA polymerase molecules at different developmental stages. It is highly important that a specific interaction between rifampicin and mitochondrial RNA polymerase be well delineated as such a property could be used as a genetic probe, i.e., the search for rifampicin-resistant mutations. Although preliminary studies show that rifampicin kill curves can be established with Drosophila embryos (Nishiura unpublished) there are indications from other studies (Buss, 1978) that rifampicin may effect other processes directly in higher organisms such as cytoplasmic protein synthesis. This might complicate assigning a genetic locus for an altered polymerase subunit using rifampicin resistance as phenotype. However, if such a relationship does exist, it could provide, as such mutations have with bacterial cells (Ishihama, 1979) a powerful tool for understanding the control of mitochondrial transcription.

Functional: One series of experiments that should be performed with the purified polymerase is the testing of specific transcription of isolated mitochondrial genes. Both the fidelity and symmetry of transcription could be assayed in such a system, and should be rigorously analyzed as to the effect of salt and ionic optima, as well as with both crude and pure enzymes. The search for promoter

regions as well as regulatory factors could be incorporated into such experiments. Also, if large amounts of the mitochondrial RNA polymerase can be purified, affinity columns with the polymerase attached to an immobilized matrix might be used to recover proteins which may play a role in transcription (as assayed in the reconstituted system) as well as specific membrane proteins which may be responsible for the specific translocation of the RNA polymerase into the mitochondria.

Lastly, the specific effects of mitochondrial RNA polymerase on DNA initiation might be assayed. Though this would require in vitro DNA replication system (which several labs are working on), the incorporation of deoxynucleotides by this activity might be a starting point. In light of the previous discussion concerning a possible regulation by the aggregate monomer transition, this should be investigated as well.

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