THE ISOLATION, PURIFICATION AND CHARACTERIZATION OF THREE RNA POLYMERASES FROM NOVIKOFF HEPATOMA ASCITES TUMOR

Thesis by

Stanley Charles Froehner

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To Sandy

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Abstract

DNA-dependent RNA polymerase has been isolated from nuclei of Novikoff hepatoma ascites tumor cells and resolved into three activities, designated Ia, Ib, and II, by a combination of phosphocellulose and DEAE cellulose chromatography. Ia and Ib have been further purified by sucrose density centrifugation. Both gradient profiles exhibit coincidence of the polymerase activity and protein peaks, suggesting that the two may be homogeneous enzymes. Ia migrates as a single species on non-denaturing polyacrylamide gel electrophoresis. SDS polyacrylamide gel electrophoresis indicates that Ia contains subunits of 170,000, 125,000, 69,000, 49,000, 44,000 and 37,000 molecular weights in equimolar ratios except for the 69,000 and 37,000 dalton subunits which may be present in two copies per enzyme molecule. A molecular weight of 600,000 for the enzyme calculated from the molecular weights of the subunits is in good agreement with that determined by exclusion chromatography. The probable molecular structure of Ib is subunits of 190,000 and 135,000 daltons, each present twice per enzyme molecule. The enzymological characterization of these three enzymes suggests that Ia and Ib are the nucleolar polymerases while II is nucleoplasmic. Ia and Ib are most active at low ionic strength with Mg^{++} on native DNA and are insensitive to α-amanitin.

II prefers Mn^{++}, high ionic strength, a denatured template and is inhibited by low concentrations of α-amanitin. A factor present in the material which does not bind to the DEAE cellulose column used in the purification scheme, stimulates the activity of all three of the enzymes. Ia and Ib are inactive at low enzyme concentrations in the absence of this factor. The active agent in the factor is probably a protein, since it is heat sensitive, and may be a subunit of the enzyme.

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CHAPTER I

GENERAL INTRODUCTION

Within the past few years, it has become evident that the DNA-dependent RNA polymerase of prokaryotes has the capability of doing more than simply catalyzing the polymerization of nucleotides into a faithful copy of the template. In several cases, the enzyme apparently selects certain sequences on the DNA which are to be transcribed and, in this way, plays a central role in the control of gene activity. It appears likely that this specific transcription can also be modified by alterations in the molecular structure of the polymerase, thus introducing a mechanism by which genes can be switched on and off, or by which the template preference of the polymerase can be changed. Since this work provided the stimulus for the investigations presented here, a review of these findings is in order.

The RNA polymerase of Escherichia coli has been the most thoroughly studied one, mainly because of its availability, ease of isolation and purification, and stability [see Burgess (1971) for a recent review]. The core enzyme consists of one β' subunit (M = 165,000), one β subunit (M = 155,000), and two α subunits (M = 40,000) and has been shown to initiate randomly and symmetrically on the template in vitro. If another subunit σ (M = 90,000), which is lost during phosphocellulose chromatography (Burgess et al., 1969), is added,

the transcription appears to be assymetric and specific, at least on phage DNA (Sigiura *et al.*, 1970). There has been much speculation over the past two years concerning the role of sigma in the control of gene activity (Travers, 1970; Summers and Siegel, 1969; Losick and Sonenshein, 1969). However, in retrospect, it is possible that these ideas resulted from preconceptions dictating interpretations of experiments. There is no doubt that the sigma factor is important in the proper functioning of the RNA polymerase *in vitro* but there appears to be no evidence for *different* sigma factors directing initiation of RNA synthesis at different sites on the DNA template.

The role that RNA polymerase plays in the temporal control of RNA synthesis during phage infection is being elucidated in the T4 and T7 bacteriophage systems. After T4 infection, there is a shut-off of host RNA synthesis and a sequential expression of phage genes necessary for replication of the phage genome, synthesis of the structural proteins and enzymes necessary for phage morphogenesis, and lysis of the host cell. The molecular structure of the host RNA polymerase is modified soon after infection and it appears that this modification is instrumental in the change in template specificity of the enzyme and the temporal expression of phage genes (Schachner *et al.*, 1971). The T4-modified (by 10 min after infection) and host polymerases have been compared by fingerprint mapping

(Schachner and Zillig, 1971) and differences in all three of the subunits of the core enzyme have been found. The α-subunit of the modified enzyme shows all of the peptides found in the host α-subunit digest plus an additional one which may have an adenylic group attached (Weber and Goff, 1970). The β and β' subunits both exhibit a large number of differences in the modified and host enzymes. Another indication of the differences between the two enzymes is that the T4-modified polymerase loses its sigma subunit when purified by chromatography on DEAE-cellulose. It is believed that these alterations are responsible for the changes in specificity of RNA synthesis exhibited by the polymerase upon infection.

Recently, it has been reported that three small polypeptides are associated with the host RNA polymerase after T4-infection (Stevens, 1972). By use of a double-label technique involving labeling of the polymerase with H^3 before infection and with C^{14} after, the host core enzyme ($\alpha_2 \beta \beta'$) is shown to be labeled only before infection. After infection, the ^{14}C counts which purify with the enzyme are present in polypeptides of 22,000, 14,000 and 10,000 molecular weights. When a gene 55 mutant of T4 is used, the peptide of 22,000 molecular weight is missing. Pulitzer and Geiduschek (1970) have shown that the product of gene 55 is required continuously for transcription of late messenger during T4 infection.

It seems possible that these small "subunits" may prove important in the regulation of RNA synthesis by T4 phage.

The synthesis of RNA in T7-infected cells is also under temporal control and RNA polymerase is again implicated. However, in contrast to the T4 system, an entirely new and different RNA polymerase is synthesized (Chamberlin, et al., 1970). The host polymerase is able to transcribe gene 1 of the phage genome, the product of which is an RNA polymerase of 107,000 molecular weight containing a single polypeptide. This phage-induced polymerase is rifampicin-insensitive (in contrast to the host enzyme) and appears to be responsible for the synthesis of messenger RNA for late bacteriophage functions. A temperature-sensitive mutation in gene 1 leads to reduced enzymatic activity in vitro at elevated temperatures, indicating that gene 1 is the structural gene for T7 polymerase. This explains the pleiotropic control of late T7 functions exerted by gene 1. Earlier experiments proposed the appearance of a new T4 σ-factor specific for the genes transcribed late after infection, but stimulation of the host polymerase by this "σ-factor" (which turned out to be the new polymerase) was due simply to stabilization of the T7 polymerase by added protein (Summers and Siegel, 1969).

Some of the most interesting work implicating RNA polymerase in the control of gene expression has been done with Bacillus subtilis, a sporulating bacteria. New genes

are turned on during the process of spore formation and others which are active during vegetative growth are repressed in the spores. The virulent subtilis phage $\phi\varepsilon$ is able to infect vegetative cells and multiply, but if the phage genome is injected into spore cells, no replication or lysis occurs until the spores are germinated. Losick and Sonenshein (1969) have shown that the RNA polymerases of vegetative and spore cells differ in their ability to transcribe $\phi\varepsilon$ DNA: vegetative polymerase uses the phage template effectively while spore polymerase does not. This difference in template specificity suggests a mechanism by which a change in RNA polymerase (either a modification or a complete replacement of the existing one) causes the changes in gene expression necessary for sporulation or germination. The enzymes from both stages have been purified and two differences found. The subunit structure appears to be extremely similar to that of the E. coli polymerase except that the σ subunit has a molecular weight of 57,000, (Losick and Sonenshein, 1970). Sigma is necessary for the transcription of $\phi\varepsilon$ DNA and apparently is not present in the sporulation enzyme, at least it does not appear in the phosphocellulose column run-off. Also, the β subunit has a molecular weight of 155,000 in the vegetative enzyme but only of 110,000 in the spore polymerase. Leighton et al. (1971) have shown that this difference is due to the action of a serine

protease which appears during the sporulation process. The induction of this protease may, in fact, be the trigger for the initiation of sporulation by modifying the vegetative RNA polymerase, thereby changing its template specificity and allowing the transcription of sporulation genes. Temperature-sensitive protease mutants have been isolated which do not sporulate at 47°C. Neither the modification of the β subunit nor the loss of ability to transcribe DNA occurs at the nonpermissive temperature in these mutants. The significance of the loss of sigma at this stage is unknown.

These three examples, T4, T7 and B. subtilis, indicate the importance of RNA polymerase in the control of gene expression in prokaryotes. At the time this work was begun, one could only speculate about the existence of such mechanisms in eukaryotic cells. The present studies on mammalian RNA polymerase were therefore started with the possibility in mind that the transcription enzyme might play an important role in the regulation of gene activity.

Until very recently, all of the studies of eukaryotic DNA and chromatin transcription have utilized bacterial RNA polymerases mainly because a suitable preparation of the mammalian enzyme was not available. Though there are some indications that E. coli RNA polymerase initiates specifically on mammalian DNA (Smith, et al., 1969), several problems have become evident in studies with heterologous

(bacterial) polymerases. Dahmus (1969) found that RNA transcribed from rat liver DNA hybridizes to 2-6% of the DNA (under conditions in which only those sequences transcribed from the repetitive segment of the DNA hybridize), while RNA transcribed from chromatin hybridizes to 5-10% of DNA. This result is somewhat unexpected in view of the fact that the template activity of liver chromatin is about 20% that of DNA in this transcription system. Apparently, the DNA is transcribed at a limited number of sites, possibly even the same sites as are transcribed in chromatin, since there is considerable homology between the RNA transcribed from chromatin and that transcribed from DNA, as determined by competition hybridization (Maurice Dupras, personal communication). A likely explanation for this problem is that the heterologous transcription system is not functioning properly. The most obvious approach to the solution of this problem is to develop an isolation and purification procedure for the mammalian polymerase and thus eliminate the problems due to the heterologous transcription system.

Earlier studies of mammalian RNA polymerase used either an "aggregate enzyme" preparation (Weiss, 1960), which was probably chromatin-bound, or isolated nuclei. Even though these systems are extremely crude, they yielded information which is the basis for the present understanding of the eukaryotic transcription machinery. The RNA

synthesized by rat liver nuclei in the presence of Mg^{++} at low ionic strength has a base composition similar to that of ribosomal RNA (Widnell and Tata, 1966) while a DNA-like RNA is produced under conditions of high ionic strength in the presence of Mn^{++}. These results, when considered together with autoradiographic data indicating that the Mg^{++}/low salt activity is localized in the nucleolus (Pogo et al., 1967; Maul and Hamilton, 1967), suggests the existence of two RNA polymerase activities, one localized in the nucleolus and responsible primarily for ribosomal RNA synthesis (Mg^{++}/low salt) and the other (probably nucleoplasmic) yielding a product with a base composition more like that of the DNA (Mn^{++}/high salt). Weiss' "aggregate enzyme" is probably the nucleoplasmic one since it is stimulated by added ammonium sulfate. These experiments have been questioned since the effects of ionic strength could be due to alterations in the template. Subsequent experiments on isolated chromatin (Smart and Bonner, 1971) indicate that the ionic strength used (1.2M) is sufficient to remove approximately 50% of the histone from the chromatin. However, Stirpe and Fiume (1967) showed that the toxin α-amanitin inhibits the Mn^{++}/high salt activity but not the Mg^{++}/low salt one in isolated nuclei, lending much support to the two polymerase theory. Direct evidence with soluble enzymes free of chromatin was still necessary to rule out template effects of salt.

One of the first chromatin-free mammalian RNA polymerase preparations reported involved the isolation of nuclei in hypertonic sucrose and then leakage of polymerase out of the nuclei in an isotonic solution of sucrose (Liao et al., 1968). This enzyme is completely dependent on added DNA for activity and can be resolved into two components by sucrose density gradient centrifugation, one dependent on Mn^{++} and the other on Mg^{++} (Liao et al., 1969). The preparations are, however, of extremely low activity and are quite likely not representative of the polymerase present in vivo (Roeder, 1969).

Seifart and Sekeris (1969) developed a procedure for sonication of rat liver nuclei in 0.75 M NaCl followed by dilution to 0.125 M NaCl which precipitates chromatin and leaves the RNA polymerase soluble. This enzyme again is totally dependent on added template, prefers Mn^{++} to Mg^{++}, and is stimulated by the addition of ammonium sulfate to the reaction mixture. Chromatography on DEAE-cellulose yields a single peak of activity. Though this procedure produces no evidence of two RNA polymerase activities, it has served as the basis for most extraction methods used to date. The important step appears to be the sonication, which reduces the viscosity of the lysed nuclei and solubilizes the chromatin-bound polymerase. This would appear to be a good procedure for the solubilization of many chromatin-bound enzymes.

Roeder and Rutter (1969) modified the above procedure slightly (0.3 M $(NH_4)_2SO_4$ as the sonication medium) and used it to isolate RNA polymerase from rat liver and sea urchin nuclei. Chromatography of these preparations on DEAE-Sephadex yields multiple activities, two in rat liver and three in sea urchin. Rechromatography of the enzymes separately shows no indication of interconversion or artifactual resolution due to reversible aggregation. Form I (from both rat liver and sea urchin) elutes from the column at about 0.15 M $(NH_4)_2SO_4$ and shows maximum activity at low ionic strength in the presence of Mg^{++}. Form II elutes at 0.25 M $(NH_4)_2SO_4$, prefers Mn^{++} to Mg^{++}, and is stimulated by the addition of $(NH_4)_2SO_4$ up to 0.1 M. Both prefer denatured to native DNA as a template. Form III is very unstable and is usually present in very small amounts; as a result, it is not well characterized. It does prefer native DNA as template and has a very broad ionic strength optimum. By extracting the enzyme from purified rat liver nucleoli, Roeder and Rutter (1970) demonstrated the localization of Form I in this organelle, while Form II (and III, though in a very small amount) appear to be nucleoplasmic, although leakage from the nucleoli during isolation can not be ruled out. Finally, II from calf thymus (Kedinger et al., 1970) and rat liver (Lindell et al., 1970) was shown to be extremely sensitive to α-amanitin. Subsequently, Chambon et al. (1970) showed,

by using labeled α-amanitin, that the inhibitor binds to the enzyme, probably stoichiometrically, and acts like streptolidigin: the elongation step of RNA synthesis is affected but not the initiation of the enzyme on the template.

This represents the extent of the information on mammalian RNA polymerase available at the time this research project was begun. Since that time, many other tissues have been studied, both plant (Mondal et al., 1970; Mondal et al., 1972) and animal, and the results are consistent with those described above (volume 35 of the Cold Spring Harbor Symposium on Quantitative Biology contains most of this information and, in addition, discusses other aspects of RNA synthesis in both prokaryotes and eukaryotes). Likewise, multiple forms of nuclear RNA polymerase have been demonstrated in such lower eukaryotes as Dictyostelium discoideum (Brian Seed, personal communication), Saccharomyces cerevisiae (Dezelee et al., 1972), Blastocladiella emersonii (Horgen and Griffin, 1971), Achyla bisexualis (Timberlake et al., 1972), as well as Drosophila melanogaster (Steven Harris, personal communication). Thus, the existence of RNA polymerases with very similar properties has been demonstrated for a wide variety of eukaryotes. The nomenclatures used for the enzymes is summarized in Table 1. It is interesting that mitochondria from Saccharomyces cerevisiae (Tsai et al., 1971) and from

TABLE 1

Multiple forms of mammalian RNA polymerase*

Nomenclature	Sensitivity to α-amanitin	Location in nucleus
A {AI / AII; I {Ia / Ib	-	Nucleolus
AIII, III	-	Nucleoplasm
B {BI / BII; II {IIa / IIb	+	Nucleoplasm

*Adapted from Butterworth et al. (1971).

rat liver (W.J.Rutter, seminar at CIT) also contain multiple RNA polymerase activities. In the latter, the three activities are quite analogous to the nuclear enzymes; the second form is even sensitive to α-amanitin.

One approach to the study of these RNA polymerases is to purify the enzymes and to compare their subunit structures. This has been accomplished for Form II with both calf thymus and rat liver tissues. In three papers published simultaneously (Kedinger et al., 1971; Mandel and Chambon, 1971; Chesterton and Butterworth, 1971), polymerase II was further resolved into two components on DEAE cellulose. After purification by a series of other steps, the subunit structure of these two partially resolved Form II polymerases was investigated on SDS polyacrylamide gels. One possesses two subunits of molecular weights 200,000 and 160,000 while the other contains one of 180,000 and one of 160,000 molecular weight in equal molar proportions. Weaver et al. (1971) have also purified polymerase II and presented good evidence that the enzyme (in this case, the two forms of II were not resolved) contains subunits of molecular weights of 190,000, 170,000, 150,000, 35,000, and 25,000. The three smallest subunits are each present in equal molar ratios, while the sum of the molar ratios of the two large subunits equals the ratios of each of the other subunits. They observed that the relative amounts of the two large subunits are not constant from

one preparation to another; the amount of the 170,000 molecular weight subunit seems to increase, with a corresponding decrease in the 190,000 subunit when preparations are allowed to "age" at 4°C in the crude state. If phenylmethyl sulfonylfluoride (PMSF), a serine protease inhibitor, is present during the isolation, the amount of the 190,000 molecular weight subunit is greater than in the control preparations. These results were interpreted to indicate the modification of polymerase II by a protease. It is interesting that no further degradation of the enzyme can be detected and that no detectable change in the specific activity of the polymerase occurs upon modification of the large subunit. This suggests that the alteration may have biological significance and is not simply an artifact of the isolation procedure. No preparations of rat liver polymerase II have been obtained, even in the presence of PMSF, in which the 170,000 dalton subunit is completely absent, suggesting that both forms of polymerase II may exist *in vivo*. One can only speculate on the biological role of the "protease modification", although a role might be inferred because of the parallel with the findings with *Bacillus subtilis* RNA polymerase (Leighton *et al.*, 1971).

The transcription of mammalian chromatin by the homologous RNA polymerase has only recently been studied (Butterworth *et al.*, 1971). Chromatin contains endogenous

RNA polymerase activity which is totally α-amanitin-sensitive, at least when assayed at high ionic strength. Rifampicin AF/0-13, an inhibitor of chain initiation, has no effect on the endogenous activity, suggesting that the chromatin-bound enzyme has already initiated synthesis and the activity measured is the elongation of nascent chains present in complex with the polymerase and DNA. Chromatography on DEAE cellulose confirms that the endogenous polymerase is Form II. The chromatin-bound activity, which is quite low, can be supplemented by the addition of purified polymerase II. In contrast, the addition of polymerase I to chromatin does not increase the amount of RNA synthesized over the endogenous level, suggesting that Form I cannot use chromatin as a template. It is not known whether this indicates the absence or unavailability of nucleolar DNA in the chromatin, or whether the Form I polymerase is lacking some factor necessary for synthesis of RNA on a chromatin template. If the former proves correct, it would appear that the nucleolar polymerase has the ability to recognize its putative <u>in vivo</u> initiation sites. The ionic strength and metal ion requirements for the transcription of chromatin are extremely complicated due to differential effects on the template and the polymerase (Henk van den Broek, personal communication) and need to be thoroughly studied before investigations of the RNA produced can be conducted.

It has been reported that E. coli and mammalian RNA polymerase II initiate at different sites on chromatin (Butterworth et al., 1971). This was determined by "saturation" of the chromatin with bacterial enzyme, followed by the addition of mammalian polymerase, which results in increased synthesis of RNA. The converse is also true: pre-saturation with mammalian enzyme does not prevent RNA synthesis by E. coli polymerase. Several considerations affect the interpretation of these experiments: (1) The chromatin was not completely saturated with the first enzyme added; (2) the possibility of exchange of the two polymerases at the binding sites cannot be ruled out; (3) no evidence was presented indicating whether either or both of the polymerases transcribe both strands of the DNA. In fact evidence obtained from the comparison, by competition hybridization, of RNA transcribed from chromatin by bacterial and mammalian polymerases (Smith et al., 1969) indicates that those sequences transcribed from the middle-repetitive fraction of the DNA are quite similar. This problem is worthy of further investigation.

The Novikoff hepatoma ascites tumor of the rat has been used as a source of RNA polymerase studied in the work presented here. This tissue was chosen for several reasons. First, the synthesis of RNA in vivo has been thoroughly studied in ascites because it is possible to label the various types of RNA to a relatively high specific activity.

This should prove useful in comparison of RNA produced *in vitro* from chromatin and DNA with that synthesized *in vivo*. Secondly, ascites tumor chromatin is easily obtainable and has been well-characterized (Dahmus and McConnell, 1969), providing a background for the investigation of transcription *in vitro*. Also, large amounts of tissue can be obtained (approximately 100 ml of packed cells from 25-30 rats). Finally and most important of all, ascites contains a large amount of isolatable RNA polymerase activity. A survey of various tissues, including rat liver, rat brain, calf thymus, and ascites tumor, indicated that the hepatoma yields the largest amount (in some cases, an order of magnitude more) of RNA polymerase per unit of work. This is probably because the cells are rapidly-dividing (once every 17 hr; Mayfield and Bonner, 1971) and are synthesizing RNA at a high rate.

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CHAPTER II

THE ISOLATION AND PURIFICATION OF NOVIKOFF HEPATOMA ASCITES TUMOR RNA POLYMERASES

INTRODUCTION

The thorough characterization, both enzymologically and physically, of prokaryotic RNA polymerase has suggested mechanisms of control of gene expression which directly involve the transcription enzyme (see Burgess, 1971, for a recent review). The extrapolation of these mechanisms to the eukaryotic systems may be possible, but requires a comparable characterization of the eukaryotic RNA polymerases. Also, the study of gene control using isolated chromatin has been complicated by the use of E. coli RNA polymerase for in vitro transcription studies. It is questionable whether the comparison of derepressed genes in isolated chromatin with those expressed in vivo by the use of a heterologous system (bacterial RNA polymerase and mammalian chromatin) yields results which can be interpreted unambiguously. The use of homologous RNA polymerase in chromatin transcription may, at least, simplify the problems involved.

As a beginning to the solution of these problems, RNA polymerase from Novikoff hepatoma ascites tumor nuclei has been solubilized and purified extensively. During the purification, multiple activities have been resolved and the subunit structure of two of the activities (probably the nucleolar RNA polymerases) has been determined. The

molecular structures are different from each other and from the nucleoplasmic RNA polymerase (Weaver, *et al.*, 1971). All three may have one subunit in common. Thus, the three RNA polymerases of ascites tumor are physically distinct enzymes and may be responsible for the synthesis of different types of RNA *in vivo*.

MATERIALS AND METHODS

Maintenance of cell line

The Novikoff hepatoma ascites is a liver-derived tumor grown in the intraperitoneal cavity. Male Sprague-Dawley rats (150-250 g) are injected with 0.5 ml of fluid with an 18-gauge needle and then killed by etherization after 6-7 days. The yield is approximately 20-25 ml of ascites fluid per rat. Bacterial contamination is less than 100 cells per ml as judged by colony growth on media containing proteose peptone yeast extract and glucose.

RNA polymerase assay

The reaction mixture contains in a total volume of 0.50 ml: 25 µmoles Tris-HCl, pH 7.9; 5 µmoles $MgCl_2$; 2.5 µmoles dithiothreitol (DTT); 0.50 µmoles ATP, UTP, and CTP (P.L. Laboratories); 0.05 µmoles ^{3}H-GTP (100 µc/µmole) (Schwarz/Mann); 50 µg DNA; and enzyme. After incubation at 37°C, usually for 10 min, the reaction is stopped by the addition of 1.0 ml of 0.1 M EDTA, pH 8, 1% SDS, 500 µg/ml carrier RNA. Approximately 10 ml of cold TCA-pyrophosphate [100% TCA (w/v): saturated sodium pyrophosphate (room temperature): cold H_2O, 1:2:7, made up fresh each time] is added and the tubes allowed to stand for about 10 min. The precipitates are collected on Selectron membrane filters (Schleicher and Schuell, B-6,

25 MM, 0.45 micron). The tubes are rinsed three times with cold TCA-pyrophosphate and the filters are washed 6-10 times with the same solution. The filters are then placed in glass scintillation vials, 0.5 ml 5% TCA is added, and the capped vials heated for 15 min at 95°C. After cooling, 10 ml of Aquasol (New England Nuclear) is added and the samples counted in a Beckman LS-200B liquid scintillation counter.

Preparation of nuclei for enzyme extraction

The ascites fluid is diluted with an equal volume of cold TNKM (0.05 M Tris, pH 6.7, 0.13 M NaCl, 0.0025 M $MgCl_2$, 0.025 M KCl) and centrifuged for 10 min at 2 K rpm in International No. 284 swinging bucket rotor. The cells are then resuspended in 5 volumes of cold deionized H_2O and centrifuged at 2 K rpm for 6 min. This preferentially lyses the red blood cells. TNKM is added and the cells recentrifuged as above. To lyse the ascites cells, the white pellets are suspended in 5 volumes of 0.01 M Tris, pH 7.9, 0.01 M $MgCl_2$, 0.025 M NaCl, 0.33 M sucrose and the suspension is made 0.25% Triton X-100. After stirring in the cold for 15 min, the nuclei are pelleted by centrifugation at 2K rpm for 20 min.

Extraction of RNA polymerase from nuclei

The crude nuclear pellet is suspended in an equal volume of 0.02 M Tris, pH 7.9, 0.01 M $MgCl_2$, 0.01 M DTT,

2.0 M sucrose and then made 0.30 M $(NH_4)_2SO_4$ by the addition of 1/9 volume of 3.0 M $(NH_4)_2SO_4$, pH 7.9. After thorough mixing, the viscous solution is sheared in 50 ml batches with a Polytron (Brinkman Instruments) twice for 45 sec each at a setting of 3, with cooling on ice and mixing between. The sheared solution is allowed to stir for 20-30 min to insure maximum extraction. The salt concentration is then diluted to 0.1 M $(NH_4)_2SO_4$ by adding the extract to 2 volumes of 0.05 M Tris, pH 7.9, 0.005 M $MgCl_2$, 0.1 mM EDTA, 5 mM DTT, 25% glycerol (TGMED) while stirring with a glass rod. The precipitated chromatin is then removed by centrifugation at 30 K rpm for 4-5 hr in a Spinco 30 rotor. The supernatant, which contains 90% of the activity, is precipitated by the addition of 1/2 volume of saturated $(NH_4)_2SO_4$, pH 8 (saturated at 4^oC) with stirring. After diluting the precipitated solution with about 1/2 volume of 33% $(NH_4)_2SO_4$ to lower the density (otherwise, the precipitate floats), the pellets are collected by centrifugation at 12 K rpm in a Sorvall GSA rotor for 20 min and discarded. The supernatant is made 50% $(NH_4)_2SO_4$ by the addition of 1/3 volume saturated $(NH_4)_2SO_4$, pH 8 and allowed to stand overnight. The pellets are collected as above and dissolved in 0.05 M Tris, pH 7.9, 0.1 mM EDTA, 5 mM DTT, 25% glycerol (TGED) +0.15 M KCl at a concentration of 5-7 mg/ml protein. This crude extract is dialyzed against 2 changes of 10 volumes TGED

+0.15 M KCl for 6-8 hr each. Precipitated chromatin is then removed by centrifugation at 50 K rpm for 4 hr in a Ti50 rotor.

Phosphocellulose chromatography

Whatman P-11 cellulose phosphate is precycled by suspension in 0.1 N HCl followed by washing with water and then with 0.1 N KOH. After excess KOH is removed by filtration and washing with H_2O, the resin is equilibrated in TGED + 0.15 M KCl and the pH adjusted to 7.9. A 2.5 x 45 cm column is poured and equilibrated with TGED + 0.15 M KCl until the effluent is pH 7.9 and .15 M KCl (checked by conductivity). The sample (30-60 ml; < 5 mg/ml protein) is applied at a flow rate of 0.2 ml/min, the column washed with one column volume of TGED + 0.15 M KCl (at a flow rate of 0.5 ml/min) and then developed with a gradient of TGED + 0.15 M KCl to TGED + 0.80 M KCl (300 ml each). Approximately 100-9 ml fractions are collected, the A_{280} profile determined and 100 λ of every other fraction assayed for activity. The appropriate fractions are pooled, an equal volume of saturated $(NH_4)_2SO_4$, pH 8 added and the solution allowed to precipitate overnight at 4°C. The precipitates are collected by centrifugation at 10 K rpm for 20 min in a Servall swinging bucket rotor and redissolved in a few ml of TGMED + 0.05 M $(NH_4)_2SO_4$. After dialysis against 50 volumes of TGMED + 0.05 M $(NH_4)_2SO_4$

for 5-8 hr, the samples can be used directly for further purification or stored at -70°C.

DEAE cellulose chromatography

DEAE cellulose (Type 20; Schleicher and Schuell) is precycled by suspension in 0.25 N NaOH, removal of excess base by washing with water on a Buchner funnel and then resuspension in 0.25 N HCl. After removal of excess acid, the resin is suspended in TGMED + 0.05 M $(NH_4)_2SO_4$ and the pH adjusted to 8. A 1.5 x 30 cm column is poured and washed with TGMED + 0.05 M $(NH_4)_2SO_4$ until the eluate is pH 7.9 and 0.05 M $(NH_4)_2SO_4$. The sample (3-7 ml at 1 mg/ml protein) is applied at a flow rate of 0.2-0.3 ml/min and allowed to stand for about 30 min. After washing with one column volume of TGMED + 0.05 M $(NH_4)_2SO_4$, a gradient of TGMED + 0.05 M $(NH_4)_2SO_4$ to TGMED + 0.50 M $(NH_4)_2SO_4$ (100 ml of each) is applied at a flow rate of 0.6 ml/min and 100 drop fractions (3 ml) are collected. The A_{280} profile is determined and 100 λ of every second fraction is assayed for activity. The appropriate fractions are combined, an equal volume of saturated $(NH_4)_2SO_4$, pH 8 added, and the solution is allowed to precipitate overnight at 4°C.

Sucrose density gradient centrifugation

The precipitated protein from the DEAE cellulose column is collected by centrifugation at 25 K rpm for 1 hr in an

SW25.1 rotor and the tubes wiped free of $(NH_4)_2SO_4$. The pellet is dissolved in 100-200 λ of 0.05 M Tris, pH 7.9, 0.005 M $MgCl_2$, 0.1 mM EDTA, 10 mM DTT, 5% glycerol and the final $(NH_4)_2SO_4$ concentration, due to that remaining in the pellet, is determined by conductivity (10λ diluted into 6 ml of H_2O and compared to a standard curve). The volume of the sample is adjusted so that the $(NH_4)_2SO_4$ concentration is less than 0.6 M. The sample is then layered on a 5.0 ml 5-20% sucrose gradient (containing 0.05 M Tris, pH 7.9, 0.005 M $MgCl_2$, 0.1 mM EDTA, 10 mM DTT, 0.2 M $(NH_4)_2SO_4$, 10% glycerol), overlayered with parafin oil, and centrifuged for 10 hr at 65 K rpm in an SW65 rotor. Approximately 25 fractions (10 drops) are collected by puncturing the bottom of the tube and 20λ is assayed for activity. Protein concentrations are determined on 20 λ of each fraction by the method of Bramhall et al. (1969).

Polyacrylamide gel electrophoresis

For gel electrophoresis under non-denaturing conditions, the method of Davis (1964) is used (5% acrylamide, pH 8.7; with stacking gel, pH 6.9; Tris-glycine reservoir buffer, pH 8.3). Approximately 10 µg of protein in 100λ TGMED + 0.05 M $(NH_4)_2SO_4$ is applied and electrophoresed for 2 hours at 3 ma per gel. SDS gel electrophoresis is performed according to Shapiro et al. (1967). Samples are dialyzed against 0.01 M sodium phosphate, pH 7.1, 0.1% SDS, 0.1% β-mercaptoethanol, 10% glycerol and up to 100λ applied to

the gel. For the analysis of the sucrose gradient fractions, 100 λ of each fraction are diluted with 100 λ of dialysis buffer and then dialyzed overnight against 500 ml. 50 λ is applied to each gel. Electrophoresis is for 2½ hours at 6 MA per gel. Both the native and SDS gels are stained with coomasie blue and destained according to Elgin and Bonner (1970).

BioGel A-1.5 M chromatography

BioGel A 1.5 M (BioRad) is equilibrated in TGMED + 0.2 M $(NH_4)_2SO_4$ and poured into a 2.2 x 79 cm column. 2.0 ml of sample are applied and 100 drop (2.4 ml) fractions are collected at a flow rate of 9.6 ml/hr. For calibration, Blue Dextran and H^3GTP are used to determine the void volume and included volume. E. coli RNA polymerase (M = 500,000), catalase (M = 247,000), BSA dimer (M = 134,000), hemoglobin (M = 66,000), and ovalbumin (M = 45,000) are used to calibrate the column for molecular weight determination.

RESULTS

Isolation procedure

The isolation procedure employed (Fig. 1) is a modification of that used by Roeder and Rutter (1970a). Even though the shearing procedure results in foaming and extensive protein denaturation, the yield of polymerase activity is not affected as compared to that achieved by gentler (and more time-consuming) methods such as sonication. The activity which remains in the supernatant after precipitation of the sheared chromatin by dilution represents about 90% of the isolatable activity. It is extremely important in this and all subsequent steps that the dithiothreitol is not oxidized since the enzyme is sensitive to air oxidation. Dithiothreitol is susceptible to auto-oxidation, particularly in a medium of Mg^{++} and pH 8, and deteriorates over a period of a few days under these conditions. For best results the dithiothreitol should be added to the buffers immediately before use.

Ammonium sulfate precipitation

Precipitation with $(NH_4)_2SO_4$ has proven to be the most convenient method for concentration of the enzymes. Also, considerable purification is obtained by taking the 33-50% cut, as is shown in Fig. 2. The recovery of activity is 90-95% at this purification step. Best results are obtained

Fig. 1

Flow diagram of RNA polymerase isolation procedure

See Methods section for details.

Isolation of RNA Polymerase from Novikoff Hepatoma Ascites Cells

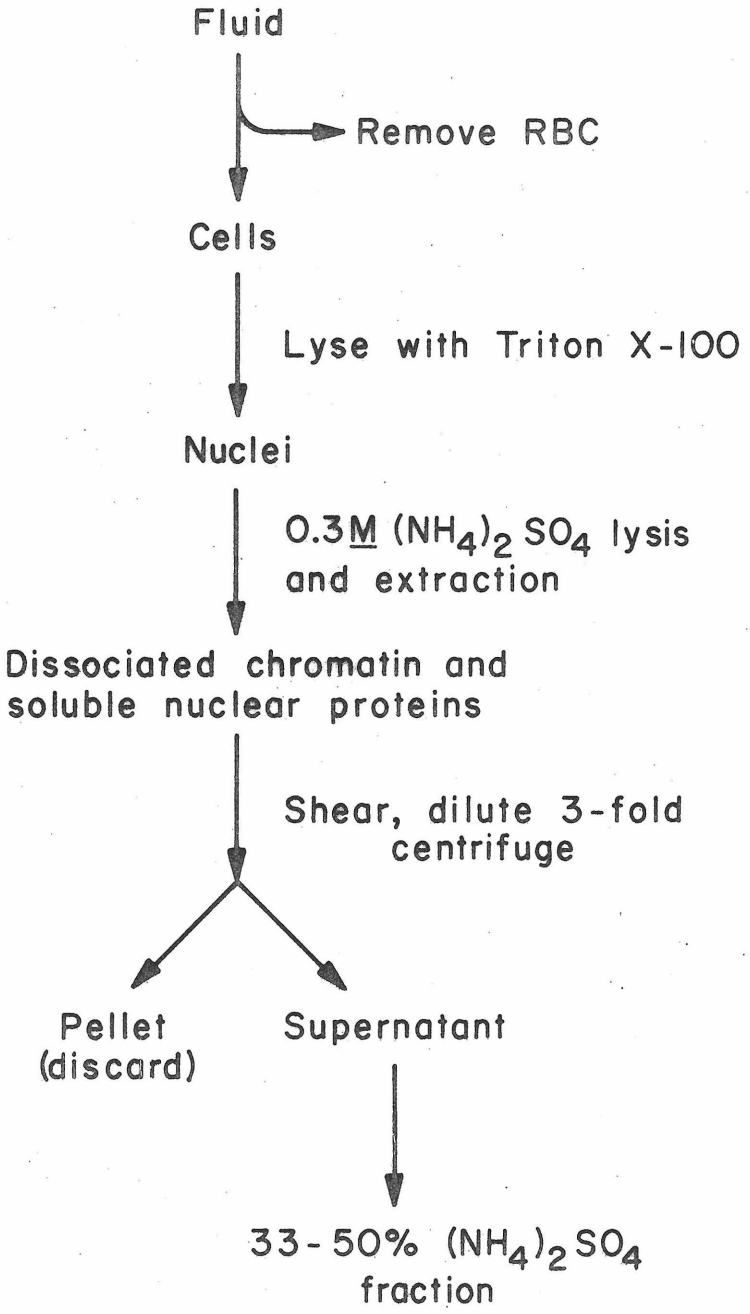
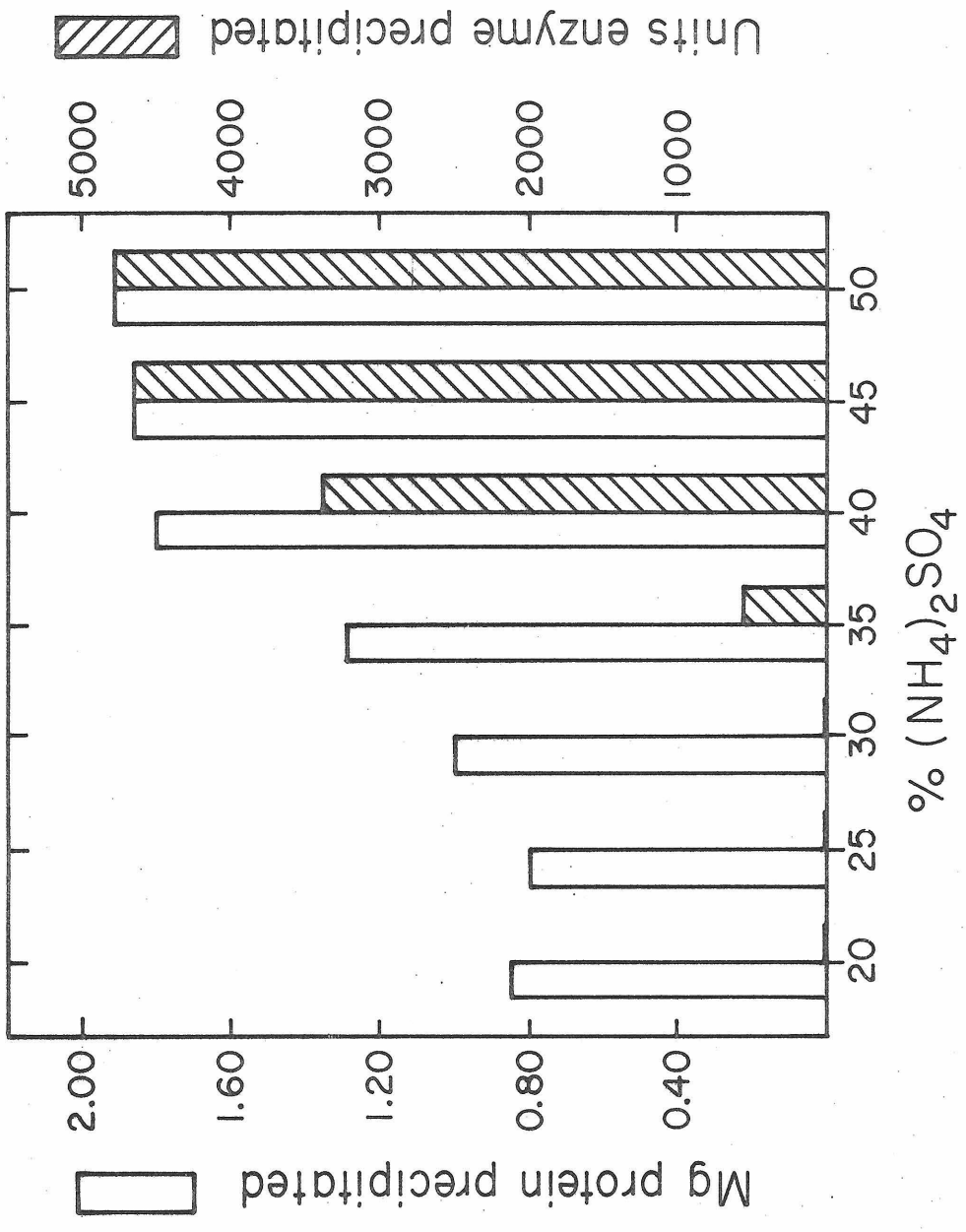


Fig. 2

Ammonium sulfate precipitation of crude extract

The appropriate amount of saturated $(NH_4)_2SO_4$, pH 7.9, was added to 0.5 ml of crude extract (purified through the 30 K rpm centrifugation) and allowed to precipitate at 4^oC overnight. The precipitates were collected by centrifugation and redissolved in TGMED + 0.05 M $(NH_4)_2SO_4$. After dialysis, the fractions were assayed for polymerase activity and the protein concentrations determined.


Units enzyme precipitated
5000
4000
3000
2000
1000
Mg protein precipitated
2.00
1.60
1.20
0.80
0.40
20
25
30
35
40
45
50
% $(NH_4)_2SO_4$

when the precipitation is allowed to proceed for at least 10 hr. After redissolution of the pellets in TGED + 0.15 M KCl, it is necessary to remove the excess $(NH_4)_2SO_4$ by dialysis, since the salt concentration can be as high as 0.5 M if this is omitted and the enzyme will not bind to the phosphocellulose column. Because of the viscosity of the medium (25% glycerol) and its high protein concentration (often 8-10 mg/ml), the dialysis rate is quite slow. Two 6-hour periods are sufficient, although it is advisable to monitor the salt concentration by conductivity. Small amounts of residual chromatin precipitate during dialysis and these are removed by high speed centrifugation. After this centrifugation the ratio $A_{280/260}$ is about 1.0, indicating a very low content of nucleic acid. This crude extract is stable and can be stored for at least several months at -70°C.

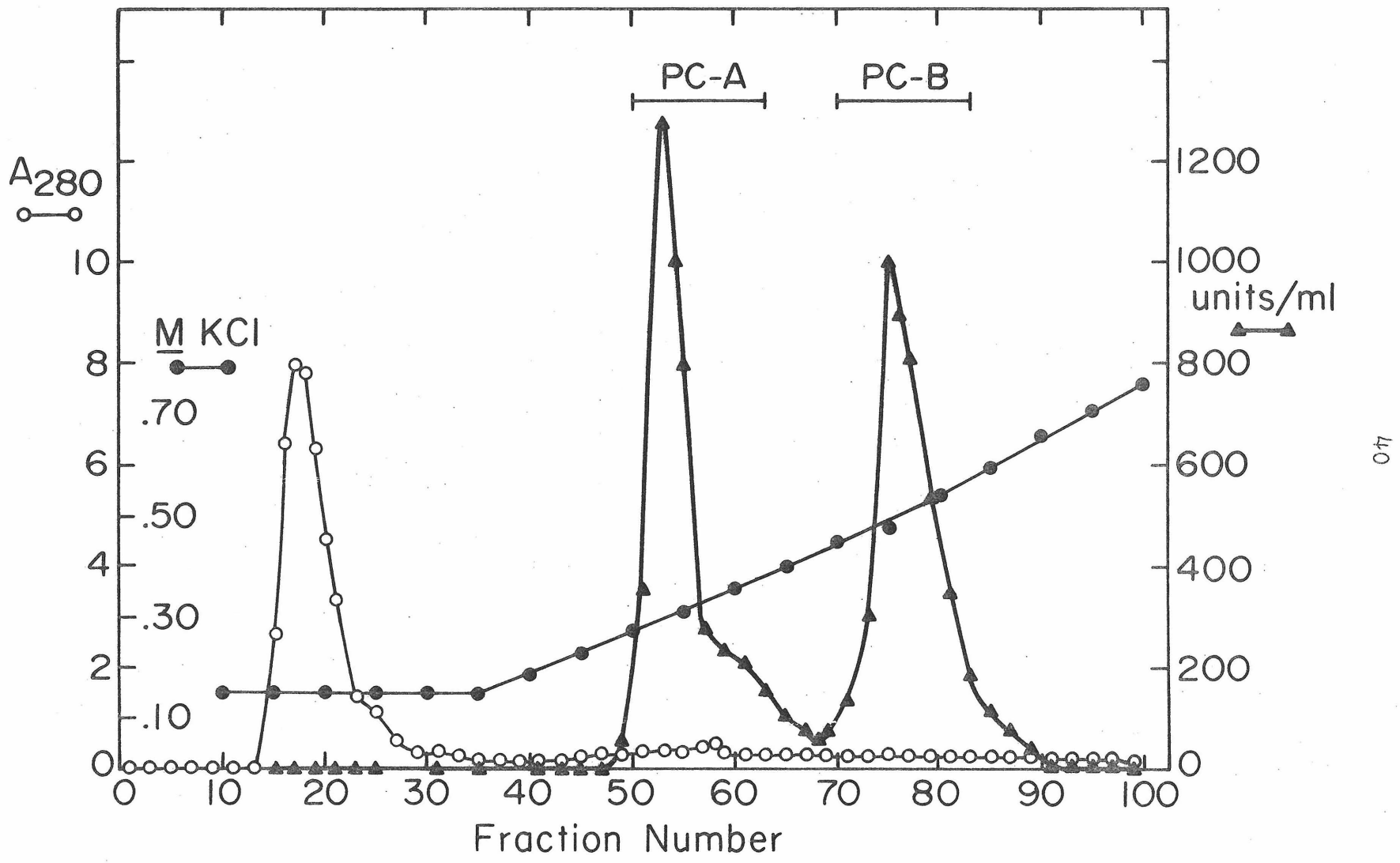
Phosphocellulose chromatography

Considerable purification is achieved by chromatography on phosphocellulose as shown in Fig. 3, while the yield of activity is 60-90%. Also, the resolution of two RNA polymerase activities is accomplished, one peak eluting at about 0.30 M KCl (PC-A) and the other at 0.50 M KCl (PC-B). The shoulder on the PC-A activity peak (eluting at about 0.35 M KCl) is reproducible and may indicate the presence of a third form of the enzyme. PC-B never constitutes more than about 50% of the total activity and usually less; some

Fig. 3

Phosphocellulose chromatography of ammonium sulfate fraction

25 ml of a crude extract containing 170 mg of protein and 291,000 units of RNA polymerase were applied at 10.2 ml/min to a 2.5 x 45 cm column equilibrated in TGED + 0.15 M KCl. The column was washed with one volume of TGED + 0.15 M KCl at 0.5 ml/min and then eluted with a linear gradient of 300 ml each of TGED + 0.15 M KCl and TGED + 0.80 M KCl. Approximately 100-8.8 ml fractions were collected. 100 λ aliquots were assayed for activity and the salt gradient determined by conductivity measurements.


PC-A
PC-B
A280
M KCl
units/ml
Fraction Number
0
10
20
30
40
50
60
70
80
90
100
.10
.30
.50
.70
200
400
600
800
1000
1200

preparations contained essentially no PC-B. It is not known if this represents instability of the activity or variations in the cells or isolation procedure. Often, PC-B elutes as a broad peak rather than the sharp symmetrical one shown here. Samples as large as 90 ml can be applied to the column with good resolution of the two peaks if the flow rate during application is slow and the salt concentration low enough (0.15 M KCl). Columns can be reused several times, with sufficient buffer equilibration between runs if care is taken to avoid bacterial growth. Larger columns generally result in lower yields of activity. The concentrated peak fractions can be stored at -70°C for several months with very little loss of activity.

DEAE cellulose chromatography

Further purification of both PC-A and PC-B can be achieved by chromatography on DEAE cellulose (Figs. 4 and 5). PC-A is resolved into two activities, eluting at 0.15 M $(NH_4)_2SO_4$ (Ia) and 0.22 M $(NH_4)_2SO_4$ (II). Peak II is relatively small and constitutes less than 10% of the original activity when assayed under these conditions (i.e., no Mn^{++}). However, its appearance is quite reproducible though its amount varies considerably. Activity yield is about 25-50%. PC-B elutes as a single peak at about 0.11 M $(NH_4)_2SO_4$ and is termed Ib. Yields as high as 98% have been obtained. After this step, Ia and Ib are quite unstable and considerable losses of activity occur when the fractions

Fig. 4

DEAE cellulose chromatography of PC-A

4.1 ml of PC-A containing 9.7 mg of protein and 46,400 units of RNA polymerase were applied at a flow rate of about 0.2 ml/min to a 1.5 x 30 cm column equilibrated in TGMED + 0.05 M $(NH_4)_2SO_4$. The column was then washed with one volume of TGMED + 0.05 M $(NH_4)_2SO_4$ and developed at a flow rate of 1.0 ml/min with a linear gradient of 100 ml each TGMED + 0.05 M $(NH_4)_2SO_4$ and TGMED + 0.50 M $(NH_4)_2SO_4$. Approximately 80-2.8 ml fractions were collected. 100 λ aliquots were assayed for enzyme activity and the salt gradient determined by conductivity measurements.

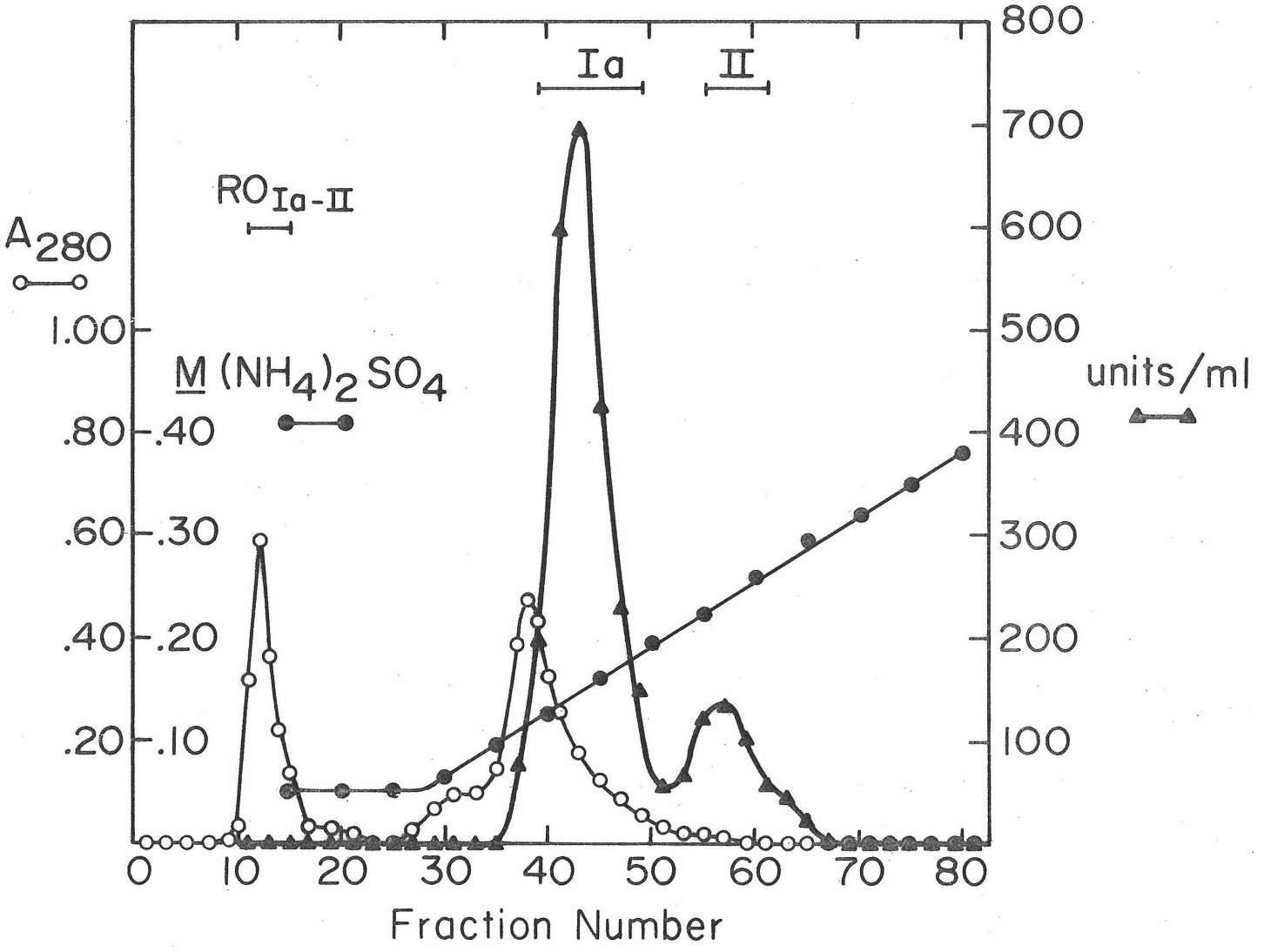
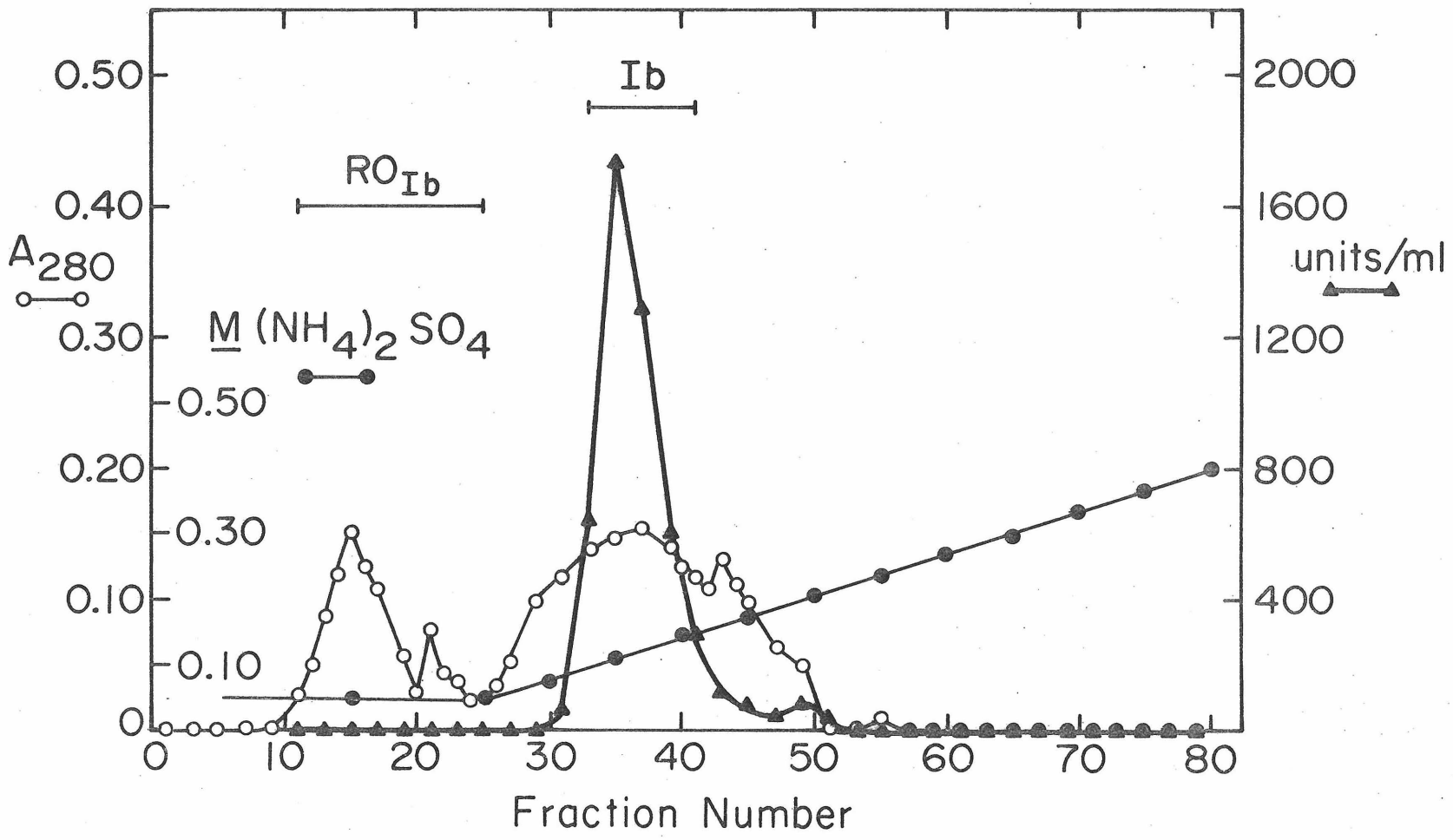

Ia
II
RO Ia-II
A280
1.00
.80
.60
.40
.20
M (NH4)2 SO4
.40
.30
.20
.10
800
700
600
500
400
300
200
100
units/ml
0
10
20
30
40
50
60
70
80
Fraction Number

Fig. 5

DEAE cellulose chromatography of PC-B

6.0 ml of PC-B containing 6.0 mg of protein and 30,750 units of RNA polymerase were applied at a flow rate of 0.3 ml/min to a 1.5 x 30 cm column equilibrated in TGMED + 0.05 M $(NH_4)_2SO_4$. The elution and analysis procedures were as described in Fig. 4.


Ib
RO Ib
A280
M (NH4)2 SO4
units/ml
0.50
0.40
0.30
0.20
0.10
0
0.50
0.30
0.10
2000
1600
1200
800
400
0
10
20
30
40
50
60
70
80
Fraction Number

are concentrated with $(NH_4)_2SO_4$, although the recovery of protein appears to be quite high. Other methods of concentration, such as pressure dialysis, are also unsatisfactory. This instability may be due, at least in part, to the loss of a factor in the run-off of the DEAE cellulose columns, termed RO_{Ia-II} and RO_{Ib} (See Ch. IV). Any attempts to dialyze the enzymes after this purification step result in almost complete loss of activity.

Comparison of the elution positions of these enzymes from DEAE cellulose with those of Roeder and Rutter (1970a) suggests that Ia and Ib are equivalent to their nucleolar enzymes while Form II is their α -amanitin-sensitive nucleoplasmic polymerase. Enzymological studies presented below confirm this conclusion (Ch. III). Chesterton and Butterworth (1971) have reported the resolution of polymerase I into two species by phosphocellulose chromatography though a different isolation procedure was used. Also it is apparent from the DEAE-Sephadex chromatography of sea urchin RNA polymerase (Roeder and Rutter, 1970b) that polymerase I is composed of at least two forms.

Sucrose density gradient centrifugation

Ia and Ib can be further purified by sucrose gradient centrifugation in high salt $\left[0.2\ M\ (NH_4)_2SO_4\right]$. The inclusion of glycerol in the gradient serves to stabilize the enzyme and is a modification of the procedure of Seifart and Sekeris (1969). The high salt prevents

aggregation, as occurs with E. coli RNA polymerase (Berg and Chamberlain, 1970). The two enzymes have a similar sedimentation velocity and are slightly larger than catalase (11 S) or about 14-16 S. This is in agreement with the findings of Chesterton and Butterworth (1971) who obtained sedimentation coefficients of 16 S for both enzymes and calculated a molecular weight of 600,000.

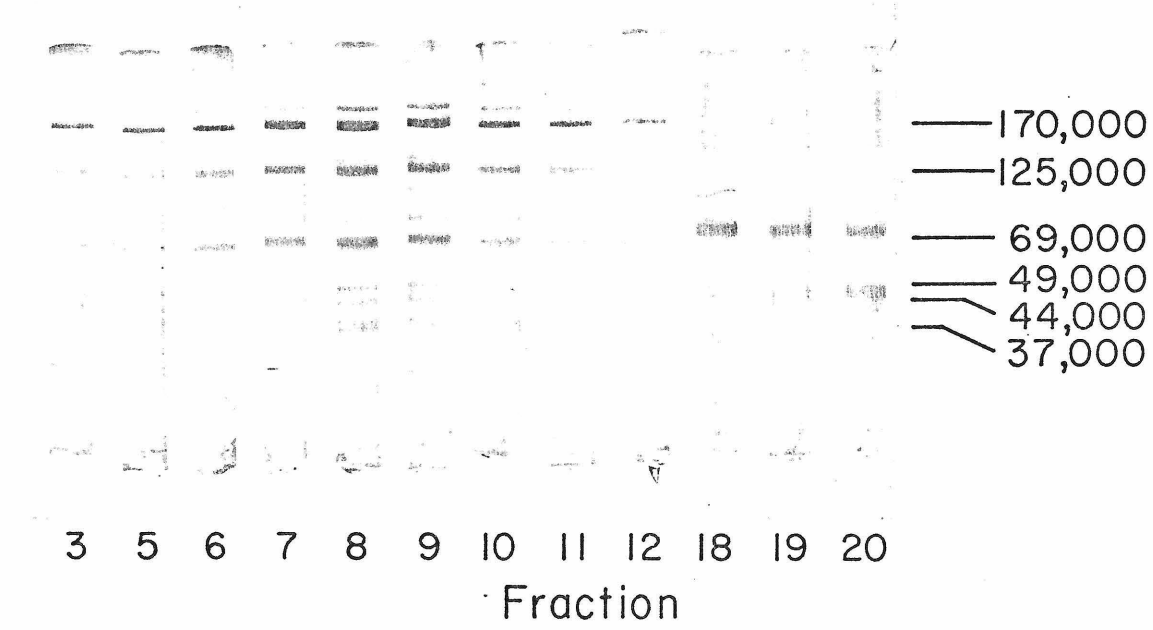
State of purity and subunit structure of Ia and Ib

Examination of the sucrose gradient profiles reveals coincidence of the protein and RNA polymerase activity peaks, particularly so in the case of Ia, indicating a relatively high state of purity of the enzymes, although the presence of contaminating protein of similar sedimentation characteristics cannot be eliminated. To investigate the subunit structures of the two enzymes, aliquots of each fraction were analyzed by SDS polyacrylamide gel electrophoresis. The results are shown in Figures 6 and 7. Ia appears to have a complex subunit structure: polypeptides of 170,000, 125,000, 69,000, 49,000, 44,000, and 37,000 molecular weight are present in the fractions which contain enzyme activity. The amounts of these six bands are highest in fraction 8 (the one with the highest enzyme activity) and decrease on either side of the peak with approximate correspondence to the activity, as would be expected if these polypeptides constitute the enzyme. Fractions 18 and 19, which do not contain activity, show an entirely

Fig. 6

Sucrose density gradient centrifugation of Ia

The ammonium sulfate precipitate of Ia from the DEAE cellulose column was collected by centrifugation at 25 K rpm for 1 hr in an SW 25.1 rotor and redissolved in 100 λ of 0.05 M Tris, pH 7.9, 0.005 M $MgCl_2$, 0.1 mM EDTA, 5 mM DTT, 5% glycerol. The sample was layered on a 5 ml 5-20% (w/v) sucrose gradient containing 0.05 M Tris, pH 7.9, 0.005 M $MgCl_2$, 0.1 mM EDTA, 5 mM DTT, 10% glycerol, 0.20 M $(NH_4)_2SO_4$, and centrifuged for 10 hr at 65 K rpm in a SW65 rotor. 10-drop fractions were collected and 20 λ aliquots were assayed for RNA polymerase activity. Protein concentrations were determined on 20 λ aliquots. SDS gel electrophoresis was conducted as described in MATERIALS AND METHODS.


170,000
125,000
69,000
49,000
44,000
37,000
3 5 6 7 8 9 10 11 12 18 19 20
Fraction

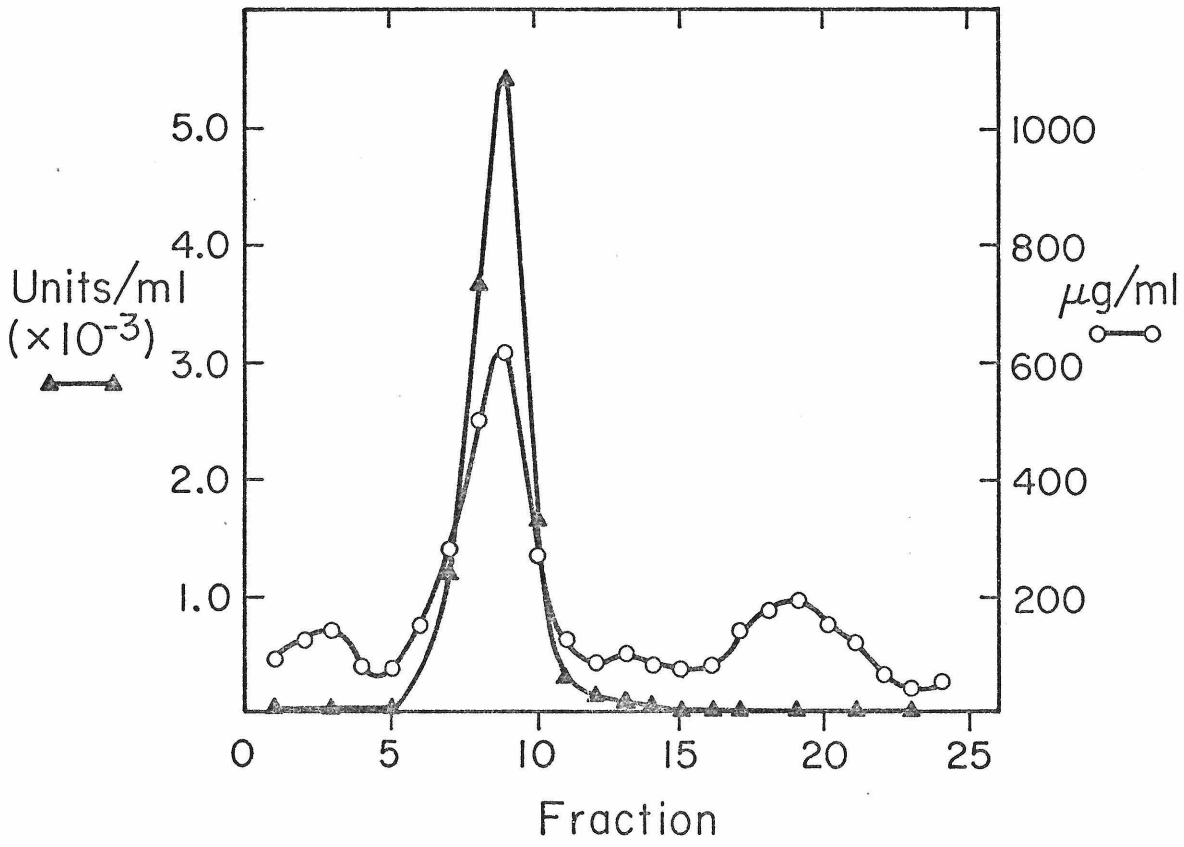

Units/ml
(×10⁻³)
5.0
4.0
3.0
2.0
1.0
1000
800
600
400
200
μg/ml
0 5 10 15 20 25
Fraction

Fig. 7

Sucrose density gradient centrifugation of Ib

All procedures were identical to those described in Fig. 6 except the centrifugation was for 10½ hr at 65 K .

Fig 2 Robbins & Bonner

top

different spectrum of polypeptides, except that a protein of approximately 70,000 molecular weight is present. It is not possible to determine whether this is identical to that present in fractions 6-11.

If these subunits are all part of RNA polymerase, the relative molar ratios should be integral numbers. These ratios were determined by scanning the gels and evaluating the relative areas under the peaks after a computer gaussian fit to each. The results are shown in Table 1. Within a reasonable margin of error, the putative subunits are all present in equimolar ratios, with the exception of the 69,000 and 37,000 molecular weight polypeptides. These latter two have calculated molar ratios of about 1.50. It is possible that these polypeptides have abnormal affinities for the dye used to stain the gels. However, even if this is assumed, it is still difficult to assign an integral molar ratio to these subunits; each enzyme molecule may contain either one or two molecules each. It is important that the molar ratios for all six polypeptides remain constant (within the margin of error) across the peak of RNA polymerase activity, as would be expected if they sedimented as one complex. (Table 1).

Some preparations of Ia, including the one shown here, contain a subunit of 190,000 daltons which co-migrates with the active complex. The molar ratio of this subunit varies considerably from one preparation to another, although

TABLE 1

Molar ratios of the subunits of polymerase Ia

Subunit (molecular weight)	Molar Ratio				
	Fraction 7	Fraction 8	Fraction 9	Fraction 10	Avg ± S.D.
170,000	1.00	1.00	1.00	1.00	1.00 ± 0
125,000	0.91	0.93	0.73	0.94	0.88 ± .10
69,000	1.56	1.44	1.32	1.40	1.43 ± .10
49,000	0.83	0.92	0.87	0.86	0.87 ± .12
44,000	1.25	1.26	1.12	0.92	1.14 ± .16
37,000	1.49	1.53	1.38	1.63	1.51 ± .10

The fractions refer to the sucrose density gradient fractions of Ia (Figure 6). The molar ratios were calculated from spectrophotometric scans of the SDS polyacrylamide gels. Gaussian curves were fitted to the peaks and the areas determined by a computer program.

it is never greater than about 0.3. Weaver et al. (1971) reported the presence of a 190,000 dalton subunit in polymerase II from rat liver and calf thymus which appears to be converted to 170,000 daltons by a protease present in the crude extract. It seems likely that a similar situation may exist with polymerase Ia, although experiments designed to inhibit the presumptive protease with phenyl methyl sulfonyl fluoride (PMSF) were not definitive.

The minor bands which appear on the SDS gels and which have been ignored to this point, namely those of approximately 100,000, 80,000, and 55,000 molecular weight, are thought to be contaminants on the basis of their low molar ratios. It should be mentioned that minor bands tend to be over emphasized when coomassie blue stain is used since staining with this dye is nonlinear with amount of protein at low protein concentrations (Elgin, 1971). Since large amounts of protein were applied to the gels shown (10-20 μg), even minor contaminants would be visible because the detection limit of gels stained with coomasie blue is about 0.5 μg per band. Thus, the appearance of a large number of bands on the SDS gel is not a reliable indication of the purity of the preparation.

The most convincing evidence for the high state of purity of Ia is obtained by analytical gel electrophoresis in the absence of denaturants. Ia electrophoreses as a single major component with a very minor amount of

contamination (Figure 8a). It can only be assumed that the major component is the RNA polymerase, since attempts to elute the enzyme from the gel for assay were unsuccessful. Also, the possibility that aggregation occurs during electrophoresis cannot be eliminated, although essentially all of the protein migrated into the gel.

The subunit structure of polymerase Ib has also been investigated by SDS gel electrophoresis. The sucrose gradient profile of Ib (Fig. 7) again shows some coincidence of protein and polymerase activity peaks, particularly in fractions 7, 8 and 9. The SDS gels of these fractions exhibits three major bands of molecular weight 190,000, 135,000 and 67,000 daltons. The 67,000 molecular weight polypeptide clearly does not correspond in intensity to the activity peak and probably is not a subunit of Ib. The molar ratios of the two large subunits are approximately 1.0. Fig. 8 shows SDS gels of Ia and Ib, electrophoresed separately and together. Except for the 190,000 molecular weight subunit, none of the bands appear to co-migrate, although the 125,000 dalton subunit (Ia) and the 135,000 dalton subunit (Ib) may be analogous.

Determination of the molecular weight of Ia

Ia (purified through the DEAE cellulose chromatography step) was chromatographed on a calibrated BioGel A-1.5 M column to estimate its molecular weight (Fig. 9). As judged by its position of elution relative to E. coli RNA

Fig. 8

Native and SDS gel electrophoresis of Ia and Ib

a) Native gel of Ia (13 μg)

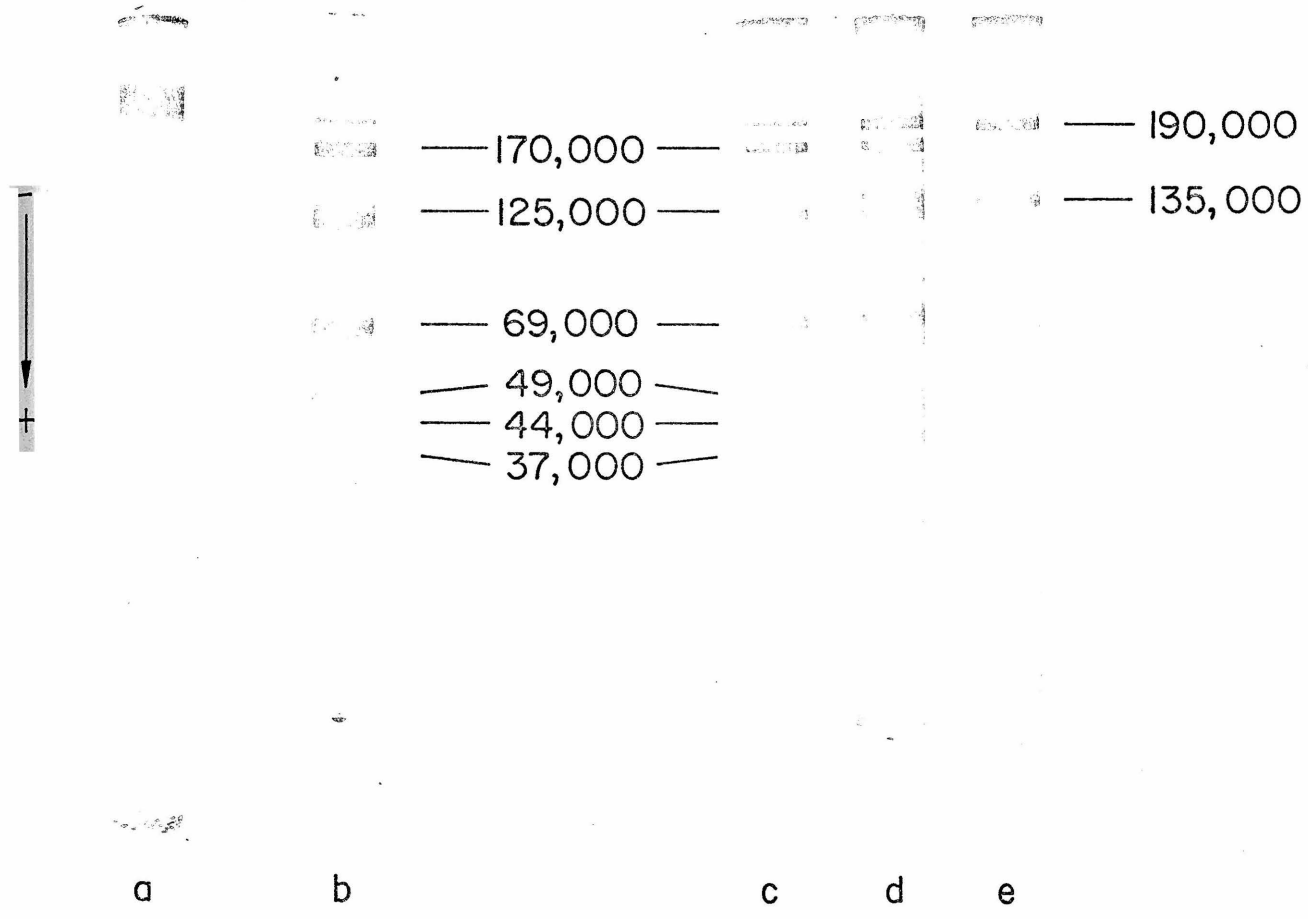
b) SDS gel of Ia (13 μg); same preparation as (a).

c) SDS gel of Ia (10 μg); different preparation from (a) and (b)

d) SDS gel of Ia (10 μg) + Ib (5 μg)

e) SDS gel of Ib (5 μg); same preparation as (d)

Procedure for electrophoresis is described in MATERIALS AND METHODS. c, d, and d e were electrophoresed simultaneously.

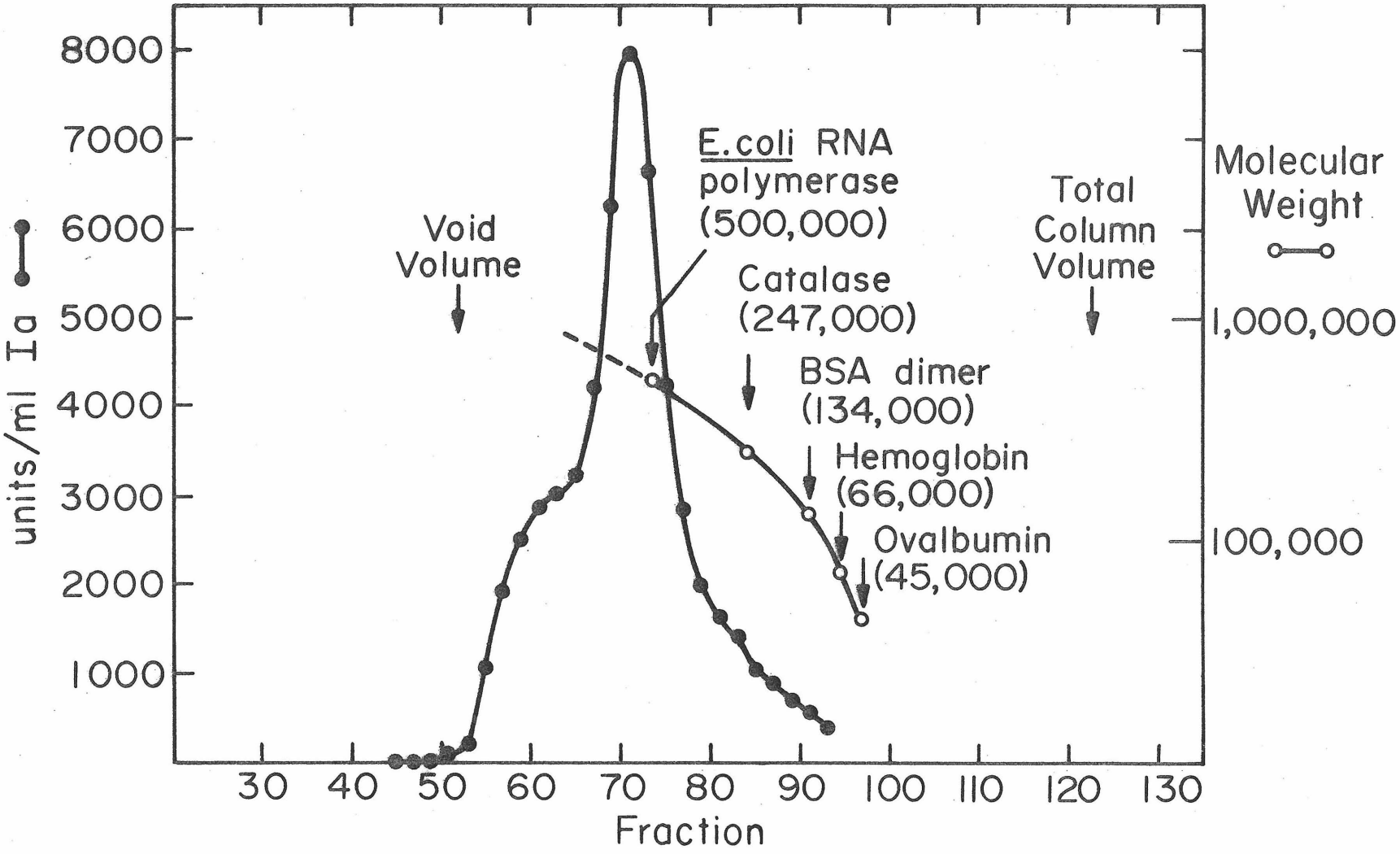

190,000
170,000
135,000
125,000
69,000
49,000
44,000
37,000
a
b
c
d
e

polymerase (500,000 daltons), the molecular weight of Ia appears to be about 600,000 (Fig. 9). This is consistent with the molecular weight determined by Chesterton and Butterworth (1971) by sucrose density gradient centrifugation. Although the chromatography was done at an ionic strength of about 0.60, the activity did not elute as a symmetrical peak. The shoulder present on the high molecular weight side of the activity peak is of about 1.2 million molecular weight and may represent a dimer of the enzyme. E. coli RNA polymerase is known to aggregate reversibly (Berg and Chamberlin, 1970), although at this ionic strength it exists in the monomer form.

Fig. 9

Bio-Gel A 1.5 M chromatography of Ia

2.0 ml of Ia containing 2.5 mg of protein was applied to a 2.2 x 79 cm Bio-Gel A-1.5 M column equilibrated in TGMED + 0.20 M $(NH_4)_2SO_4$. Approximately 120-2.4 ml fractions were collected at a flow rate of 9.6 ml/hr. 100 λ aliquots were assayed for RNA polymerase activity.


8000
7000
6000
5000
4000
3000
2000
1000
units/ml Ia
30
40
50
60
70
80
90
100
110
120
130
Fraction
Void Volume
E.coli RNA polymerase (500,000)
Catalase (247,000)
BSA dimer (134,000)
Hemoglobin (66,000)
Ovalbumin (45,000)
Total Column Volume
Molecular Weight
1,000,000
100,000

DISCUSSION

The data presented suggest several possibilities concerning the subunit structures of these two (nucleolar?) RNA polymerases. If Form Ia is homogeneous, as the native gel electrophoresis would seem to indicate, then the subunit structure is complex. The presence of large subunits (170,000, 125,000) has precedent not only in the bacterial RNA polymerase but also in the mammalian form II enzyme which also has a 170,000 dalton subunit plus others of 150,000, 35,000 and 25,000 daltons (Weaver *et al.*, 1971). Recent work with RNA polymerase isolated from T4-infected *E. coli* has shown the presence of three small subunits apparently complexed with the enzyme and these were implicated in the control of gene expression (Stevens, 1972). Thus, the seemingly large number of subunits indicated by the SDS gels does not appear to be unreasonable. Assuming one of each subunit per enzyme molecule, the predicted molecular weight would be 494,000, which is somewhat lower than that indicated by exclusion chromatography. It is possible that there are two moles of the 69,000 dalton subunit and two moles of the 37,000 dalton subunit per mole of enzyme which would bring the calculated molecular weight of the total enzyme (600,000) very near to that estimated by exclusion chromatography and sucrose density gradient centrifugation. An alternative is that the smaller subunits

are contaminants (either adventitiously bound to the polymerase or part of another enzyme complex which has similar sedimentation characteristics and that Ia consists of two subunits of molecular weights 170,000 and 125,000 daltons, each present twice per enzyme complex, resulting in a molecular weight of 590,000 daltons. It is noteworthy that the 125,000 dalton polypeptide has at times been resolved into two components on SDS gels.

Polymerase Ib exhibits two major bands on SDS gel electrophoresis of molecular weights 190,000 and 135,000 daltons. The most likely structure, assuming a total molecular weight of about 600,000 for the enzyme (since it has approximately the same S value as Ia), is two moles of each subunit per mole of enzyme. The other polypeptides present, however, cannot be ruled out. The only common structural property between Ia and Ib is the 190,000 dalton subunit; thus, these are two distinct enzymes and not simply different aggregation states of one enzyme.

Weaver et al. (1971) proposed a proteolytic conversion of the 190,000 dalton subunit to 170,000 daltons for polymerase II. It is interesting that form Ia contains a small amount of a 190,000 dalton polypeptide (much less than one mole per mole of enzyme), suggesting that the same modification may occur with form I polymerases. No modification of Ib has been observed. In view of the role of alterations in the molecular structure of prokaryotic RNA polymerase in

the control of gene expression, this proteolytic modification may be biologically significant and not simply an artifact of the isolation procedure. The use of protease inhibitors to obtain unaltered enzyme should yield some insight into this question.

Though these enzymes have been extensively purified, the specific activities are still quite low. There are several possible explanations for this fact. First of all, the preparations may contain enzyme molecules which are inactive due to denaturation. This seems likely since the recovery of enzyme activity from the sucrose gradient step is quite low while the recovery of protein is high. Also, it is possible that some necessary cofactor has been removed during the purification. Thirdly, at low enzyme concentrations, the activity is not proportional to concentration (in fact, there is no activity at very low enzyme concentrations), making the determinations of the specific activity difficult (See Ch. IV). Finally, protein factors necessary for optimal transcription (either by increasing the number of chain initiations or by increasing the rate of chain elongation) may have been lost during the purification.

Any discussion of the functions of these two enzymes at the present time can only be speculation. If they are both located in the nucleolus, then at least one may be involved in the synthesis of ribosomal RNA. Since little

is known about other types of RNA produced in the nucleolus, it is difficult even to postulate what other genes these polymerases may transcribe.

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CHAPTER III

ENZYMOLOGICAL CHARACTERIZATION OF THREE RNA POLYMERASES OF NOVIKOFF HEPATOMA ASCITES TUMOR

INTRODUCTION

RNA polymerase has been isolated from nuclei of Novikoff hepatoma ascites tumor and resolved into three activities (Ia, Ib, II) by a combination of phosphocellulose and DEAE cellulose chromatography. After further purification of Ia and Ib by sucrose density gradient centrifugation, the molecular structure of these two enzymes was shown to be different. The subunit structure of II, as determined by Weaver *et al.* (1971), is different from either Ia or Ib, although all three may share a common subunit of 190,000 daltons. These enzymes have been characterized according to their enzymological properties to differentiate them further and to obtain some information about their functions *in vivo*. The ionic strength optimum conditions, metal cation requirements and sensitivity to α-amanitin of these three polymerases confirm the earlier proposal (Chapt. II) that Ia and Ib are localized in the nucleolus while II is nucleoplasmic. Template studies suggest major differences between the nucleoplasmic and nucleolar polymerases while Ia and Ib differ only slightly in this respect.

MATERIALS AND METHODS

Preparation of enzymes

Polymerases Ia and Ib were purified through the sucrose gradient centrifugation step and II through the DEAE cellulose chromatography as described in Chapt. II. Dilutions were made in the presence of 100 µg/ml BSA (final conc.) to minimize loss of activity due to denaturation.

RNA polymerase assay:

Activity was assayed as described in Chapt.II. For time course experiments, the DEAE filter paper was used (Litman, 1968). The specific radioactivity of the H^3 GTP was increased 10-fold to 1000 uc/umole and 50 λ was removed from the reaction mixture at the appropriate times. The aliquots were pipetted onto DE81 filter papers (Whatman, 2.3 cm) and after 15-30 seconds, the filters were submerged in 5% Na_2 HPO_4 (5-10 ml/filter). The filters were then washed collectively 6 times with 5-10 ml/filter 5% Na_2HPO_4 for 15-30 minutes each wash. After rinsing twice with deionized H_2O to remove excess Na_2HPO_4, the filters were washed twice with 95% ETOH and once with ether. After drying, the discs were hydrolyzed and counted as described in Chapt. II.

Preparation of poly dT:H^3 poly rA hybrid

To hybridize poly dT and poly A, 0.20 ml of poly dT (5 A_{260} units/ml in .01 M Tris, pH 7.9; molecular weight = 1-5 x 10^6; General Biochemicals, Chagrin Falls, Ohio) plus 0.05 ml of H^3 poly A (10 µc/ml in .12 M phosphate buffer, pH 6.8, [equimolar quantities of mono and dibasic $NaPO_4$]; 51.0 mc/mmole polynucleotide phosphorous; Schwarz/Mann) were diluted to 1.0 ml with .12 M phosphate buffer, pH 6.8 and heated at 95°C for 3 minutes. The solution was then placed at 60°C for 2 hours to allow reannealing. 98% of the H^3 counts were precipitable with TCA.

Inhibitor solutions

α-amanitin (Henley Co.) was dissolved in .01 M Tris, pH 7.9 at a concentration of 2,000 ug/ml. Rifamycin SV (a gift of Dr. Dan McMahon) was dissolved in 95% ETOH at a concentration of 5.0 mg/ml. Rifamycin AF/0-13 (a gift of Brian Seed) was dissolved in dimethyl sulfoxide at a concentration of 35 mg/ml. Cycloheximide was dissolved in H_2O at 1 mg/ml.

Preparation of rat liver chromatin

Chromatin was prepared from frozen rat liver by the method of Elgin and Bonner (1970) and stored at -90°C in .01 M Tris, pH 7.9. After thawing, the chromatin was

homogenized and then used in the reaction. The spectral properties of the chromatin were:

$$A_{280}/A_{260} = 0.59$$

$$A_{230}/A_{260} = 0.74$$

$$A_{320}/A_{260} = 0.006$$

The total protein:DNA ratio was 2.14:1.00. For the assays using chromatin as a template, the Mg^{++} concentration was lowered to 5 mM since at the normal concentration (10 mM), aggregation of the chromatin occurs.

RESULTS

Characteristics of reaction product

As shown in Table 1, the product synthesized by the crude total enzyme (ammonium sulfate precipitated fraction) is > 99% sensitive to KOH hydrolysis and is 95% sensitive to digestion by ribonuclease A. This indicates that the product of the reaction as measured by TCA precipition is RNA. The 5% of the RNA which is apparently insensitive to RNase may reflect double-strandedness of the product. Also, as shown in Table 1, the rate of incorporation is not affected by the presence of inorganic phosphate, which inhibits polynucleotide phosphorylase at 0.8 mM (Burgess, 1969). Finally, under the assay conditions used (no Mn^{++}), the activity is 98% insensitive to α-amanitin, indicating that very little form II RNA polymerase is present (Kedinger et al., 1970; Lindell et al., 1970).

Optimum salt conditions

The multiple forms of mammalian RNA polymerases have been shown to differ in the ionic strength necessary for optimal activity (Roeder and Rutter, 1969). Figure 1 illustrates the differential effects of $(NH_4)_2SO_4$ concentration on the three forms of ascites RNA polymerase. Forms Ia and Ib show optimum activity at low ionic strength (less than .03 M $(NH_4)_2SO_4$) while polymerase II requires

Table 1

Characterization of reaction product

Treatment or Additions	Relative Activity
Control	1.000
+ 8 ug/ml α-amanitin	0.980
+ 0.8 mM inorganic phosphate	0.997
RNase treatment*	0.047
KOH hydrolysis‡	0.004

The reaction mixtures contained 36.6 µg of crude total enzyme, purified through the ammonium sulfate precipitation. Incubation was for 20 min at 27°C.

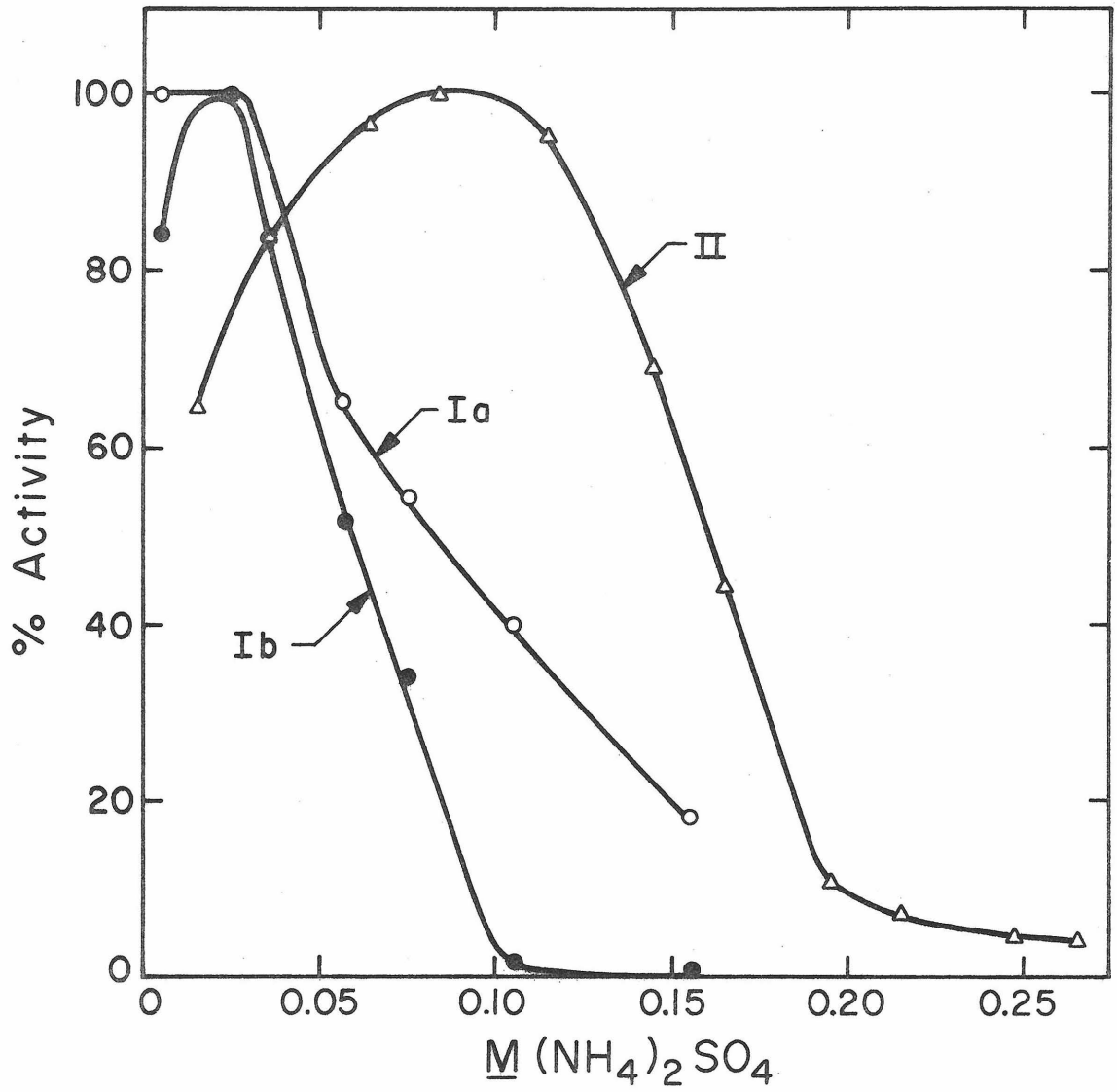
*After the incubation, the reaction mixture was heated for 5 minutes at 95°C to destroy the polymerase activity, 10λ of RNase A (5.0 mg/ml) added, and the incubation continued for 1 hr at 37°C.

‡After incubation, KOH was added to 0.25 M and the incubation continued for 18 hours at 37°C.

Fig. 1

Ionic strength optimum for Ia, Ib and II

Assays were conducted as described in MATERIALS AND METHODS, all at 10 mM $MgCl_2$ with calf thymus DNA. Enzyme concentrations were 17.5 µg/ml of Ia, 8 µg/ml of Ib, and 5.5 µg/ml of II. Incubations were for 30' at 37°C.


100
80
60
40
20
0
% Activity
0
0.05
0.10
0.15
0.20
0.25
M (NH4)2 SO4
II
Ia
Ib

about 0.1 M $(NH_4)_2SO_4$. Taking into account the other ions present in the reaction mixture, the optimum ionic strengths are about 0.10 for form I and about 0.40 for form II.

Divalent metal ion requirements

Ia and Ib show a strong preference for Mg^{++} as a divalent cation; in fact, no RNA synthesis occurs if Mn^{++} is substituted, at least under the reaction conditions used here. Polymerase II, in contrast, is active only in the presence of Mn^{++} (Table 2). The concentrations of Mg^{++} and Mn^{++} which result in optimum activity are also quite different (10 mM and 2 mM, respectively). This may indicate different roles for the two ions.

Inhibitor studies

Several inhibitors have been tested with the three polymerase activities under investigation here.

1) α-amanitin

As shown in Figure 2, polymerase II is extremely sensitive to α-amanitin, a bicyclic polypeptide isolated from the mushroom Amanita phalloides (Wieland, 1968). At a concentration of 0.01 µg/ml, II is inhibited by 70% while forms Ia and Ib are virtually unaffected at concentrations 100-fold higher. α-amanitin binds directly to the enzyme (with a binding constant of 10^{-8}) and inhibits

Table 2

Divalent cation requirements for Ia, Ib and II

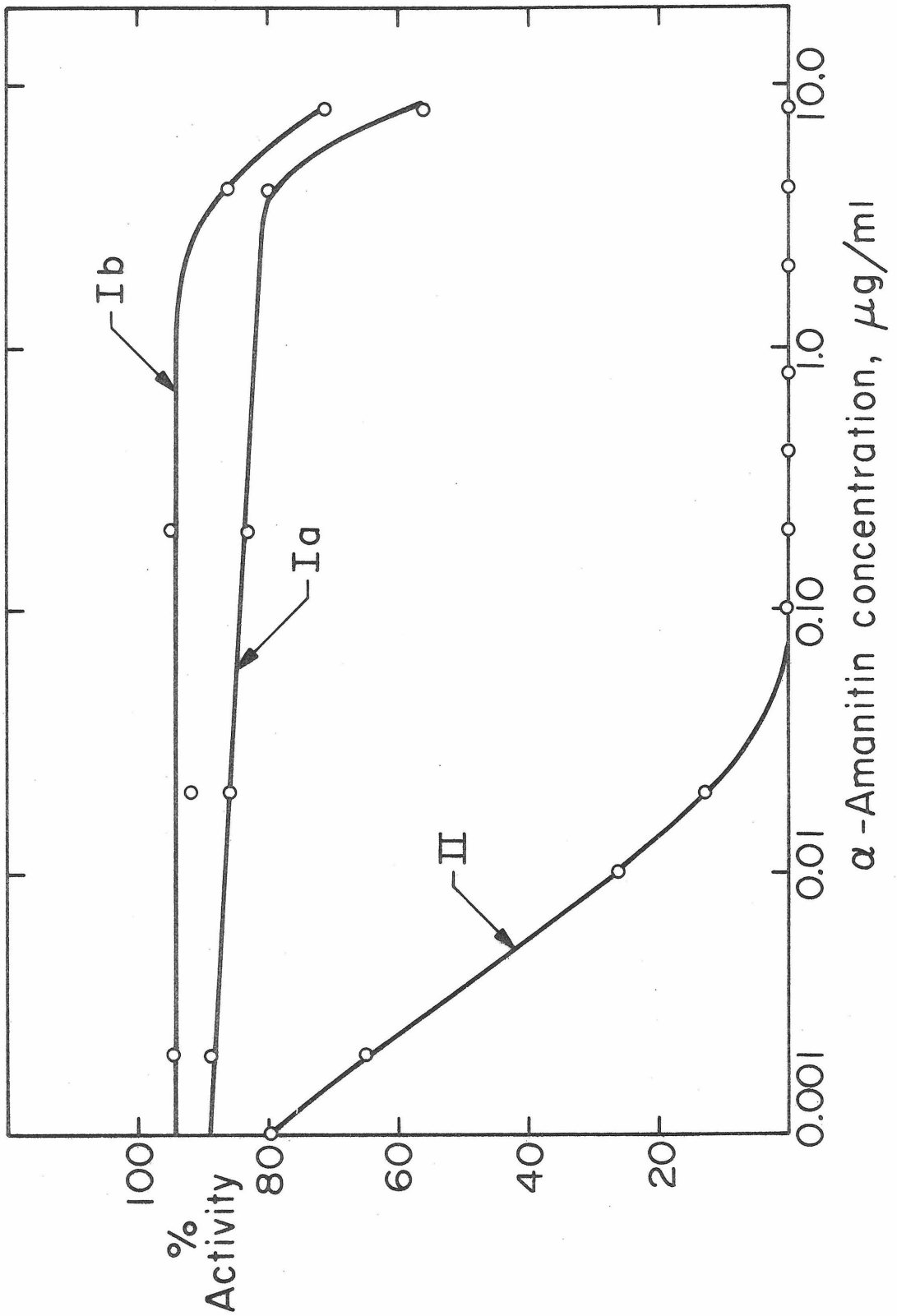
Enzyme	pmoles GMP incorporated/min/ml	
	2 mM $MnSO_4$	10 mM $MgCl_2$
Ia	0	1.02
Ib	0	1.28
II	2.00	0

Reaction mixtures containing Ia (7.0 µg/ml), Ib (6.5 µg/ml) and II (2.0 µg/ml) were incubated for 30 min at 37°. II was assayed at 0.1 M $(NH_4)_2SO_4$.

Fig. 2

<u>α-amanitin inhibition of Ia, Ib and II</u>

Ia (10 µg/ml) and Ib (5 µg/ml) were assayed at 10 mM $MgCl_2$, II (1.8 µg/ml) at 2 mM $MnSO_4$ plus 0.10 M $(NH_4)_2SO_4$. α-amanitin was dissolved in deionized H_2O. Incubations were for 30 min at 37°C.


Ib
Ia
II
100
80
60
40
20
% Activity
0.001
0.01
0.10
1.0
10.0
α-Amanitin concentration, μg/ml

the elongation step in the reaction (Chambon, et al., 1970), as does streptolidigin (Cassani et al., 1970).

2) Rifamycin

Rifamycin has been useful in the study of prokaryotic RNA polymerase because it binds directly to the enzyme and inhibits initiation of RNA synthesis (diMauro et al., 1969). Many derivatives of rifamycin exist and one of these (AF/0-13) has been reported to inhibit polymerase II though polymerase I was apparently not tested (Butterworth, et al., 1971). Fig. 3 shows the effect of rifamycin AF/0-13 on the RNA polymerases of ascites tumor. All three forms are extremely sensitive to this rifamycin derivative, while rifamycin SV, which inhibits bacterial RNA polymerase, has very little effect, at much higher concentrations. Even at 64 μM, rifamycin SV shows only about 25% inhibition.

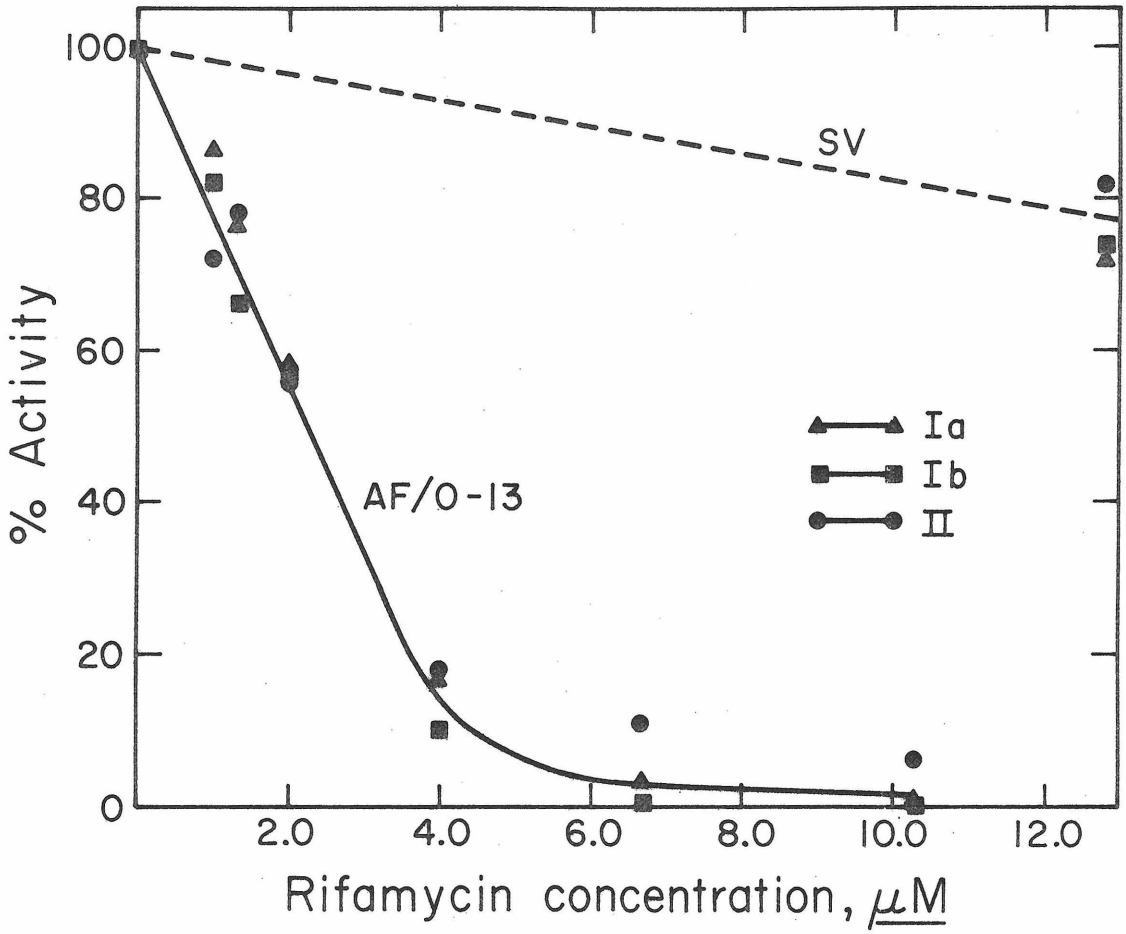
3) Cycloheximide

There have been reports that polymerase I from two aquatic fungi (Blastocladiella emersonii and Achlya bisexualis) is inhibited by low concentrations of cycloheximide (Horgen and Griffin, 1971; Timberlake, et al., 1972). This is interesting in view of the inhibition of ribosomal RNA synthesis in vivo in HeLa cells by cycloheximide (Muramatsu, et al., 1970). However, cycloheximide has no effect on either Ia or Ib from ascites tumor at

Fig. 3

Rifamycin inhibition of Ia, Ib and II

Ia (20 μg/ml) and Ib (5 μg/ml) were assayed at 10 mM $MgCl_2$; II (1 μg/ml) was assayed at 2 mM $MnSO_4$ plus 0.10 M $(NH_4)_2SO_4$. Rifamycin SV was dissolved in 95% ethanol at 10 mg/ml and rifamycin AF/0-13 was dissolved at 35 mg/ml in dimethyl formamide. The organic solvents were included in the control (no rifamycin) and had no effect on the activity.


100
80
60
40
20
0
% Activity
SV
AF/O-13
Ia
Ib
II
2.0
4.0
6.0
8.0
10.0
12.0
Rifamycin concentration, μM

concentrations up to 10 μg/ml (data not shown).

Template requirements

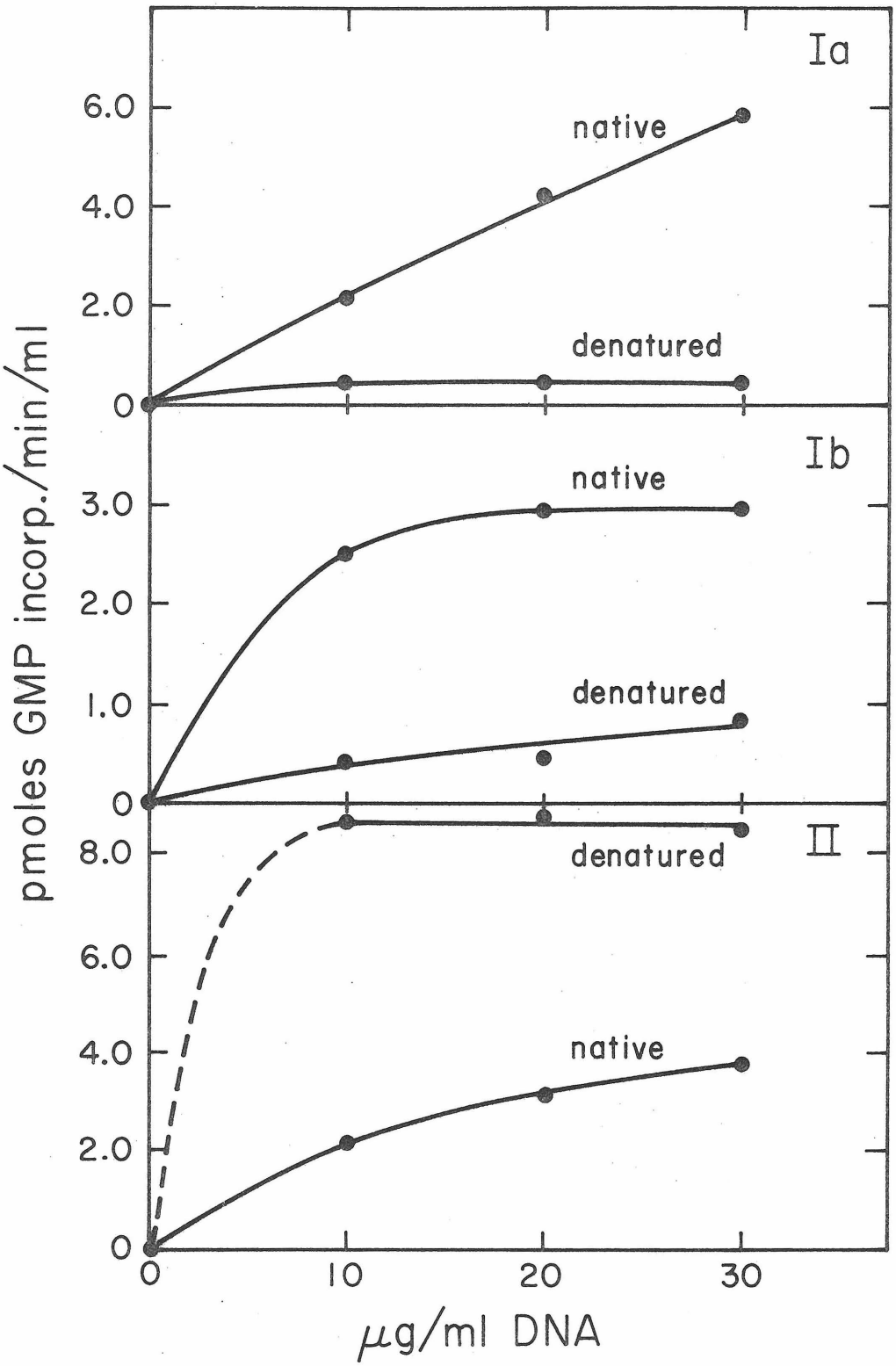
All three of the RNA polymerases studied here are dependent on a template for activity (Fig. 4). Form II not only shows a higher rate of RNA synthesis on denatured template but also has a much higher affinity for single-stranded DNA. Polymerases Ia and Ib, in contrast, prefer double-stranded DNA, using denatured DNA as a template relatively poorly. Ib uses denatured template several fold better than Ia.

In connection with these results, it is important to consider the findings of Hausen and Stein (1970). A novel ribonuclease (RNase H or hybridase), which degrades only RNA which is part of an RNA:DNA hybrid, was discovered in calf thymus and shown to contaminate crude preparations of RNA polymerase. If denatured DNA is used as a template, the resultant RNA synthesized remains hybridized to the template (Bishop, 1969; Hayashi et al., 1965; Sinsheimer and Lawrence, 1964), providing a suitable template for the hybridase, and resulting in very little net synthesis on denatured DNA. The RNA synthesized on native DNA, on the other hand, remains unhybridized and thus is not susceptible to degradation by the hybridase. Thus, in the presence of RNase H, RNA polymerase may appear to prefer native DNA as a template simply because the product

Fig. 4

Activity of Ia, Ib and II on native and denatured rat DNA

Ia (20 μg/ml) and Ib (5 μg/ml) were assayed at 10 mM $MgCl_2$; II (1.0 μg/ml) was assayed at 2 mM $MnSO_4$ plus 0.10 M $(NH_4)_2SO_4$. Rat DNA was denatured by heating at 95°C for 5 min and then cooled in ice. Incubations were for 30 min at 37°C.


Ia
native
denatured
Ib
native
denatured
II
denatured
native
6.0
4.0
2.0
0
3.0
2.0
1.0
0
8.0
6.0
4.0
2.0
0
0
10
20
30
pmoles GMP incorp./min/ml
μg/ml DNA

synthesized on denatured DNA is rapidly destroyed. All three enzymes from ascites tumor were tested for hybridase activity, using a poly dT:H^3 poly rA synthetic hybrid, and found to contain none (Table 3). It appears that the ability of Ia and Ib to use native templates is a property of the polymerases and not due to contaminating enzymes.

Chromatin has been shown to be a restricted template (relative to DNA) for the in vitro synthesis of RNA using E. coli RNA polymerase (Bonner et al., 1968; Georgiev, G.P., 1969; Smith et al., 1969). Some problems, due to the use of this heterologous system (mammalian chromatin and bacterial polymerase) have become apparent (Dahmus, 1969), although there is some evidence that the RNA transcribed from the middle-repetitive DNA (rat liver chromatin) is the same when either mammalian or bacterial polymerase is used (Smith et al., 1969). The use of mammalian RNA polymerases may yield insight into problems involved with this type of study (see Chapt. I). Figure 5 shows the transcription of rat DNA and rat liver chromatin by polymerases Ia and Ib. Both have extremely limited activity on chromatin relative to DNA, although chromatin has about twice the template activity with Ib (about 5% of DNA) as with Ia (2-2.5% of DNA). It is difficult to determine the K_m for the chromatin since the activity on chromatin is so low. However, for DNA, the

Table 3

RNase activity of Ia, Ib and II

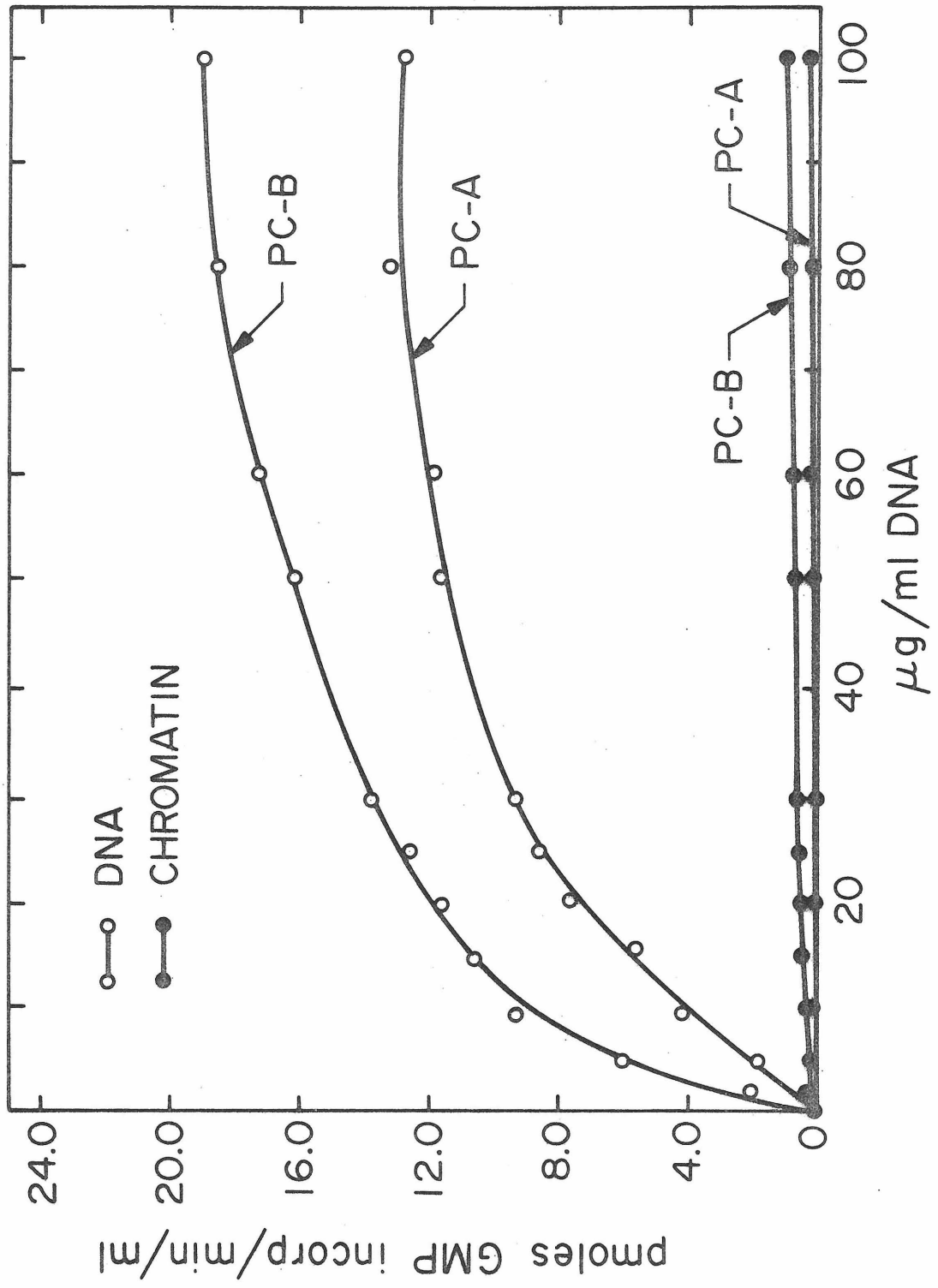
Additions	Substrate		
	H^3 poly A (cpm)	poly dT:H^3 poly A (cpm)	H^3 RNA (cpm)
None	3050	3170	12,507
Ia	3065	3089	12,491
Ib	3043	3143	13,070
II	3048	3028	11,731

H^3 poly A and poly dT:H^3 poly A hybrid were prepared as described in MATERIALS AND METHODS. H^3 RNA (~80,000 cpm/μg) was synthesized *in vivo* with *E. coli* RNA polymerase on native rat liver DNA and subsequently purified (gift of Maurice Dupras) 50 λ yielded 12,208 cpm (precipitable with TCA). 10 λ of H^3 poly A or poly dT:H^3 poly A hybrid or 50 λ H^3 RNA was incubated with Ia (20 μg/ml), Ib (5 μg/ml) or II (1 μg/ml) in the standard assay mix (H^3 GTP was replaced with unlabeled nucleotide) for 30' at 37°C. The samples were then processed as described in MATERIALS AND METHODS for RNA synthesis assays.

Fig. 5

Template activity of chromatin with PC-A and PC-B

PC-A (57 μg/ml) and PC-B (43 μg/ml) were assayed at 5 mM $MgCl_2$ with both chromatin and DNA in the presence of 1 μg/ml α-amanitin. Incuations were for 30 min at 37°C.


pmoles GMP incorp/min/ml
24.0
20.0
16.0
12.0
8.0
4.0
0
20
40
60
80
100
μg/ml DNA
DNA
CHROMATIN
PC-B
PC-A
PC-B
PC-A

K_m with polymerase Ib appears to be lower than with Ia. The template activity of rat liver chromatin using polymerase II from rat liver is about 20% that of DNA (H. van den Broek, personal communication). Approximately the same value is obtained with E. coli RNA polymerase (Bonner et al., 1968).

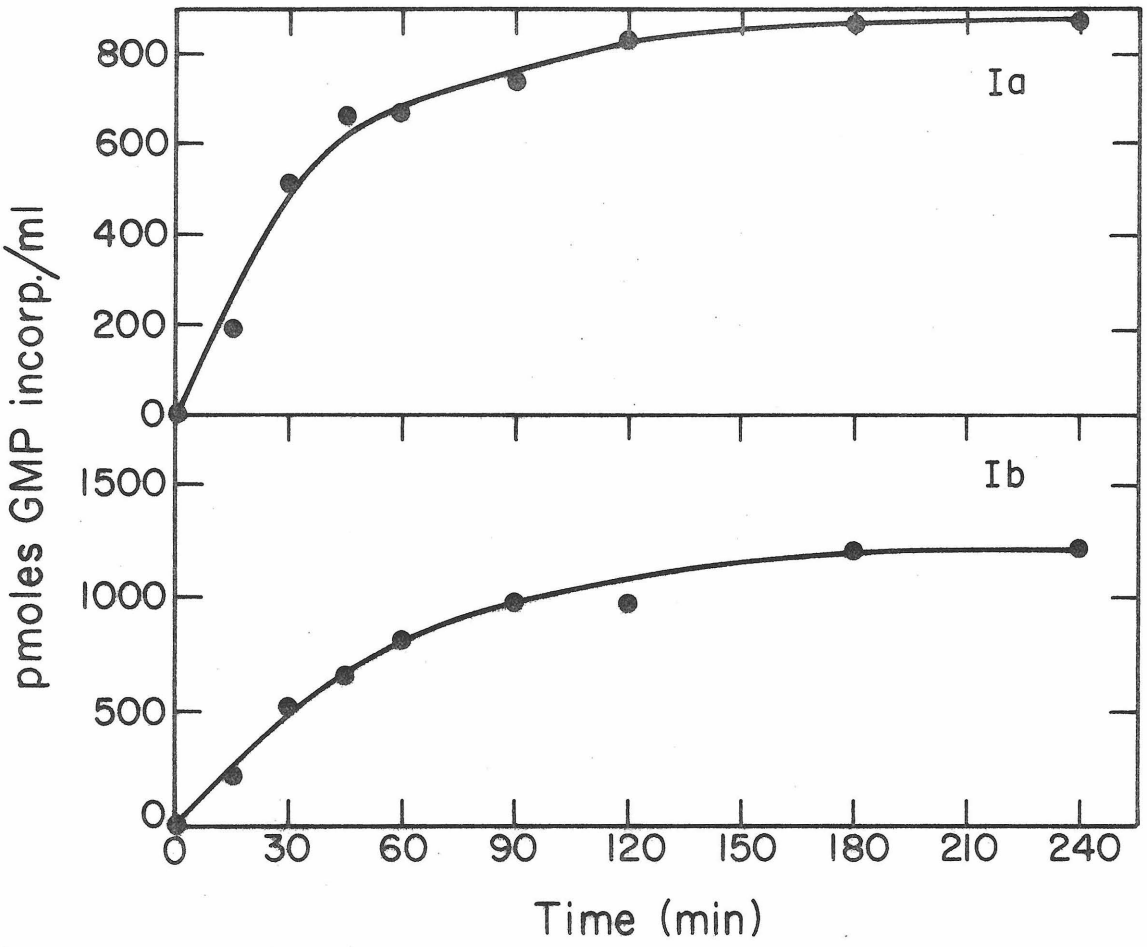
Kinetics of the reaction

The time course of RNA synthesis by forms Ia and Ib is shown in Figure 6. Both show linear rates of synthesis for at least 30 min and continue for at least 1 hour. In contrast, E. coli RNA polymerase, when assayed at an ionic strength comparable to the one used here, stops synthesis after about 10 min. (McConnell, 1970), while at higher salt concentrations, the enzyme is capable of reinitiation and further synthesis. The kinetics displayed by Ia and Ib at low ionic strength indicate that both of these mammalian enzymes are capable of reinitiation. The cessation of synthesis is probably due to inactivation of the enzyme at 37°C, since none of the substrates have been depleted, although product inhibition is also possible. The initial rate of synthesis can be doubled by increasing the GTP to 1 mM; a concentration of 0.1 mM has been used to increase the sensitivity of the assay, since the specific radioactivity of the H^3 GTP can be increased 10-fold. (The increase in sensitivity is 5-fold). None of

Fig. 6

Kinetics of RNA synthesis by Ia and Ib

Ia (17.5 μg/ml) and Ib (16.5 μg/ml) were incubated with 10 mM $MgCl_2$ and rat liver DNA (100 μg/ml) at 37°C.


pmoles GMP incorp./ml
800
600
400
200
0
Ia
1500
1000
500
0
Ib
0
30
60
90
120
150
180
210
240
Time (min)

the results presented here, other than the initial rate of synthesis, is affected by lowering the GTP concentration from 1 mM to 0.1 mM.

After synthesis of RNA ceases, there is no indication of a decrease in the amount of RNA present upon further incubation (Fig. 6). Thus, there appears to be no contamination by RNase in either the reaction components or the enzymes. This conclusion is substantiated by incubation of labeled RNA with the enzymes (Table 3).

DISCUSSION

The RNA polymerase activities isolated from ascites tumor cells by the method described in Chapt. II appear to be DNA-dependent RNA-synthesizing enzymes. The product is totally sensitive to KOH hydrolysis, and destroyed to a large extent by ribonuclease treatment. The activity is not inhibited by inorganic phosphate, ruling out the presence of polynucleotide phosphorylase.

The results presented here concerning the enzymological characteristics of polymerases Ia, Ib, and II from ascites tumor support the conclusion drawn earlier that Ia and Ib are localized in the nucleolus, while form II is nucleoplasmic. Roeder and Rutter (1969) first showed that form I is nucleolar by isolation of RNA polymerase from purified nucleoli and then chromatography on DEAE Sephadex. Essentially all of the activity elutes between 0.10-0.15 M $(NH_4)_2SO_4$, which is defined as polymerase I. Both Ia and Ib from ascites tumor elute from DEAE cellulose between 0.11-0.15 M $(NH_4)_2SO_4$. The following information is also consistent with Ia and Ib being comparable to the nucleolar enzyme of Roeder and Rutter (1969).

1) Ia and Ib show optimum activity at low ionic strength, while II exhibits maximal incorporation at 0.10 M $(NH_4)_2SO_4$.

2) II prefers Mn^{++} as the divalent cation while Ia and Ib work best with Mg^{++}.

3) II is extremely sensitive to α-amanitin while Ia and Ib are inhibited only at extremely high concentrations.

The inhibition of all three ascites tumor polymerases by rifamycin AF/0-13 brings up several interesting points. If rifamycin binds to the enzyme, as is the case with the bacterial RNA polymerase, then all three mammalian enzymes must have some common site(in the 190,000 dalton subunit?) since they exhibit approximately the same affinity for the inhibitor. This is in contrast to α-amanitin which only binds to and inhibits form II. Secondly, rifamycin SV strongly inhibits the bacterial enzyme but has no effect on mammalian RNA polymerase. The difference in structure of rifamycin SV and AF/0-13 is quite small: AF/0-13 is 3-formyl rifamycin SV:O-n-octyloxime. This may indicate that the active site of rifamycin is the aromatic ring (for more information on the active site, see Green et al., 1972). Also, the lack of significant inhibition of the ascites polymerase by rifamycin SV reduces the possibility that any of the three enzymes are bacterial in origin (from ascites cell contaminated with bacteria, for example). Finally, polymerase II is not totally inhibited by rifamycin AF/0-13 at concentrations up to 10 μM, while forms Ia and

Ib show no activity at this concentration. This could indicate the existence of a small number of very stable rifamycin-resistant initiation complexes which may represent enzyme molecules which have initiated correctly, i.e., at specific initiation sites (Dausse et al., 1972). Since polymerase II prefers single-stranded templates, it is likely that transcription in vitro results from non-specific initiation because mammalian DNA contains nicks and single-stranded regions. Preincubation of polymerase II with DNA prior to the simultaneous addition of nucleotide triphosphates and rifamycin AF/0-13 may lead to the selection of specific initiation sites and assymetric transcription of the template. Such experiments have been conducted with T_7 DNA and E. coli RNA polymerase (Dausse et al., 1972). It would also be of interest to compare the number of rifamycin AF/0-13 resistant initiations on DNA and chromatin with polymerase II since it is possible that initiation is more specific on chromatin.

Since the ascites tumor enzyme preparations used here are free of ribonuclease H activity, the preference of Ia and Ib for native templates may indicate that these enzymes are capable of specific initiation, given the correct conditions and template. It is noteworthy, in this regard, that both Ia and Ib use chromatin as a template but only to a very limited extent as compared to deproteinized DNA.

The percentage of the DNA in the chromatin preparation used here which is nucleolar in origin is probably very small. Therefore, if these enzymes are able to recognize *in vitro* only those transcription sites utilized *in vivo*, then a very low template activity would be expected. Again, it would be of interest to determine the number of rifamycin-resistant initiations under these conditions.

Several other points of interest concerning the chromatin transcription are worthy of discussion. First, the activity of Ia and Ib on chromatin does not result from contaminating form II polymerase in the enzyme preparation or endogenous to the chromatin (Butterworth *et al.*, 1971) since sufficient α-amanitin (1 ug/ml) is included to completely inhibit polymerase II. Secondly, these results are in direct contrast to the report by Butterworth *et al.* (1971) that one form I polymerase does not use chromatin as a template. It is possible that their enzyme preparation lacks some factor necessary for chromatin transcription since the ascites tumor enzymes used in this experiment have been purified through the phosphocellulose chromatography step only. Also polymerase Ib transcribes chromatin more than twice as effectively as Ia. This is essentially the only enzymological distinction found between these two enzymes, though they differ in their chromatographic properties and molecular structure. The properties of the RNA produced

by these two enzymes using a chromatin template deserves further investigation. Finally, it is clear that the bulk of the transcription of chromatin *in vitro* is carried out by polymerase II. Butterworth *et al.* (1971) showed that the RNA polymerase endogenous to chromatin is α-amanitin-sensitive and when isolated from the chromatin, chromatographs on DEAE cellulose as expected of form II. The data presented here indicating that the template activity of chromatin with Ia and Ib is 2.5% and 5%, respectively, while with form II from rat liver, a value of about 20% is obtained (Henk van den Broek, personal communication) are consistent with this idea.

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CHAPTER IV

STIMULATION OF NUCLEOLAR RNA POLYMERASES BY A HEAT-SENSITIVE FACTOR

INTRODUCTION

An important subunit of *E. coli* RNA polymerase, sigma, is lost if phosphocellulose chromatography is included in the purification of the enzyme (Burgess *et al.*, 1969). This subunit has been reported to stimulate initiation on some templates, to mediate selection of the correct strand for transcription *in vitro*, and to select the initiation site on the DNA (Sugiura *et al.*, 1970). Thus, the investigation of transcription *in vitro* requires a preparation of *E. coli* RNA polymerase which contains the sigma subunit. Although earlier experiments implicating the sigma factor directly in the control of gene expression have not been substantiated (Summers and Siegel, 1969; Travers, 1970), other protein factors may be involved in this process (Stevens, 1972).

There is no evidence at the present that eukaryotic RNA polymerase *per se* is involved directly in the control of gene expression. One approach to the investigation of this possiblity is to look for factors which affect the synthesis of RNA *in vitro*, either by increasing (or decreasing) the rate of synthesis or by altering the type of RNA produced. Polymerase II has been shown to prefer denatured DNA as a template (Roeder and Rutter, 1969), suggesting that some component necessary for transcription as it occurs *in vivo* (presumably on native DNA) is lost

during the purification. It has been demonstrated that addition of the protein present in run-off fractions of the DEAE cellulose chromatography of calf thymus RNA polymerase greatly increases the activity of the form II enzyme. The "factor" stimulates only on native DNA and is sensitive to proteolytic enzymes (Stein and Hausen, 1970). A similar factor has been isolated from rat liver and has many of the same properties as that of the calf thymus factor (Seifart, 1970). In both cases only polymerase II is affected by the factor; form I activity is not increased upon addition of the factor. The active agent is probably a protein of approximately 70,000 molecular weight (at least, for rat liver) (Seifart, 1970).

Polymerases Ia and Ib from Novikoff hepatoma ascites tumor, although extensively purified, have low specific activity (Chapt. II). However, upon addition of proteins which do not bind to DEAE cellulose during the enzyme purification, the activity of both enzymes is greatly stimulated. Thus, it appears that a stimulating factor, similar to that isolated from calf thymus and rat liver, is also present in ascites tumor and, contrary to other reports, stimulates the activity of the nucleolar enzymes, Ia and Ib.

MATERIALS AND METHODS

Enzyme purification

Polymerases Ia, Ib, and II from Novikoff hepatoma ascites tumor are prepared as described in Chapt. II.

Factor preparation

The run-off fractions of the DEAE cellulose chromatography of PC-A and PC-B enzymes are used as the source of the factor. The fractions are pooled and precipitated by the addition of 1½ volumes of saturated $(NH_4)_2SO_4$, pH 7.9 (4°C). After precipitation overnight at 4°C, the precipitate is collected by centrifugation, dissolved in approximately 5 ml of TGMED + 0.05 M $(NH_4)_2SO_4$ and dialyzed against 100 volumes of TGMED + 0.05 M $(NH_4)_2SO_4$ for 5-6 hours. The sample is then subjected to another pass through the DEAE cellulose column (Chapt. II) to insure that all RNA polymerase activity has been removed. The run-off fractions are again precipitated with $(NH_4)_2SO_4$, redissolved, dialyzed as described above, and stored at -90°C in TGMED +0.05 M $(NH_4)_2SO_4$.

Assays

The assays for RNA polymerase activity and for RNase H activity were conducted as described in Chapt. III.

Isolation of DNA from reaction mixture

For the experiment shown in Fig. 4, two-1.0 ml reaction mixtures, containing 200 μg/ml rat DNA, were incubated for 45 min at 37°C. One contained Ia (5 μg/ml) while the other contained Ia (5 μg/ml) plus $RO_{Ia\text{-}II}$ (69 μg/ml). After the incubation, 20 λ of 20% SDS plus 110 λ 10M LiCl were added and the solution extracted with 1.0 ml phenol. After separation of the phases by centrifugation and removal of the aqueous phase, the phenol phase was re-extracted with 0.5 ml 0.01 M Tris, pH 7.9, and the two aqueous phases combined. The extraction procedure was then repeated. Two volumes of 95% ethanol were added to the final aqueous phase and the precipitated DNA wound out on a small glass rod. After redissolution in 200 λ 0.01 M Tris, pH 7.9, the DNA samples were dialyzed against 250ml 0.01 M Tris, pH 7.9. The spectra of the DNA samples were typical of rat liver DNA (A_{280}/A_{260} = .52; A_{230}/A_{260} = .47). Approximately 45% of the original DNA in the reaction mixture was recovered.

RESULTS

The rate of RNA synthesis by the ascites RNA polymerases as a function of the enzyme concentration has been determined. The results for both Ia (Fig. 1) and Ib (Fig. 2) are not as expected for a typical enzyme; the rate of synthesis does not increase linearly with enzyme concentration but shows a very pronounced concave shape. In fact, at low concentrations of enzyme (less that 4 μg/ml for Ia and 3 μg/ml for Ib) no detectable synthesis of RNA occurs. At higher concentrations the activity appears to be linear with enzyme concentration. The lack of activity of Ia and Ib is not due to denaturation of the enzyme at very low concentration since the presence of BSA (final concentration of 80 μg/ml) has no effect on the curve. In contrast, polymerase II exhibits an almost linear activity vs. concentration curve, with only a slight deviation from linearity at low concentration of enzyme (less than 0.5 μg/ml) (data not shown). Thus the effect is probably not due to lack of sensitivity or some artifactual problem with the assay.

If the run-off protein from the DEAE cellulose chromatography (the run-off from the chromatography of PC-A will be designated RO_{Ia-II} while that of PC-B will be referred to as RO_{Ib}) is added to the reaction mixtures (at a constant amount), the activity vs. enzyme

Fig. 1

Effect of RO_{Ia-II} on the activity of Ia at different enzyme concentrations

Reaction mixtures (0.25ml) contained 10 mM $MgCl_2$, 200 µg/ml rat DNA, Ia at the indicated concentrations, plus RO_{Ia-II} (140 ug/ml) where indicated. BSA (80 µg/ml) was included if RO_{Ia-II} was not present. Rates of synthesis were determined by removing 50 λ aliquots at 0, 15, 30 and 45 min. for analysis by the DEAE filter paper method.

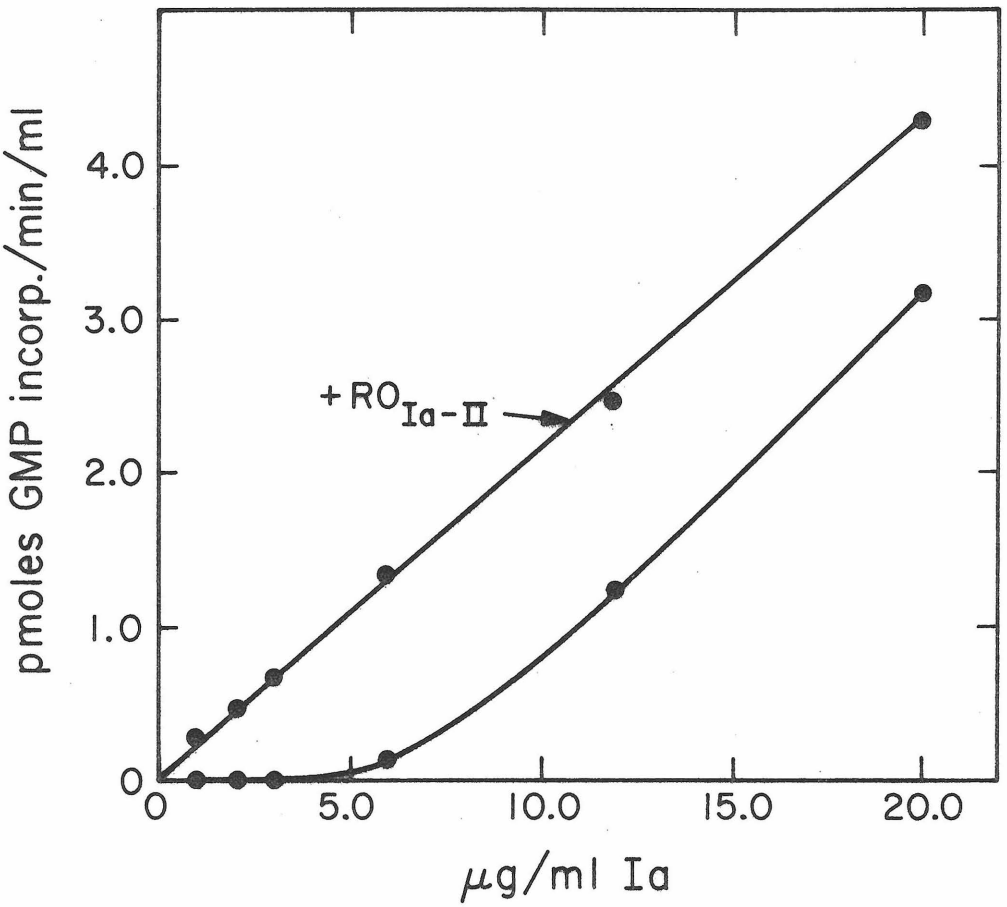
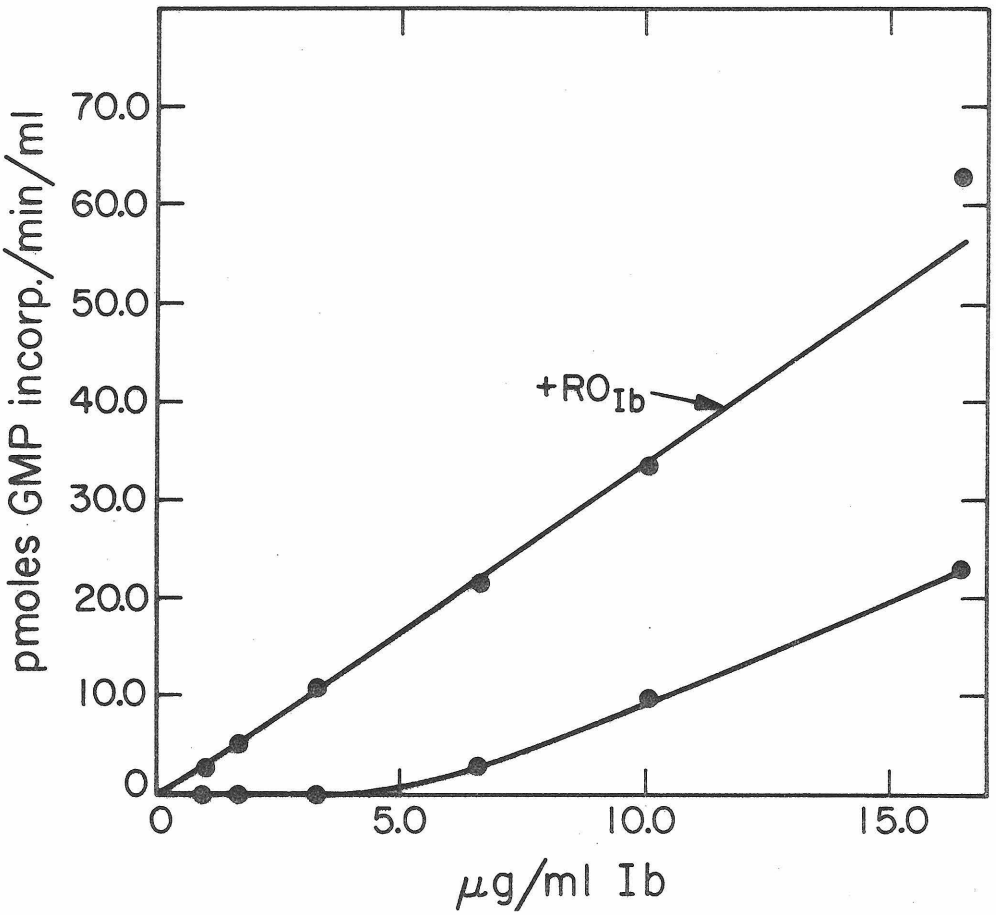

pmoles GMP incorp./min/ml
4.0
3.0
2.0
1.0
0
+ RO Ia-II
0
5.0
10.0
15.0
20.0
μg/ml Ia

Fig. 2

Effect of RO_{Ib} on the activity of Ib at different enzyme concentrations

The procedure was identical to that of Figure 1. Where indicated, RO_{Ib} was included at 129 µg/ml.


70.0
60.0
50.0
40.0
30.0
20.0
10.0
0
pmoles GMP incorp./min/ml
+RO Ib
0
5.0
10.0
15.0
μg/ml Ib

concentration curves are then linear (Figs. 1 and 2), at least over the enzyme concentration ranges tested. Thus, at low enzyme concentrations, RO_{Ia-II} and RO_{Ib} greatly increase the rates of RNA synthesis by Ia and Ib, respectively. In fact, at very low concentrations of enzyme, Ia and Ib show an absolute requirement for the factor for activity. As the enzyme concentration is increased, the stimulation decreases until at 20 μg/ml Ia, the stimulation is only 30%. This low amount of stimulation at high concentrations is probably not due to a limiting amount of RO_{Ia-II} as shown by the saturation curve of Ia with RO_{Ia-II} (Fig. 3). Ib is stimulated several-fold by RO_{Ib} at relatively high enzyme concentrations, although not as much as at low concentrations.

It has been reported that the stimulating factor from calf thymus is stable to heating (5 min at 80°C has little effect on the activity while 15 min at 100°C results in less than 50% decrease in the activity) (Stein and Hausen, 1970). However, after some purification, the factor is heat labile (W.J. Rutter, personal communication). If RO_{Ia-II} from ascites tumor is subjected to 85°C for 5 minutes, 90% of the activity is lost (Table 1). (Note also that RO_{Ia-II} alone has no RNA polymerase activity.)

The activity of E. coli core RNA polymerase can be increased by alterations in the DNA. Introduction of single-stranded nicks into the template, for instance,

Fig. 3

Reaction mixtures (0.25 ml) contained 10 mM $MgCl_2$ and 200 µg/ml rat DNA. Rates of synthesis were determined as in Fig. 1.

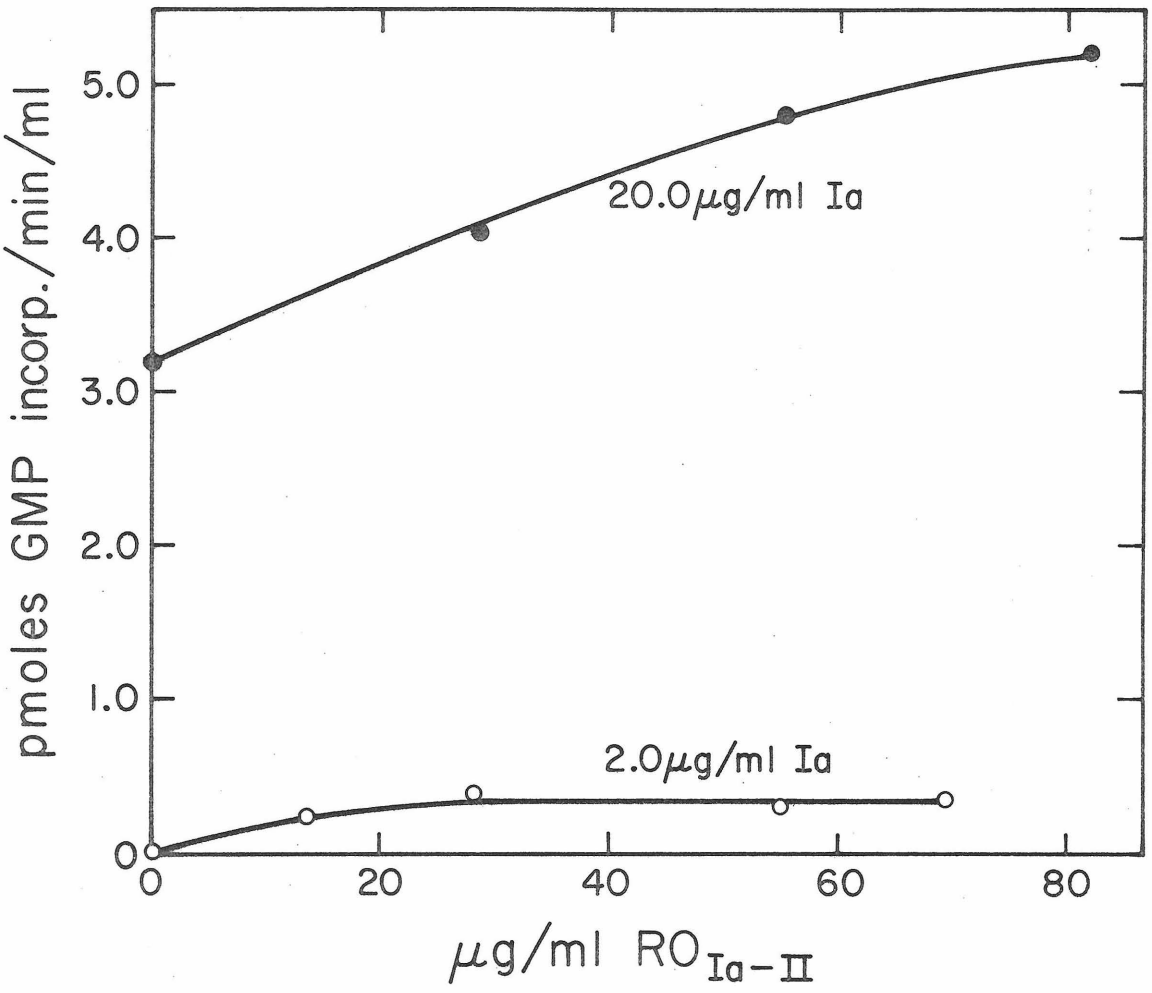

pmoles GMP incorp./min/ml
5.0
4.0
3.0
2.0
1.0
0
20.0μg/ml Ia
2.0μg/ml Ia
0
20
40
60
80
μg/ml RO Ia-II

Table 1

Heat lability of RO_{Ia-II}

Additions	Rate of RNA synthesis (pmoles GMP incorp./min/ml)
Ia	0
RO_{Ia-II}	0
Ia + RO_{Ia-II}	3.34
Ia + RO_{Ia-II}(heated)	0.33

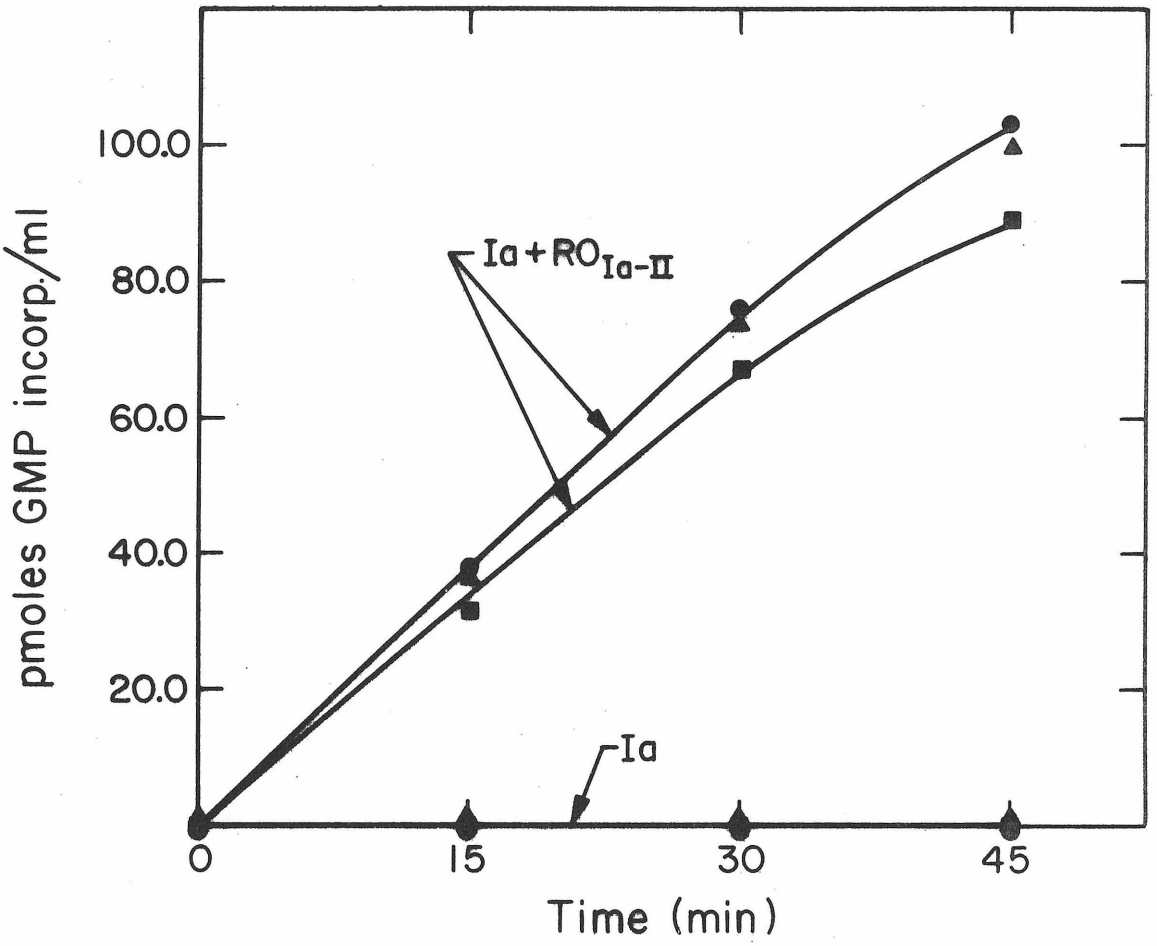
Reaction mixtures contained 2 µg/ml Ia and 69 µg/ml RO_{Ia-II}. Where indicated RO_{Ia-II} was heated for 5 min at 85°C, cooled and centrifuged to remove precipitated protein.

produces artifactual initiation sites for the polymerase, thus increasing the RNA synthesizing activity (Vogt, 1969). Therefore, one explanation for the stimulation of Ia by RO_{Ia-II} might be that the latter alters the template to make it more available for transcription. To investigate this possibility, two reaction mixtures were prepared, one containing Ia and the other Ia plus RO_{Ia-II}. After incubation as usual, the DNA from these reactions was isolated by phenol extraction and then used again in identical reaction mixtures. As shown in Fig. 4, the DNA is not permanently "activated" by RO_{Ia-II} for transcription. In fact, the amount of stimulation of Ia by RO_{Ia-II} is the same on either of the re-isolated DNA preparations or on DNA which has not been subjected to pre-incubation and re-isolation. It is possible, of course that the DNA is nicked and then subsequently repaired by contaminating ligase or that the "activated" DNA has been selectively lost during the isolation from the reaction mixture.

The factors isolated from calf thymus (Stein and Hausen, 1970) and from rat liver (Seifart, 1970) stimulate the activity of polymerase II on native DNA only. This property has also been investigated for RO_{Ia-II} and RO_{Ib} from ascites tumor. Since mammalian DNA contains highly repetitive sequences which reanneal almost

Fig. 4

Reaction mixtures contained Ia (5 μg/ml), plus RO_{Ia-II} (69 μg/ml) where indicated. Three DNA samples (90μg/ml) were used: (1) DNA isolated from a reaction mixture which contained Ia (5 μg/ml) (■——■); (2) DNA isolated from a reaction mixture which contained Ia (5 μg/ml) plus RO_{Ia-II} (69 μg/ml) (●——●); (3) DNA which had undergone no previous treatment (▲——▲). Reaction rates were determined as described in Fig. 1.


pmoles GMP incorp./ml
100.0
80.0
60.0
40.0
20.0
Ia + RO Ia-II
Ia
0
15
30
45
Time (min)

instantaneously (Britten and Smith, 1969), rat DNA is not a suitable template for this investigation. Any stimulation on "denatured" DNA could be due to the selective utilization of renatured, double-stranded regions of template. Therefore, ØX174 RF (double stranded) and SS (single-stranded) DNA was used (Fig. 5). Previous results which indicated that Ia and Ib prefer native DNA, while II uses denatured DNA are confirmed; Ia and Ib both show a higher rate of RNA synthesis with ØX174 RF DNA while II prefers ØX174 SS DNA. RO_{Ia-II} stimulates synthesis by Ia and II only on RF DNA, as does RO_{Ib} with Ib. Not only does RO_{Ia-II} not stimulate synthesis on SS DNA, the activity of Ia and II alone on SS DNA is completely inhibited by the addition of RO_{Ia-II}. RO_{Ib} slightly inhibits the activity of Ib on SS DNA. This suggests that the factor may limit the initiation of the enzymes to certain sites. However, another explanation is possible. If RNase H (Hausen and Stein, 1970) is contaminating RO_{Ia-II}, then any synthesis which occurs on SS DNA will remain as a DNA-RNA hybrid and be degraded by the hybridase.

RNase H activity of RO_{Ia-II} and RO_{Ib} was assayed and the results are shown in Table 2. Both RO_{Ia-II} and RO_{Ib} contains significant amounts of RNase H as demonstrated by their ability to degrade H^3 poly rA which is in the form of a hybrid with poly dT. Though H^3 poly rA alone is not degraded, this is not an indication of the absence of

Fig. 5

Reaction mixtures contained 10 mM $MgCl_2$ for Ia (10 µg/ml) or Ib (6.6 µg/ml). II was incubated at 2 mM $MnSO_4$ plus 0.10 M $(NH_4)_2SO_4$ at 1 µg/ml. The DNA concentration (for both RF and SS) was 60 µg/ml. The ØX-174 RF DNA was at least 80% form II (nicked circles). RO_{Ia-II} (140 µg/ml) and RO_{Ib} (129 µg/ml) were included where indicated.

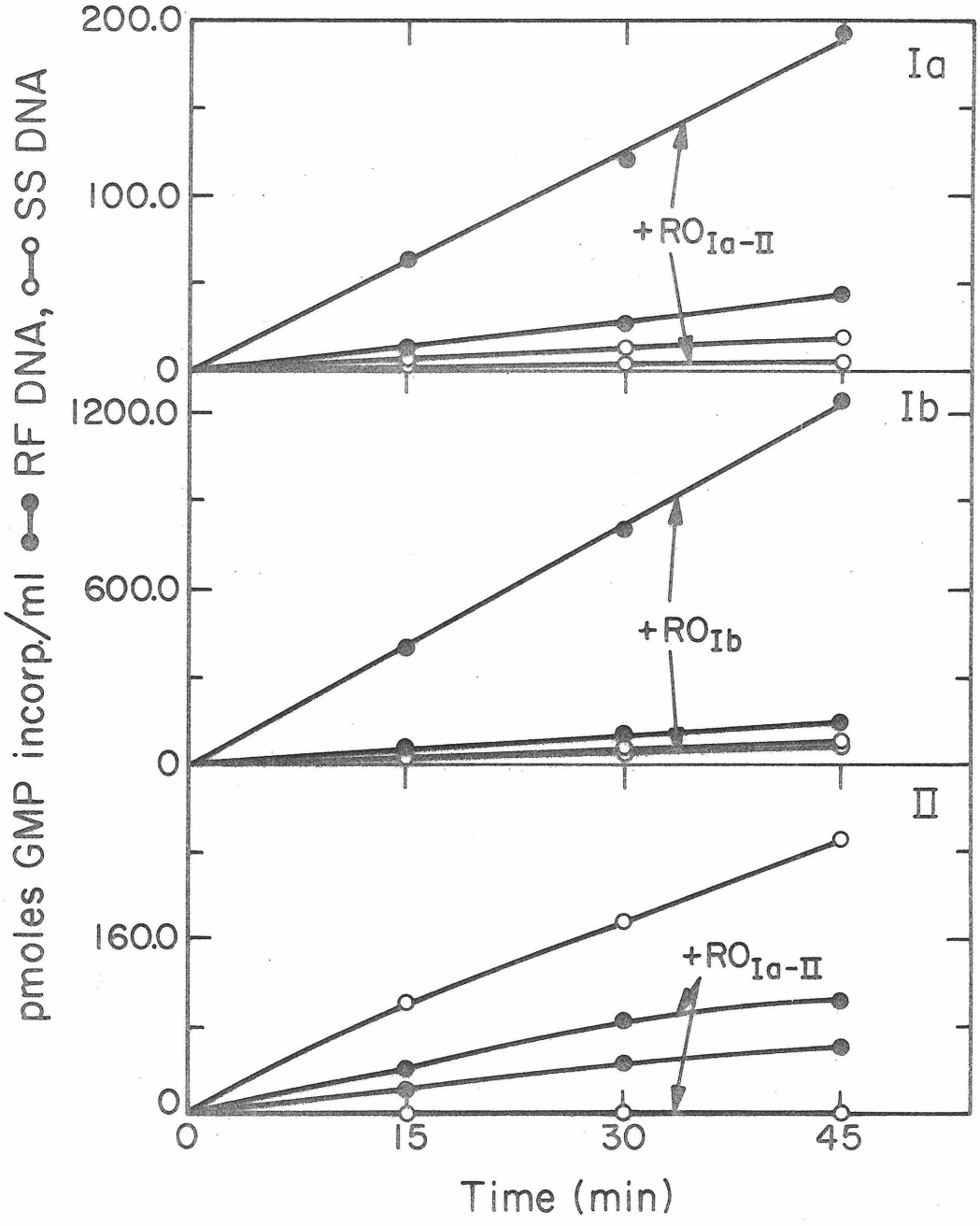

200.0
100.0
0
Ia
+RO Ia-II
1200.0
600.0
0
Ib
+RO Ib
II
160.0
0
+RO Ia-II
0
15
30
45
Time (min)
pmoles GMP incorp./ml ●—● RF DNA, ○—○ SS DNA

Table 2

RNase H activity of RO_{Ia-II} and RO_{Ib}

Additions	Substrate	
	poly dT:H^3poly rA (cpm)	H^3poly rA (cpm)
None	3332	2949
RO_{Ia-II}	379	2700
RO_{Ib}	1009	2928

Assays were carried out as previously described (Ch.III) with either 35 µg/ml RO_{Ia-II} or 65 µg/ml RO_{Ib}. Incubations were for 45 min. at 37°C.

other types of RNase since poly A is insensitive to RNase digestion at the salt concentration used here (Darnell, et al., 1971). Stein and Hausen (1970) and Seifart (1970) did not report the RNase H activity of their factor preparations.

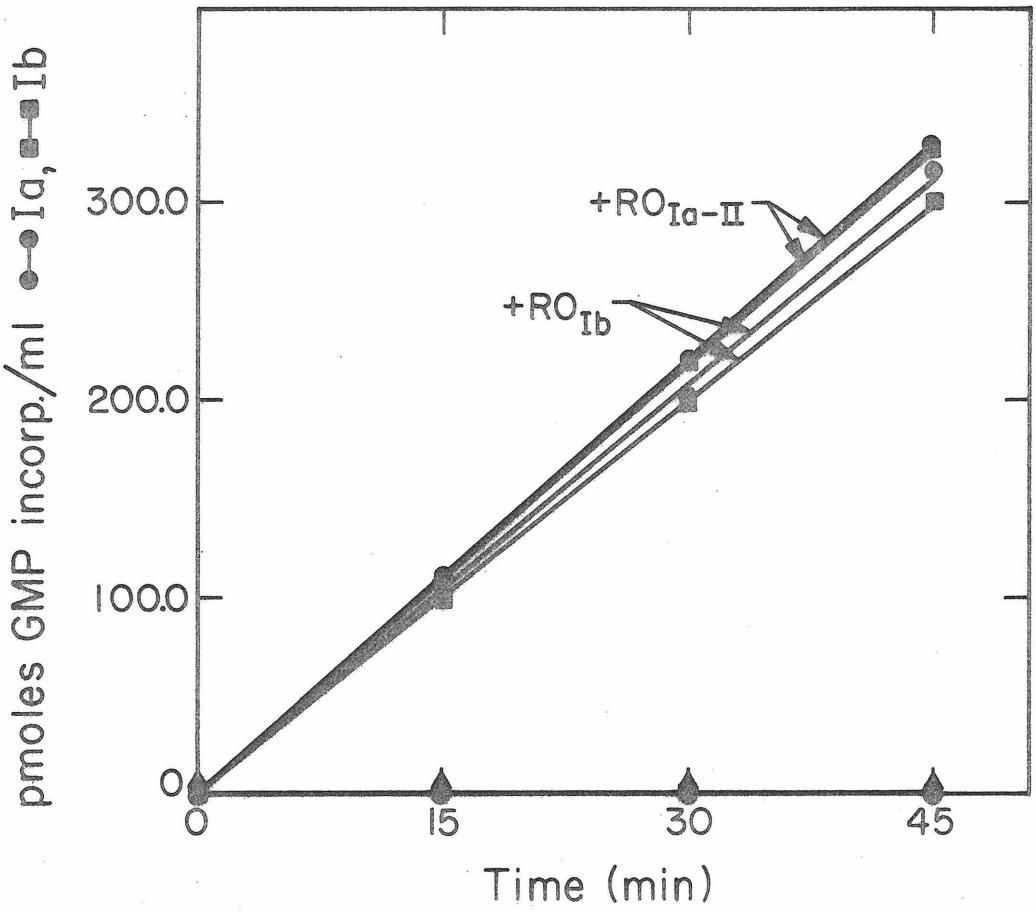
From the information presented so far, it is clear that the run-off fractions of the DEAE cellulose chromatography of both PC-A and PC-B contain a factor(s) which stimulates the activity of Ia and Ib respectively. It was of interest to determine if RO_{Ia-II} is specific for Ia or if it also stimulates Ib and if RO_{Ib} stimulates Ia. As shown in Fig. 6, at saturating factor concentrations, RO_{Ia-II} and RO_{Ib} stimulate Ia and Ib equally well, and independently of which fraction is used with a given enzyme.

Other steps in the enzyme purification procedure removed large amounts of protein which contain no RNA polymerase (Chapt. II), particularly the phosphocellulose chromatography and sucrose density gradient centrifugation. These non-active fractions have also been tested for stimulation of RNA polymerase activity. Neither the run-off of the phosphocellulose column nor the slowly-sedimenting protein in the sucrose gradient of Ia have any effect on the rate of RNA synthesis by Ia (data not shown). Thus, it appears that the factor under investigation chromatographs on phosphocellulose along with the enzyme

and is lost only when applied to DEAE cellulose.

Fig. 6

Reaction mixtures contained Ia (8 µg/ml) or Ib (1.6 µg/ml), plus either RO_{Ia-II} (69 µg/ml) or RO_{Ib} (129 µg/ml).


pmoles GMP incorp./ml ●—● Ia, ■—■ Ib
300.0
200.0
100.0
0
+RO Ia-II
+RO Ib
0
15
30
45
Time (min)

DISCUSSION

Both Ia and Ib show very strong cooperative effects, i.e., the activity increases non-linearly with enzyme concentration. No activity is observed at low enzyme concentrations. One interpretation of this phenomenon is that at low enzyme concentrations, one of the subunits dissociates from the enzyme, yielding an inactive enzyme. At higher concentrations, the equilibrium is shifted toward association of the subunit and the rest of the complex, resulting in an active enzyme. Certain trivial explanations have been ruled out. Small amounts of RNase present in the assay reagents could destroy small amounts of product synthesized at low enzyme concentrations. At higher enzyme concentrations, more RNA is synthesized and the RNase is not able to destroy it completely during the time course of the experiment, resulting in net synthesis. This is probably not the case since neither the reagents nor Ia or Ib show any trace of RNase activity (Chapt. III, Table 3). It is also possible that the enzymes become inactive at low concentrations simply due to dilution of the protein. However, BSA has been included to avoid this.

Whatever the explanation, the non-linearity of dose response curves makes calculations of yields and specific activities very difficult since the activity is extremely dependent on the RNA polymerase concentration. It is also

possible that earlier reports that the run-off factor does not stimulate polymerase I (Stein and Hausen, 1970; Seifart, 1970) can be explained by the fact that stimulation is dependent on enzyme concentration. No significant stimulation would be observed at high enzyme concentrations, at least with polymerase Ia.

It is impossible to determine at present if the stimulation is due to one or more factors, since RO_{Ia-II} and RO_{Ib} are not homogeneous preparations. However, the active agent is probably a protein since it is heat-sensitive and is destroyed by treatment with proteolytic enzymes (Michael E. Dahmus, personal communication). Also, preliminary experiments indicate that RO_{Ia-II} can be purified by chromatography on carboxymethyl cellulose, although the purified factor is unstable.

At present, the mechanism of action of the factor is unknown. The experiments involving re-isolation of the DNA suggest that permanent physical alteration of the template is not involved. Any proposed mechanism involving recognition of native DNA must be viewed with caution since stimulation by the factor on denatured DNA cannot be ruled out because of contaminating RNase H. Several lines of evidence, however, suggest that the factor, whatever its function, is a subunit of RNA polymerase. First, the factor chromatographs with the enzyme on phosphocellulose, though this may be due either to its association with the

enzyme or with the phosphocellulose or both. It is noteworthy that RO_{Ia-II} and RO_{Ib} appear to chromatograph with PC-A and PC-B, respectively, which are well resolved on phosphocellulose, suggesting that they do, in fact, remain associated with the enzyme during this purification step. Secondly, the cooperative effect of Ia and Ib is abolished by the addition of RO_{Ia-II} and RO_{Ib}, respectively, yielding a linear dose response curve. If the cooperativity is due to the dissociation of a subunit from the complex, the addition of this subunit should shift the equilibrium toward association and, thus, toward active enzyme. At high enzyme concentrations, little dissociation occurs and addition of the subunit would have little effect. The reduction of stimulation at high enzyme concentrations (especially with Ia) is consistent with this proposal. Finally, Seifart (1970) has reported a molecular weight of 70,000 for the rat liver factor. It is interesting that polymerase Ia preparations from ascites tumor contains a polypeptide of 69,000 molecular weight, present in a molar ratio of about 1.5 when compared to the other subunits. Whether RO_{Ia-II} is identical to this subunit awaits purification of the factor.

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