STUDIES ON BRAIN NUCLEAR RNA POLYMERASE AND CHROMATIN TRANSCRIPTION

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ABSTRACT

In order to elaborate the nature of the mechanisms controlling the transcription of genes in the cells of higher organisms, studies have been carried out on brain nuclear RNA polymerase and the transcription of brain chromatin.

Foremost, suitable conditions were developed for the solubilization of RNA polymerase in high yields from purified nuclei of beef brain. The solubilized enzyme was partially purified by ammonium sulphate fractionation followed by DEAE-cellulose chromatography. By this procedure, two DNA-dependent RNA polymerase activities, designated as RNA polymerase I and RNA polymerase II, were resolved. These were partly characterized on the basis of their differing catalytic properties.

RNA polymerase II exhibits a preferential requirement for Mn^{++} as the divalent cation and heat-denatured DNA as the template, is markedly stimulated by 0.2 M KCl and selectively inhibited by the toxin, α-amanitin. On the other hand, polymerase I prefers Mg^{++} as the divalent cation and native DNA as the template, is considerably inhibited by 0.2 M KCl and is not affected by α-amanitin.

The capacity of RNA synthesis in vitro by RNA polymerase isolated from brain nuclei was markedly enhanced by polyamines such as spermidine or spermine. Spermidine exerted a much more pronounced effect on polymerase II than on polymerase I. Evidence is presented suggesting that RNA polymerase II activity may be

preferentially stimulated by spermidine. Yeast RNA inhibited the activity of polymerase II and spermidine counteracted this inhibition almost completely, indicating that spermidine may act by circumventing the product-inhibition. The product of the polymerase II reaction sedimented at around 18 S in a sucrose-density gradient and appeared to be a complex of the type Enzyme-DNA-RNA.

The template activity of the isolated brain chromatin for brain nuclear RNA polymerase II and E. coli RNA polymerase was less than 25% than that of the pure calf thymus DNA. This greatly repressed template capacity of the chromatin was probably due to the acid-soluble chromosomal proteins. The brain polymerase II was 3-4 times more active with acid-treated chromatin than pure DNA as template whereas the E. coli enzyme was almost equally active with either of these two templates. The RNA synthesised on either native or acid-treated chromatin as template by brain polymerase II was somewhat smaller in size than the RNA made on pure DNA as template.

It appears that the control of transcription of genes in mammalian cells could be mediated by the multiplicity of the transcriptive enzyme, RNA polymerase, and by the physiological state of the template as well.

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ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
cpm	Counts per min
CTP	Cytidine-5'-triphosphate
CT-DNA	Calf thymus DNA
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GTP	Guanosine-5'-triphosphate
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per min
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TDG	0.05 M Tris-HCl, pH 8.0, 0.5 mM DTT, 30% (V/V) glycerol
Tris	Trishydroxymethylaminomethane
UMP	Uridine-5'-monophosphate
UTP	Uridine-5'-triphosphate

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CHAPTER ONE: INTRODUCTION

1.1. GENERAL

Genetic information, carried in DNA nucleotide sequences, in living cells is expressed by the mechanisms of "transcription" and "translation". The transmission of DNA-coded messages into RNA molecules is known as transcription, whereas translation results in the formation of specific cell proteins whose properties are determined by these molecules, i.e. DNA $\longrightarrow$ RNA $\longrightarrow$ Protein. The whole process is most commonly known as the "Central Dogma" of molecular biology (Crick, 1958). Thus, one of the most important aspects of DNA function is the production of RNA. Consequently, an understanding of the molecular mechanism of the control of transcription is important in defining cellular processes.

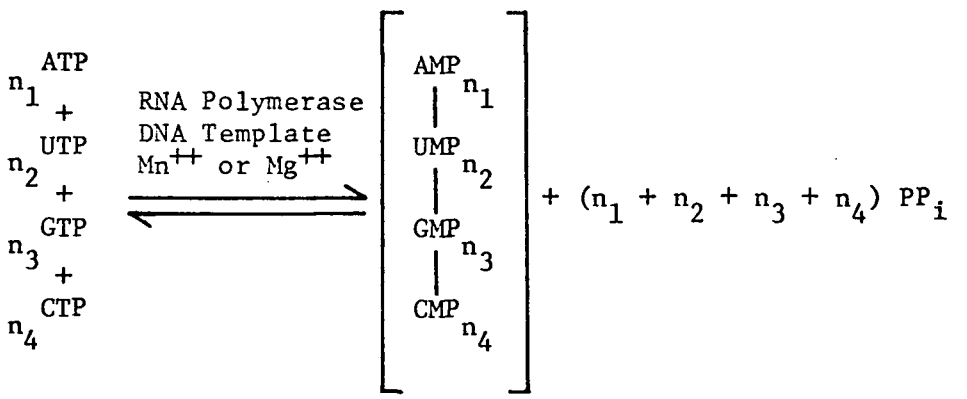
In the cells of higher organisms, the nucleus is the principal site of genetic information. A large body of evidence has shown that the biosynthesis of the major portion of cellular RNA is restricted to the cell nucleus in close association with DNA (Prescott, 1964). Since the protein synthesizing machinery is localized in the cytoplasm (see Zamecnik, 1970), the RNA molecules directly involved in protein synthesis have to be transported from the nucleus into the cytoplasm before they can manifest any function. Thus, the regulation of expression of genetic

message in mammalian cells may occur at more than one level: (1) at the level of transcription of DNA by the selective repression or activation of RNA synthesis, (2) at the level of intranuclear processing and selective transport of RNA molecules across the nuclear membrane into the cytoplasm, or (3) at the level of translation of RNA molecules into specific proteins in the cytoplasm.

From the viewpoint of cellular differentiation, it is generally believed that the control of gene expression in higher organisms is at the transcriptional level, and it remains to be seen if translational control is an important feature (see Watson, 1970). This thesis is devoted to the study of regulation occurring at the level of RNA polymerase.

1.2. FUNCTIONAL CONSIDERATIONS OF RNA POLYMERASE IN THE TRANSCRIPTION OF DNA

In the cell, the synthesis of RNA of specific base compositions is catalyzed by the transcriptive enzyme, called RNA polymerase (nucleoside triphosphate:RNA nucleotidyl transferase, EC 2.7.7.6) and proceeds on a DNA template according to the following reaction (Scheme 1):



Scheme 1. General Reaction of DNA-Dependent RNA Polymerase

RNA polymerase was first demonstrated independently by Weiss and Gladstone (1959) in rat liver nuclei and Hurwitz et al. (1960) and Stevens (1960) in Escherichia coli. Since then its wide occurrence among animals, plants and micro-organisms has been shown (see Hurwitz & August, 1963). In view of its function, this enzyme would be expected to be present ubiquitously in nature. In bacteria, the synthesis of all cellular RNA (rRNA, mRNA and tRNA) is mediated by a single RNA polymerase. Thus, DNA-dependent

RNA polymerase unequivocally plays a central role in the process of genetic transcription.

Since RNA polymerase from bacterial sources is a very complex molecule (see Burgess, 1971), it is important to discuss the structural and functional characteristics of this enzyme.

A. STRUCTURAL CHARACTERISTICS OF BACTERIAL RNA POLYMERASE

The bacterial RNA polymerase is a large molecule of 360,000-490,000 molecular weight and has a very complicated subunit structure (Burgess et al., 1969; Zillig et al., 1970; Burgess & Travers, 1970; Chamberlin, 1970; Burgess, 1971). The active form contains five subunits: one beta prime subunit (β'), one beta subunit (β), one sigma subunit (σ), two alpha subunits (α) and one omega subunit (ω), designated in order of increasing electrophoretic mobility. The polymerase molecule can be dissociated into its subunits, in the presence or absence of sulfhydryl reagents like β-mercaptoethanol, by treatment with a variety of denaturing agents, such as SDS, 8 M urea or guanidine hydrochloride (Walter et al., 1968; Burgess, 1969). The subunits can be separated analytically by SDS-polyacrylamide gel electrophoresis and the estimated values for their molecular weights are: $\beta' = 165,000 \pm 15,000$, $\beta = 155,000 \pm 15,000$, $\sigma = 95,000 \pm 5,000$,

α = 39,000 ± 2,000 and ω = 9,000 ± 2,000. The attachment of one particular subunit (i.e. the sigma) to the other subunits in the complete RNA polymerase molecule (i.e. the holoenzyme = $\beta'\beta\alpha_2\omega\sigma$) is not very firm as it is easily separated by phosphocellulose chromatography, leaving behind the minimal enzyme (i.e., the core enzyme = $\beta'\beta\alpha_2\omega$). This subunit model of RNA polymerase was originally discovered in E. coli (Burgess et al., 1969) and now known to exist in other microbial systems, such as Bacillus subtilis (Avila et al., 1970; Losick et al., 1970) and Azotobacter vinelandii (Krakow & von der Helm, 1970). Preliminary work on the subunit structure of eukaryotic RNA polymerases also reveals some similarities, but a sigma-like activity has not been detected (Chambon et al., 1970; Weaver et al., 1971).

Recently, a very careful study has resolved some important differences between holoenzyme and core enzyme in response to ionic strength (Berg & Chamberlin, 1970). These might well explain the previous confusion on the physical characteristics of bacterial RNA polymerase (see Richardson, 1969). It was found that holoenzyme has a sedimentation coefficient ($S_{20,W}$) of 14.9 S at the ionic strength (μ) of higher than 0.1 and it increases to 23 S if μ is less than 0.1. The core enzyme exhibits an $S_{20,W}$ value of 12.5 S at ionic strength of 0.26 and if the ionic strength is decreased then the $S_{20,W}$ value increases to 44-48S. However,

both the holoenzyme and core enzyme possess a sedimentation value of as low as 9 S under conditions of very high ionic strength (μ = 3.5). The sigma subunit has a $S_{20,W}$ value of 4.5 - 5.0 S. The summary of the subunit model of RNA polymerase is outlined in Scheme 2.

HOLOENZYME	⇌	SIGMA	+	CORE ENZYME
(Complete enzyme)		(Initiation factor)		(Minimal enzyme)
$\beta'\beta\alpha_2\omega\sigma$		σ		$\beta'\beta\alpha_2\omega$
490,000 MW		95,000 MW		400,000 MW
14.9 S		4.5 S		12.5 S

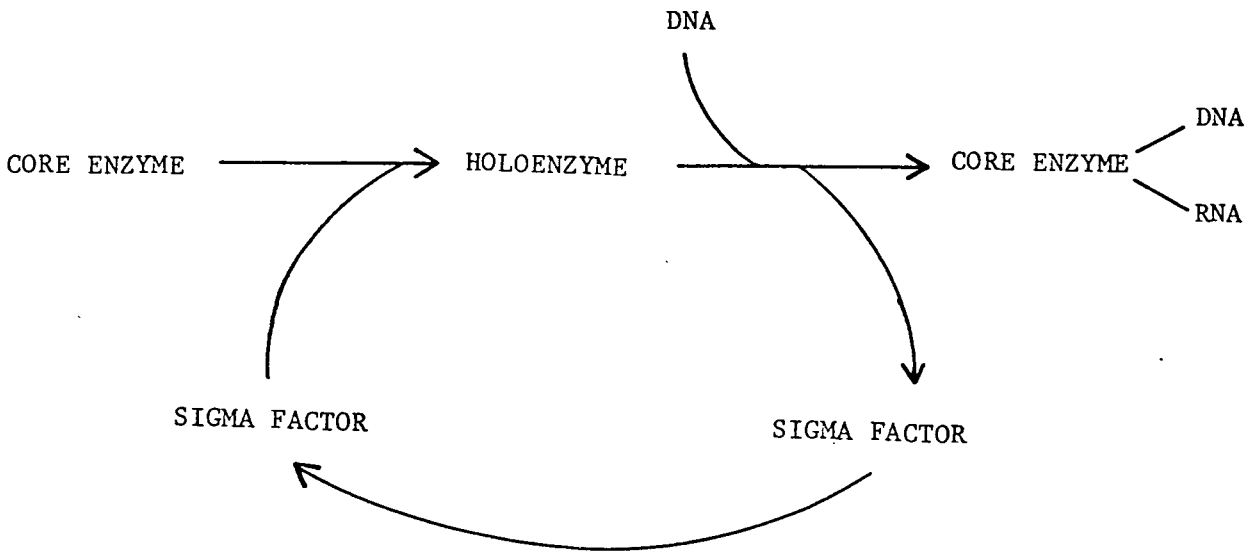
Scheme 2. Subunit Model of E. coli RNA Polymerase

B. FUNCTIONAL ASPECTS OF THE SUBUNIT MODEL OF BACTERIAL RNA POLYMERASE

Although the subunit structure of RNA polymerase appears to be defined now, at present very little is known about the function of individual subunits. Undoubtedly, the sigma component is essential for the initiation of specific RNA molecules. The beta prime and beta subunits may be involved in the primary binding of the enzyme to DNA

while nothing is known about the function of alpha and omega components.

Burgess et al. (1969) initially reported the reversible separation of two functional components of E. coli RNA polymerase and called them "core" enzyme and "sigma" factor. They found that the core enzyme is virtually inactive on phage T_4 DNA template, but the addition of sigma factor stimulates this activity. The degree of stimulation was dependent upon the quality of DNA template, being much higher with double-stranded T_4 DNA (50-75-fold) than the calf thymus or denatured DNA (1.5 - 5-fold). This finding was interpreted to mean that perhaps the core enzyme has lost the specificity while sigma factor restores it. Various lines of evidence have now established that the core enzyme transcribes DNA unspecifically while the sigma factor is required for the specific initiation of RNA chains (Bautz et al., 1969; Summers & Siegel, 1969; Sugiura et al., 1970). It has also been shown that after chain initiation sigma is released from the DNA-enzyme complexes (Travers & Burgess, 1969) and thus may be re-used in the so-called "sigma cycle", as illustrated in Scheme 3.



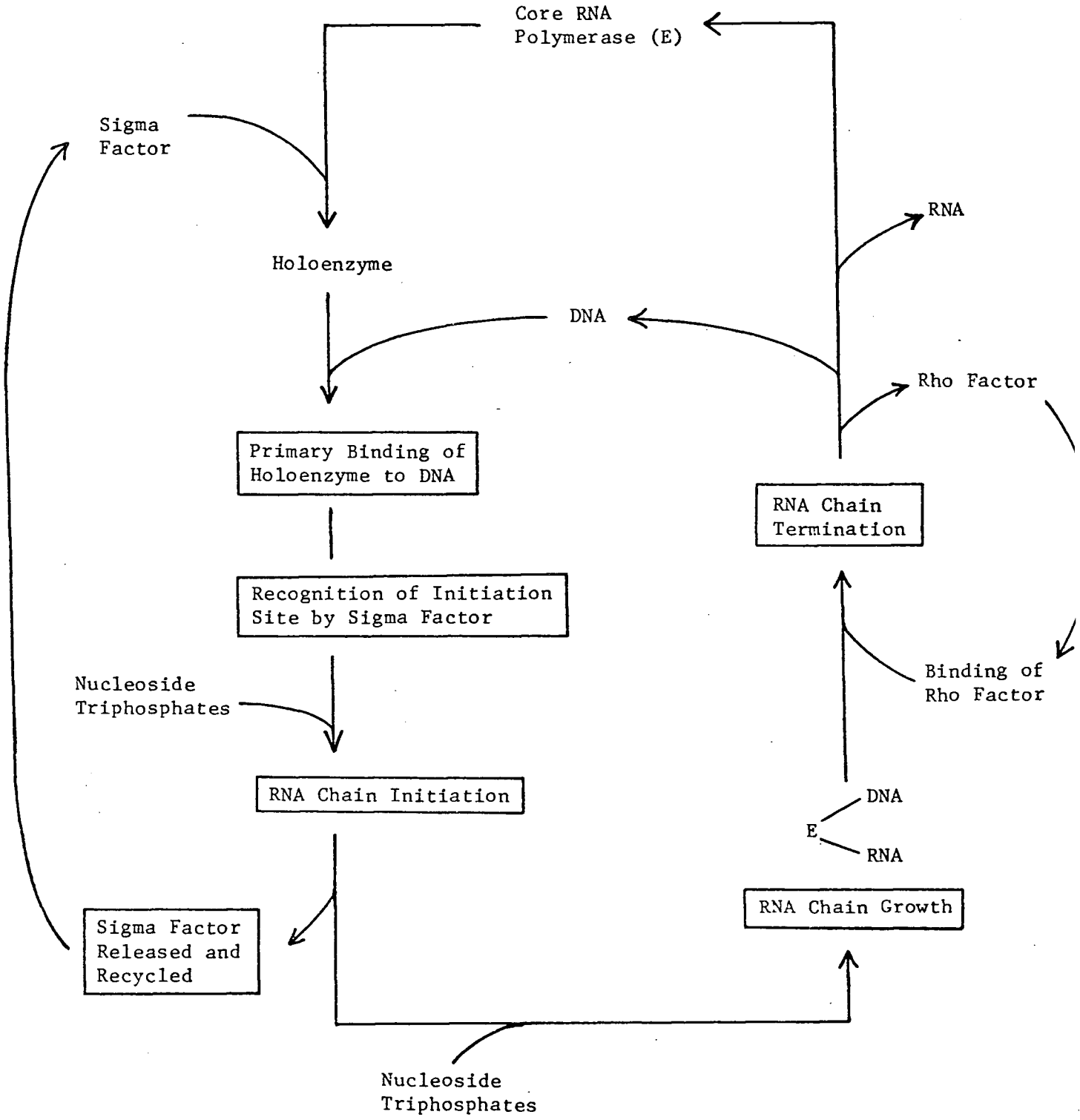
Scheme 3. The Sigma Cycle

At present, it is not clear whether the specificity of transcription is determined by sigma factor or core enzyme or both in a co-operative manner. Though the mode of action of sigma subunit remains obscure, it may be implicated in the opening of double-stranded DNA at the promotor regions (Hinkle & Chamberlin, 1970; Ishihama et al., 1971).

Both beta prime and beta subunits bind to polyanionic compounds like DNA, RNA, phosphocellulose and heparin indicating that these protein subunits may have template binding sites. Recently, the β' subunit has been designated as the DNA-binding component. This reasoning was based on the finding that the separated β' subunit, but not the other subunits, efficiently binds to DNA and is retained on membrane filters under non-denaturing conditions (Sethi et al., 1970). On the other hand, beta appears to be the catalytic subunit as suggested by the data on antibiotic-resistance. The antibiotic rifamycin inhibits the initiation step of RNA synthesis by binding to RNA polymerase rather than the template (Sippel & Hartmann, 1968; Wehrli et al., 1968; Lill et al., 1970). Using radioactive rifamycin (^{14}C-labelled), the site of action of this antibiotic was shown to be core enzyme and not the sigma component (di Mauro et al., 1969; Wehrli & Staehelin, 1970). Zillig and his co-workers, based on their recent work on the reconstitution of active enzyme from subunits isolated from rifamycin-sensitive and rifamycin-resistant mutant RNA polymerases, have clearly demonstrated that beta subunit is the site of action of rifamycin antibiotic (Rabussay & Zillig, 1969; Heil & Zillig, 1970). Similarly, another antibiotic, streptolydigin, which interacts with core RNA polymerase and prevents the elongation of RNA chains (Cassani et al., 1970), has been shown to bind β subunit (Heil & Zillig, 1970). The mechanism of action of β' and β subunits of RNA polymerase is unknown.

Besides sigma factor, several other protein factors have been described which influence the transcription reaction markedly. These are M-, psi- and rho-factors. M-Factor was isolated from a high salt-wash of E. coli ribosomes (Davison et al., 1969). It has a sedimentation value of approximately 5 S and in crude preparations it stimulates the activity of core enzyme 30-50-fold on T_4 and λ phage DNA. It is different from sigma factor and may influence a step immediately after initiation of RNA chains. The latter remains to be established. Travers et al. (1970) have isolated a protein factor from ribosome-free supernatant of E. coli cells and called it psi-factor (ψ). This factor sediments around 3 S and stimulates the synthesis of ribosomal RNA several hundred fold. The involvement of psi-factor for ribosomal RNA synthesis has now been questioned on the basis of the finding that an ordered and preferential synthesis of ribosomal RNA in vitro occurs without added psi-factor (Pettijohn, 1972). In addition, Roberts (1969) has isolated a protein factor from E. coli cells which causes the termination of RNA synthesis in vitro at distinct sites on DNA template producing RNA molecules that are probably identical to messenger RNA. This was called rho-factor (ρ). Recently, a protein has been isolated from phage T_3-infected E. coli cells, which interferes with the initiation of RNA chains, behaves like the rifamycin antibiotic does and appears to antagonize the action of sigma factor (Mahadik et al., 1972). Although cytoplasmic factors affecting the activity of mammalian RNA polymerase have been reported, their nature, function and mode of action remains to be elucidated (Stein & Hausen, 1970; Seifart, 1970).

In summary, the enzymatic machinery of DNA transcription involves the following steps (Scheme 4):



Scheme 4. The Summary of Transcriptional Events

(1) Binding of RNA polymerase to DNA template: Free enzyme reversibly binds to double-stranded DNA in a non-specific manner. Beta prime and beta subunits are involved in this function. Certain inhibitors, such as tRNA, heparin, high salt concentration and proflavin sulfate, prevent this binding. This step is sensitive to rifamycin.

(2) Initiation of RNA chains: The enzyme locates a specific binding site, perhaps a pyrimidine cluster. A highly stable complex is formed at this specific site at temperatures between 15°C - 37°C in the presence of low ionic strength. The sigma subunit plays a key role in the recognition of the initiation signal and the transcription begins with the incorporation of first 5'-ribonucleoside triphosphate, almost always a purine nucleotide.

(3) Elongation of RNA chains: The first phosphodiester bond is formed by the incorporation of the second ribonucleoside triphosphate. The sigma factor is released and utilized again in a cyclic manner. The RNA chains grow in the 5' to 3' direction only.

(4) Termination of RNA chains: The binding of rho-factor to the DNA-enzyme complex, which is already busy in making RNA, terminates the growth of RNA chains. As a consequence of this, free RNA molecules are released <u>in vitro</u>, which are probably identical to natural messenger RNA molecules.

1.3. CONCEPTS OF REGULATION OF GENETIC TRANSCRIPTION

Jacob and Monod (1961) put forward an elegantly simple model for the genetic regulation of protein synthesis, now most popularly known as the "operon" model. In essence, the operon model contains the concept of separate and specific genes (i.e. the regulatory genes) involved in the control of other genes (i.e. the structural genes). It also states that the product of the regulatory genes is a repressor molecule which will repress the gene function by binding to a specific site on DNA (operator) and thereby preventing the transcription of genes by RNA polymerase. Thus the repressor is an essential element of this control mechanism. In fact, the salient features, i.e., the structure, expression and regulation of a single operon have now been investigated (see Beckwith & Zipser, 1970), and the operon model appears to be proven at least in bacteria. In addition to this negative control, the elements of a positive control system acting at the level of initiation of gene transcription by RNA polymerase have recently been characterized (see Burgess, 1971).

By considering the involvement of formally similar mechanisms, Britten and Davidson (1969) have recently advanced a theory for the regulation of gene activity in the higher eukaryotic organisms. The key point of their proposal is that the change in gene activity during diverse states is a consequence of concerted activation of one or more sets of "producer genes" (similar to structural genes).

The function of producer genes is regulated by "activator RNA" molecules which are synthesized by "integrator gene" or genes (similar to regulatory genes). The function of integrator gene(s) linked to "sensory gene" (another sequence on DNA) is to induce the transcription of many producer genes in response to a single molecular stimulus, e.g., hormones. Since most stimuli (or inducing agents) will not bind to sensory gene DNA in a sequence-specific manner, an intermediary structure, such as a protein, will be required. Thus, in this model, the gene regulation is accomplished by sequence-specific binding of an activator RNA and not of chromosomal proteins. Although there is no proof for the occurrence of this model in higher organisms, it is subject to experimental analysis.

These considerations suggest that there are two major ways in which the control of synthesis of cellular RNA on DNA templates can be accomplished: (1) the regulation could be mediated at the level of RNA polymerase via changes in the activity or level of enzyme itself, or (2) the regulation could occur at the level of DNA template via modulations in the availability or structure of specific genes (DNA).

Recently, observations in microbial systems have, in fact, explored the possibility of regulation of gene transcription operative at the level of RNA polymerase. The subunit structure of a basic molecule of E. coli RNA polymerase was defined (Burgess

et al., 1969), and consequently, it was demonstrated that the presence of specific protein factors (regulatory subunits) is required for the transcription of specific genes during bacteriophage T_4 infection (Travers, 1971; Bautz et al., 1970; Hager et al., 1970). The synthesis of a completely new bacteriophage T_7-specific RNA polymerase has been described as another means of gene control in phage T_7-infected E. coli cells (Chamberlin et al., 1970). Phosphorylation (Martelo et al., 1970) and the interconversion between adenylated and non-adenylated forms (Chelala et al., 1971) of E. coli RNA polymerase has been reported to represent additional control mechanisms of DNA-dependent RNA synthesis.

Although there has been no evidence for the existence of similar regulatory mechanisms in higher organisms, some important questions can now be raised: (1) how RNA polymerase catalyzes the synthesis of all types of cellular RNA (i.e. rRNA, mRNA and tRNA)? (2) do many stimulators of RNA synthesis (e.g. hormones) modulate the activity or level of RNA polymerase? (3) is there any control of level or activity of RNA polymerase during cellular differentiation or virus infection? and finally (4), how precisely the control of transcription could be mediated through RNA polymerase?

Unlike bacteria and other micro-organisms, the RNA polymerase in higher organisms is localised exclusively, with the exception of mitochondria (Saccone et al., 1967; Kuntzel & Schafer, 1970; Tsai et al., 1971), in the cell nucleus in very close association with the genetic material. The chromosome of eukaryotes, as opposed to

prokaryotes, is a highly complex structure (see Hearst & Botchan, 1970) in which the nuclear genes (DNA) exist tightly-bound to an array of both basic as well as acidic proteins and RNA molecules (Bonner et al., 1968b). The structure of chromosomal DNA in higher organisms appears to be further complicated according to a recent model (Crick, 1971). One of the most prominent features of Crick's proposal (1971) is that the sites in eukaryotic DNA which are recognized by regulator molecules are not double-stranded but comprise single-stranded regions of unwound DNA. At the present time the nature of regulatory molecules is unknown, but if they are RNA as has been suggested (Britten & Davidson, 1969) then it would be very significant for the recognition sites to be single-stranded regions of DNA in order that complementary base-pairing can take place. However, the model lacks experimental support.

In view of cellular differentiation, the complex structure of eukaryotic genome, its much larger size and the occurrence of "repeated" sequences in chromosomal DNA (Britten & Kohne, 1968), the regulation of gene transcription in higher organisms would appear to be a more complicated process than is known today in the prokaryotes. The most popular hypothesis which is frequently used to explain the selective gene transcription in animal cells is the one which proposes that the synthesis of specific RNA molecules is regulated by the macromolecules associated with the DNA in the

nucleo-protein matrix (Bonner et al., 1968b; Paul et al., 1970). According to this view, the regulation is accomplished by a general repression of RNA synthesis by histone proteins, but the specificity of the repression is determined by other chromosomal components, either the acidic proteins (Paul & Gilmour, 1968; Gilmour & Paul, 1970; Spelsberg & Hnilica, 1971) or chromosomal RNA molecules (Bekhor et al., 1969; Huang & Huang, 1969) independently or both in co-operation.

From the foregoing description it would appear that the present knowledge about the regulation of DNA transcription in higher organisms is very inadequate and, in fact, does not provide any clue to the mechanisms of regulation. Nevertheless, there is a general belief among various workers in the field that the control of gene transcription in higher organisms is accomplished by mechanisms which are basically analogous, if not identical, to those found in bacteria. From this point of view, this thesis will be concerned primarily with studies of how the activity of RNA polymerase could be controlled in mammalian cells.

1.4. PRESENT STATUS OF RNA POLYMERASE IN MAMMALIAN CELLS

Until recently, most studies on DNA-dependent RNA polymerase in higher organisms have employed isolated nuclei or "aggregate-enzyme" preparations. This activity has now been reported in mitochondria (Saccone et al., 1967; Kuntzel & Schafer, 1970; Tsai et

al., 1971). In nuclear studies, the RNA polymerase activity is most frequently assayed in the absence of exogenously added DNA template because the endogenous nucleo-protein complex of the intact nuclei itself serves the function of template.

Goldberg (1961) reported that the RNA polymerase activity of "aggregate-enzyme" preparations is stimulated if the ionic strength of the reaction mixture is raised by ammonium sulphate. Subsequently, Windell and Tata (1964) found that the stimulatory effect of ammonium sulphate on RNA polymerase activity of intact nuclei is differential with respect to the divalent cation (Mn^{++} or Mg^{++}) present in the assay mixture. They showed that ammonium sulphate stimulated RNA polymerase activity of isolated rat liver nuclei in the presence of Mn^{++}, but not Mg^{++}. Moreover, they found that the RNA synthesized in the presence of Mn^{++} (plus ammonium sulphate) resembled DNA-like RNA (AU-rich) and that synthesized in the presence of Mg^{++} (minus ammonium sulphate) resembled ribosomal RNA (GC-rich) in terms of base composition and nearest-neighbour frequency (Windell & Tata, 1966). In the light of evidence that ribosomal RNA is synthesized in the nucleolus (Perry, 1962; Brown & Gurdon, 1964; McConkey & Hopkins, 1964; Liau et al., 1965) and that nucleolar preparations contain a DNA-dependent RNA polymerase (Ro et al., 1964; Tsukada & Lieberman, 1964; Liau et al., 1965; Jacob et al., 1968) and based on their own observations,

Windell and Tata (1966) proposed that the nuclei of mammalian cells contain two DNA-dependent RNA polymerase activities, namely, the Mn^{++}/ammonium sulphate-stimulated activity and the Mg^{++}-stimulated activity. In support of this proposal, the high-resolution autoradiographic studies on the intranuclear localization of these two enzymic activities demonstrated that the Mn^{++}/ammonium sulphate-stimulated reaction which makes a more DNA-like RNA occurs primarily in the extranucleolar region whereas the Mg^{++}-stimulated reaction which synthesizes ribosomal type RNA takes place primarily in the nucleolus (Pogo et al., 1967; Maul & Hamilton, 1967).

In addition, the evidence in favour of the above notion that the nuclei of animal cells would contain two types of DNA-dependent RNA polymerases comes from the studies of certain hormones which stimulate nuclear RNA synthesis, and certain inhibitors which selectively suppress the synthesis of nuclear RNA. The Mg^{++}-stimulated RNA polymerase activity is found to increase at an earlier time than does the Mn^{++}/ammonium sulphate-stimulated activity, as assayed in vitro using rat liver nuclei isolated after in vivo administration of triiodothyronine or testosterone (Tata, 1966; Liao et al., 1965). During a primary action, an increase in Mg^{++}-stimulated RNA polymerase activity only has been observed in response to various other hormones, e.g., corticosteroids (Sereni & Barnabei, 1967; Lukacs & Sekeris, 1967; Yu & Feigelson,

1971), growth hormone (Pegg & Korner, 1965; Tata, 1966; Janne & Raina, 1969), oestradiol (Hamilton et al., 1968) and hydrocortisone (Jacob et al., 1969; Sajdel & Jacob, 1971). Among the inhibitors, extremely low concentrations of a toxin, α-amanitin, which is a small bicyclic octapeptide (M.W. 1,000) from poisonous green mushroom, Amanita phalloides (see Wieland, 1968), dramatically inhibits the Mn^{++}/ammonium sulphate-stimulated activity of intact nuclei while the Mg^{++}-stimulated activity is affected very little or not at all (Stirpe & Fiume, 1967).

Although ammonium sulphate may differentially affect the putative nucleolar and nucleoplasmic (extranucleolar) RNA polymerase activities of the intact nuclei, it is also likely that ammonium sulphate simply enhances the availability of DNA for a common enzyme by the dissociation of proteins from the endogenous nucleo-protein template. The latter possibility appears to be more likely since the effect of higher concentrations of salt on the increased template activity of chromatin preparations have, in fact, been reported (Marushige & Bonner, 1966; Breuer & Florini, 1966; Georgiev et al., 1966). Thus, it is impossible to rule out the possibility of differential template efficiency in the presence of ammonium sulphate.

Until recently, the question of whether more than one functional RNA polymerase exists in the nuclei of mammalian cells has remained unresolved, primarily due to lack of methods of solubilization of this enzyme in good yields. Although some workers were able to extract nuclear RNA polymerase using buffers of low ionic strength (Chambon et al., 1965; Furth & Ho, 1965; Ballard & Williams-Ashman, 1966; Ishihama, 1967; Cunningham et al., 1968; Frederick et al., 1969; Goldberg et al., 1969) or high ionic strength (Seifart & Sekeris, 1969; Spelsberg et al., 1969), the yields are relatively low, and in each case only a single enzyme activity is reported which resembles, in catalytic properties, Mn^{++}/ammonium sulphate-stimulated activity of isolated nuclei. If more than one form of RNA polymerase did exist, as originally observed in intact nuclei by Windell and Tata (1964), then the failure to resolve them after solubilization could be due to one of the following reasons: (1) selective extraction methods or conditions may be required; (2) lack of suitable analytical procedures for selective resolution; or (3) greater instability and thus selective deterioration of one or more enzymic activities during extraction and characterisation.

Taking all these parameters into consideration, Roeder and Rutter (1969) have now described the existence of multiple forms of DNA-dependent RNA polymerase isolated from the nuclei of two eukaryotes, rat liver and sea urchin. After solubilization of nuclear enzyme activity, two major RNA polymerase activities were

resolved on a DEAE-sephadex column and were characterized based on certain catalytic properties: (1) RNA polymerase I - this enzyme activity is shown to be activated more by Mg^{++} than Mn^{++} (the Mn^{++}/Mg^{++} activities ratio at their optimum concentrations is about one), slightly stimulated by ammonium sulphate at low concentrations only (around 0.05 M) and prefers native DNA rather than heat-denatured DNA as template. (2) RNA polymerase II - this enzyme activity is activated by Mn^{++} in preference to Mg^{++} (the Mn^{++}/Mg^{++} activities ratio at their optimum concentration is about 3-5), greatly stimulated by ammonium sulphate at relatively higher concentrations only (around 0.15 M) and prefers heat-denatured DNA to native DNA as template. These workers substantiated their findings by showing that RNA polymerase I is localized in the nucleolar preparations whereas the RNA polymerase II is of nucleoplasmic origin (Roeder & Rutter, 1970). Also, it was found that very low concentrations of α-amanitin toxin inhibits RNA polymerase II almost completely whereas it has no effect on RNA polymerase I (Lindell et al., 1970; Kedinger et al., 1970). In addition to these two RNA polymerases, a minor component called RNA polymerase III has been reported in rat liver and sea urchin (Roeder & Rutter, 1969), but its characterization and functional significance remains to be learned. Similarly, the nuclei of calf thymus (Chambon et al., 1970), bovine thymus (Goldberg & Moon, 1970), Xenopus laevis (Roeder et al., 1970; Tocchini-Valentini & Crippa, 1970), yeast (Ponta et al., 1971) and maize (Strain et al., 1971) have now been shown to contain more than one form of DNA-dependent RNA polymerase.

1.5. TRANSCRIPTION OF MAMMALIAN CHROMATIN

In the interphase nucleus, the chromosomes appear in their extended form, and are of great interest from the point of view of cellular differentiation because during this period they carry out both DNA replication and RNA synthesis. Such chromosomes, in morphological terms are collectively described as "chromatin". The chromatin consists of a net-work of DNA, RNA and proteins, and thus contains the genetic material in the nucleus of all eukaryotic cells (Bonner *et al.*, 1968b).

The differentiated cells of an organism appear to contain the same genetic information (see Gurdon, 1970), but the populations of RNA transcribed vary from one tissue to another (Marushige & Bonner, 1966; Paul & Gilmour, 1966; Smith *et al.*, 1969). These observations are compelling evidence of selective gene transcription resulting from the organ-specific "masking" of genes in mammalian chromosomes (see Paul *et al.*, 1970).

Stedman and Stedman (1950) proposed that the basic proteins of cell nuclei are gene regulators. This view stands as a landmark in the advancement of the concept of differential gene activity and the regulatory role of chromosomal DNA-bound proteins. Huang and Bonner (1962) first reported that RNA synthesis *in vitro* on chromatin template is much lower than on an equivalent amount

of pure, protein-free DNA. They concluded that much of the DNA in chromatin is masked and not available for RNA synthesis. Similar observations were made by others (Allfrey et al., 1963; Paul & Gilmour, 1966). Later on, it was found that although the ability of chromatin to support RNA synthesis is greatly reduced, the full template capacity of chromatin can be restored if histone proteins are removed (Marushige & Bonner, 1966; Paul & Gilmour, 1966; Georgiev et al., 1966). Since then, a great deal of work has been done on the chemistry and biology of histone proteins (Stellwagen & Cole, 1969; DeLange & Smith, 1971; Phillips, 1971), yet their function as gene regulators is not fully understood. Present opinion, however, is that histones might provide a mechanism of general repression, but by themselves they do not determine the specificity of genome masking (see Georgiev, 1969; Allfrey, 1971; Elgin et al., 1971).

In an attempt to provide a mechanism of specificity of gene transcription, several proposals have been made:

(1) The enzymatic modifications of histone structure involving group substitutions, such as acetylation and methylation (Allfrey et al., 1964; Pogo et al., 1966; Allfrey, 1970) and phosphorylation (Kleinsmith et al., 1966; Langan, 1970) have been suggested to impose selectivity in the transcription of chromatin DNA. In view of the lack of specificity of recog-

nition of DNA regions having their histones modified, the physiological role of either of these mechanisms remains to be established.

(2) Nuclear polyanions, such as nuclear RNA, might function as de-repressors of RNA synthesis in a selective manner (Frenster, 1965). Due to lack of experimental evidence, this mechanism is a matter of speculation only.

(3) In recent years, much attention has been paid to a so-called "non-histone fraction" of chromatin. This fraction contains both acidic proteins and chromosomal RNA and evidently appears to mediate the tissue-specific restriction of chromatin transcription, as discussed below. Based on the experiments on reconstitution of chromatin, which has been pre-treated to dissociate one or the other component selectively, two opinions have emerged: (i) The regulatory molecules of tissue-specificity of gene transcription might be acidic proteins (Paul & Gilmour, 1968; Spelsberg & Hnilica, 1971). These workers have found that the non-histone fraction is essential for the reconstitution of chromatin, with the maintenance of a high degree of fidelity of transcription as measured by hybridization competition assay. (ii) The regulatory molecules of tissue-specific gene transcription might be chromosomal RNA molecules (Bekhor et al., 1969; Huang & Huang, 1969). These researchers have found that the chromosomal RNA of the non-histone fraction is necessary for the faithful reconstitution of chromatin having absolute

specificity of transcription as determined by hybridization competition assay. Some additional support in favour of each of these two opinions may be obtained by the fact that chromosomal acidic proteins (Platz et al., 1970; MacGillivray et al., 1971; Teng et al., 1971; Wang, 1971) as well as chromosomal RNA (Mayfield & Bonner, 1971) exhibit tissue-specificity. In addition, nuclear acidic proteins have been shown to stimulate the transcription of chromatin by bacterial RNA polymerase (Kamiyama & Wang, 1971; Teng et al., 1971).

1.6. POLYAMINES AND THE RNA POLYMERASE ACTIVITY

In recent years, much attention has been paid to investigating the role of polyamines because they manifest numerous effects in a variety of biological systems (see Bachrach, 1970; Cohen, 1971). The polyamines like spermidine and spermine are non-protein nitrogenous bases and occur ubiquitously in nature (Tabor & Tabor, 1964). In mammals, they are found in the highest concentrations in actively differentiating tissues, such as prostate gland (Rodes & Williams-Ashman, 1964), regenerating liver (Dykstra & Herbst, 1965; Raina et al., 1966), lactating mammary gland (Neish & Key, 1968), developing chick embryo (Caldarera et al., 1965) and developing brain (Shimizu et al., 1965a; Pearce & Schanberg, 1969). Intracellularly, polyamines are distributed preferentially in the nucleus, but considerable quantities are also found in the cytoplasm (Shimizu et al., 1965b; Stevens, 1966; Raina & Telaranta, 1967).

In addition to the above information, there is increasing evidence to permit speculation that polyamines may play some role in growth processes or cell proliferation by regulating RNA metabolism (see Bachrach, 1970). In this regard, it is interesting to note that there is a very good correlation in the changes in RNA and polyamine levels in many cases, such as developing chick embryo (Moruzzi et al., 1968), Drosophila melanogaster (Dion & Herbst, 1967), developing brain (Caldarera et al., 1969) and developing eggs of Xenopus laevis (Russell, 1970). In addition, polyamines have been shown to stimulate DNA-dependent RNA polymerase activity from microbial systems (Krakow, 1963; Abraham, 1968; Peterson et al., 1968). Similarly, the RNA synthesizing capacity of isolated nuclei of mammalian cells is also enhanced in the presence of spermidine or spermine (MacGregor & Mahler, 1967; Caldarera et al., 1968; Barbiroli et al., 1971a). By virtue of their highly cationic nature, polyamines bind strongly to nucleic acids (Tabor & Tabor, 1964) and thereby might influence RNA polymerase activity. However, this remains to be established.

The foregoing results are indicative of the fact that polyamines could play some role in the regulation of RNA synthesis, yet their physiological role is unknown. In the light of the knowledge that they are present in the mammalian cell nucleus, that they bind to nucleic acids and that they stimulate

DNA-dependent RNA polymerase *in vitro*, it is likely that they could mediate some control in the functioning of RNA polymerase. How this is accomplished is not understood. Investigations on this line of research are also described in this thesis.

1.7. THE PROBLEM OF TRANSCRIPTION IN BRAIN

Physiologically, the brain is a unique organ and in morphological terms it is a very complex and heterogeneous tissue. Attempts to investigate a molecular basis of learning and memory phenomena, highly specific of brain function, have led to the proposal that RNA may be the engram for memory (Hyden, 1960 & 1967). Two lines of biochemical evidence are in favour of possible involvement of macromolecules in brain function: (1) an increased synthesis of RNA and protein takes place in some regions of brain during learning, and (2) the consolidation of "long-term" memory is impaired by inhibitors of synthesis of RNA and protein. However, at the present time, the correlation between behavioural phenomena and the synthesis of specific macromolecules is rather feeble (see Glassman, 1969; Gaito, 1969; Becker, 1971).

Since the cellular specificity in higher organisms is thought to derive from transcriptive properties of nuclear chromatin (Bonner *et al.*, 1968b; Paul *et al.*, 1970), it is quite likely that the unique structural and functional characteristics of the brain are related to the specific properties of nuclear

chromatin, at least, in terms of distinctive patterns of RNA synthesis. Thus, the radical approach to solving the problem of neuroscience as related to molecules and behaviour will depend upon the study of transcriptional phenomena and the regulation of gene function in nerve cells.

The understanding of the mechanism of gene transcription and its control in brain is very infantile (see Mandel, 1969) and the problem still remains unexplored, possibly due to the lack of good in vitro systems. Until now, the investigations on this topic in brain have employed RNA polymerase, being assayed in intact nuclei (Bondy & Waelsch, 1965; Dutton & Mahler, 1968) or in "aggregate-enzyme" preparation (Barondes, 1964). None of these RNA polymerase preparations is dependent on exogenous DNA because the nucleoprotein complex by itself serves the function of template for RNA synthesis. Obviously, these preparations are not suitable for the purpose of studying the mechanism of RNA transcription from DNA and its control. Therefore, the elucidation of transcriptional events in the brain is of the utmost importance before any correlation between molecules and behaviour is actually conceived. This thesis is primarily devoted to such an investigation.

1.8. BIOLOGICAL SYSTEM AND STRATEGY FOR THE STUDY OF RNA POLYMERASE AND CHROMATIN TEMPLATE

For the purpose of studying the RNA polymerase and the possible involvement of this enzyme and the chromatin template in the control of RNA synthesis, beef brain has been employed as the biological system. In the light of previous knowledge on mammalian RNA polymerase (Windell & Tata, 1966), it was considered that nerve cells might contain more than one functional species of RNA polymerase. In addition, it was considered that the template could mediate a fundamental regulatory role in the transcription of genes by RNA polymerase, mainly, because the *in vivo* template in mammalian cells is a network of nuclear genes (DNA), proteins and RNA molecules rather than a naked DNA template (see Hearst & Botchan, 1970).

The primary goal of this work was two-fold. At first, the isolation of a RNA polymerase from brain which will be free of endogenous template so that the exogenously added DNA template can be used to study RNA synthesis *in vitro*. Secondly, the establishment of the existence of more than one RNA polymerase in brain, not in the intact nuclei but in the solubilized form. The experimental approach to this analysis was to obtain the complete solubilization of nuclear RNA polymerase activity, followed by the subsequent resolution of putative multiple RNA poly-

merases by chromatographic methods. Although mitochondria also contain RNA polymerase activity (Saccone et al., 1967; Kuntzel & Schafer, 1970; Tsai et al., 1971), the studies to be described in this thesis have been restricted to the nuclear RNA polymerase, largely because this enzyme is localized primarily in the nucleus of the cell. While this work using brain tissue was still in progress, the multiple RNA polymerases have recently been shown in some eukaryotic systems, such as, rat liver and sea urchin embryos (Roeder & Rutter, 1969) and thymus (Kedinger et al., 1970; Goldberg & Moon, 1970), but to date there is no such report on brain tissue. Thus, a major part of this thesis has been concerned with the analysis of RNA polymerase in brain.

The strategy for the study of chromatin transcription by a mammalian RNA polymerase, which is also an essential part of this thesis, is based on several grounds. Investigations, thus far, on the determination of transcriptional properties of chromatin have employed bacterial RNA polymerases and it has been repeatedly observed that only 5-20% of the total DNA in chromatin is available for transcription by bacterial RNA polymerase (Marushige & Bonner, 1966; Paul & Gilmour, 1966; Tan & Miyagi, 1970). These observations have implied that the remainder of the eukaryotic genome is masked by DNA-bound macromolecules (Bonner et al., 1968b; Paul

et al., 1970). For several reasons, the data obtained so far from these studies are not open to definitive interpretation.

One of the most serious drawbacks of these studies is the use of bacterial RNA polymerase for determining the transcriptional specificity of mammalian chromatin. By virtue of specificity and heterogeneity in eukaryotes, not only at cellular level but also at genomic level, it is possible that bacterial and mammalian RNA polymerases may exhibit different specificities, perhaps with respect to the initiation of RNA chains to be transcribed. It is also likely that the binding sites on the DNA for RNA polymerases of entirely different origin may be different or, at the same time, the structural components of chromatin (chromosomal protein or RNA molecules) may invoke mechanisms of absolute specificity with respect to eukaryotic rather than prokaryotic RNA polymerase. There are certain findings which might favour these considerations:

(i) Rifamycin antibiotic is known to prevent bacterial transcription by binding to bacterial RNA polymerase protein rather than DNA template, whereas it has no effect on animal RNA polymerase (Hartmann et al., 1967; Wehrli et al., 1968). On the other hand, α-amanitin toxin is known to inhibit transcription by binding to mammalian RNA polymerase rather than DNA template, but it is without any influence on the bacterial

RNA polymerase activity (Jacob et al., 1970). Differential responses of rifamycin and α-amanitin towards bacterial and mammalian RNA polymerases could, in fact, reflect some radical difference between these two enzymic proteins.

(ii) The DNA-RNA hybridization assays, which were used to characterize the number and kinds of RNA molecules transcribed in vitro by bacterial RNA polymerase from chromatin DNA, might not reflect locus specificity, because probably they are the measure of more redundant sequences present in eukaryotic DNA (Britten & Kohne, 1968; Melli & Bishop, 1969; McCarthy & Church, 1970; Kennell, 1971). Also, the physical characteristics which govern the specificity of hybridization assay are not clearly understood at the present time.

(iii) Newer data on the structure of chromatin suggest that as much as 35-50% of the DNA in chromatin may not be masked by chromosomal proteins (Clark & Felsenfeld, 1971; Itzhaki, 1971).

(iv) Just recently, some evidence has been presented that the rat liver and microbial RNA polymerases bind to and transcribe different sites on the chromatin DNA (Butterworth et al., 1971).

(v) Some workers have now begun to cast serious doubts on the biological validity of those data on chromatin transcription which were previously obtained using bacterial RNA polymerase (Clark & Felsenfeld, 1971; Butterworth et al., 1971).

The foregoing results suggest the possibility that certain distinctive characteristics of chromatin transcription may emerge if mammalian RNA polymerase, instead of bacterial enzyme, is used. This thesis will also deal with such a study. The experimental approach to this type of study was to prepare chromatin from beef brain, treat it to remove chromosomal proteins selectively and then compare the template capacity for RNA synthesis by brain RNA polymerase (a homologous system) and bacterial RNA polymerase (a heterologous system).

CHAPTER TWO: MATERIALS AND METHODS

2.1. MATERIALS

A. CHEMICALS

1. Ribonucleoside Triphosphates (ATP, GTP, CTP and UTP), disodium salts were purchased from Sigma or Calbiochem. Co. Stock solutions were prepared in distilled water and stored at -20°C.

2. [^{3}H]-Ribonucleoside 5'-Triphosphates (^{3}H-ATP, Spec. Act. 24.5 Ci/mmole; ^{3}H-GTP, Spec. Act. 1 Ci/mmole; ^{3}H-CTP, Spec. Act. 20.1 Ci/mmole; ^{3}H-UTP, Spec. Act. 18-25 Ci/mmole), tetralithium salts in ethanol were purchased from Schwarz BioResearch Corp., New York. These were stored at -20°C after desired dilutions with distilled water.

3. Calf thymus DNA was obtained from Worthington Biochemical Corporation. Stock solutions (1-2 mg/ml) in 0.01 M NaCl were prepared by gentle stirring at 4°C with the help for a small magnetic bar and were stored at -20°C.

4. Bovine serum albumin, crystalline, was obtained from Calbiochem.

5. Yeast RNA, highly polymerized, was obtained from Worthington Biochemical Corporation. A 1 mg/ml solution in distilled water was prepared fresh.

6. Calf thymus total histone fraction was obtained from Worthington Biochemical Corporation and a freshly prepared solution was used.

7. β-Mercaptoethanol was obtained from Eastman Kodak Co., New York.

8. Dithiothreitol was obtained from Calbiochem.

9. Spermine tetrahydrochloride and spermidine trihydrochloride were dissolved in distilled water just before the enzyme assay.

10. Ammonium sulphate, crystalline, enzyme grade was obtained from Mann Research Laboratories.

11. Sucrose, density-gradient grade, crystalline, ultrapure was obtained from Mann Research Laboratories. This was specifically used in the analysis of RNA product by sucrose-density gradient centrifugation.

12. DEAE-Cellulose (Cellex D) was obtained from BioRad Laboratories.

13. Ribosomal RNA was kindly provided by Dr. K. Marushige, then at the University of British Columbia, Vancouver.

14. Soluene ^{TM}-100 was obtained from Packard Instrument Co.

15. Whatman GF/C glass filters, 2.4 cm in diameter, were purchased from Reeve-Angel Co., New Jersey.

16. Scintillation fluid, a mixture of 240 g of naphthalene, 15 g of 2,5-diphenyloxazole (PPO) and 150 mg of 1,4-bis-(5-phenyl-oxazolyl-2)-benzene (POPOP) dissolved in one liter each of toluene, 1,4-dioxane and 95% ethanol.

17. Actinomycin D was a gift from Merck, Sharp & Dohme.

18. Rifamycin was purchased from Mann Research Laboratories.

19. α-amanitin was a generous gift from Dr. T. Wieland of the Max-Planck Institute, Heidelberg, Germany.

20. Pancreatic ribonuclease and deoxyribonuclease were purchased from Nutritional Biochemicals Co.

21. Escherichia coli DNA-dependent RNA polymerase (a DEAE-cellulose fraction) was kindly supplied by Dr. Shirley Su Gillam of the University of British Columbia, Vancouver.

22. All other chemicals were reagent grade.

B. BIOLOGICAL SYSTEM

Beef brain. This was generously supplied by the Intercontinental Packers Ltd., Vancouver, through the courtesy of Mr. M. Knight.

2.2. METHODS

A. PROCESSING OF TISSUE, THE CEREBRAL CORTEX

Beef brains were obtained fresh, packed in ice, from the slaughterhouse. The cerebral hemispheres were cut-off from the remaining brain. These were cleaned free of dural membranes and

blood clots, as far as possible, with the help of a forcep. The cerebral cortex was then rinsed into a large volume of ice-cold 0.25 M sucrose and blotted on Whatman paper before use.

B. ISOLATION OF NUCLEI FROM CEREBRAL CORTEX

The isolation of purified nuclei from cerebral cortex was carried out based on the method of Chauveau et al. (1956), as modified in the following manner. All experimental manipulations were done at 0-4°C. 15 g of cerebral tissue was cut into small pieces with a pair of scissors and homogenized in 3 volumes of ice-cold Medium I (2.2 M sucrose containing 1 mM $MgCl_2$) in a glass Potter-Elvehjem homogenizer fitted with a Teflon pestle (Clearance 0.006 - 0.009 inch, size C, Arthur H. Thomas Co.), employing 5 to 6 up and down motor strokes, driven at powersetting #35. The homogenate was immediately mixed with 2 more volumes of the Medium I. 25 ml aliquots of this homogenate were layered on top of 5 ml of Medium I contained in a cellulose nitrate tube for the Spinco rotor SW 25.1. After 60 min centrifugation at 22,500 rpm in a Beckman Model L Ultracentrifuge, the nuclei sedimented at the bottom of the tube as a gelatinous mass (white to light pink in colour). The nuclei were separated from cell debris and cytoplasmic components (which remain as a pellicle on the top) by decantation with the help of a spatula. The tubes were kept in an inverted position for

about 2 min to drain the adhering sucrose solution and the inside of the tubes was then wiped with cellulose wipe-paper.

The isolated nuclei were either used fresh or stored at 0°C for periods of 12-20 h.

C. SOLUBILIZATION AND INITIAL FRACTIONATION OF RNA POLYMERASE

The purified nuclei, which were isolated by the method outlined above, were used for the solubilization of RNA polymerase. The nuclei were gently suspended using a Potter-Elvehjem homogenizer in the solubilization medium which contained 0.05 M Tris-HCl buffer, pH 7.9, 0.01 M DTT, 0.2 M KCl and 30% (V/V) glycerol. After about 5 min, the viscous nuclear suspension was sonicated in 20-25 ml portions for a period of 90 sec at 0°C using a Branson Sonifier (9 bursts of 10 sec each at 4.2 amp voltage with an interval of 20 sec after each burst for cooling). The nuclear sonicate was centrifuged at 127,000 x g/h in a Spinco Rotor #50 (in routine work at 37,000 x g/1.5 h in a Sorvall Centrifuge, Model RC-2). The pellet was discarded while the clear supernatant (Fraction I) was half-saturated with respect to ammonium sulphate by the drop-wise addition of saturated ammonium sulphate solution which was pre-adjusted to pH 7.9 with liquid ammonia. While adding the ammonium sulphate, the solution was stirred slowly but constantly using a magnetic bar at 0°C. After 45 min of additional stirring, the precipitate formed was collected by centrifugation at 27,000 x g/h and sus-

pended in TDG-buffer containing 0.05 M NH_4Cl. After dialysis against the same buffer for 3-5 h at 4°C, the non-dialyzate was clarified by centrifuging at 27,000 x g/30 min to remove any residual material. The dialyzed supernatant (Fraction II) was either immediately subjected to DEAE-cellulose chromatographic analysis or stored at -65°C for periods no longer than a day.

Throughout this procedure, especially at the solubilization and ammonium sulphate fractionation steps, extra care was taken to avoid any frothing which otherwise develops and renders lower recoveries of the enzyme activity.

D. PARTIAL PURIFICATION OF RNA POLYMERASE

(a) Preparation of DEAE-cellulose:

Commercially obtained DEAE-cellulose (Cellex D), exchange capacity 0.84 meq/g, was thoroughly washed before use according to the instructions of Peterson and Sober (1962). The absorbent was repeatedly used after washing with 0.5 N NaOH.

Washed DEAE-cellulose was suspended in 0.05 M Tris-HCl, pH 8.0, decanted off and was re-suspended in the equilibrating buffer (TDG-buffer containing 0.05 M NH_4Cl).

(b) DEAE-cellulose chromatography of the enzyme:

Columns of approximately 1 cm diameter x 20 cm dimensions were used. DEAE-cellulose slurry in the equilibrating buffer was allowed to pack by gravitational force. For final equilibration of the column, 6-8 bed volumes of the equilibrating buffer were allowed to percolate through just before use.

Preparations of solubilized enzyme (Fraction II containing 6-10 mg protein/ml) were loaded on the column. After the enzyme solution had been absorbed on to the column bed, the column was washed with approximately 3 bed volumes of the equilibrating buffer, followed by the elution of enzyme routinely by stepwise increase in the concentration of NH_4Cl in TDG-buffer (at first, from 0.05 M to 0.15 M, then to 0.3 M, and finally to higher than 0.3 M). In some analytical work, the elution was carried out by a linear-gradient of NH_4Cl in TDG-buffer, produced by mixing 100 ml each of 0.05 M NH_4Cl and 0.6 M NH_4Cl contained in separate beakers (250 ml capacity) connected by a tygon plastic tube. 3 ml fractions were usually collected at a flow rate of 30-40 ml/h.

Storage of all enzyme fractions was conducted at -65°C in the presence of higher concentrations of glycerol, 30-50% (V/V), for periods of no longer than 2 weeks. They were thawed at 0°C just prior to the enzyme preparation or enzyme assay and were utilized usually within a week.

E. MEASUREMENT OF RNA POLYMERASE ACTIVITY

The determination of RNA polymerase activity was based upon the original reaction of Weiss (1960), which measures the initial rate of incorporation of radioactivity from a labelled ribonucleoside triphosphate into TCA-insoluble material (RNA).

(a) RNA Polymerase Assay:

For the standard assay, the reaction mixture contained 30 mM Tris-HCl buffer, pH 8.0, 3 mM $MnCl_2$, 6 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.6 mM each of ATP, CTP and GTP, 1 μCi of [5-^{3}H]-UTP (Spec. Act., 18-25 Ci/mmole), 100 μg of CT-DNA and the enzyme preparation in a final volume of 0.5 ml. Incubation was carried out at 37°C for 20 min.

The reaction was terminated by quick quenching of the assay tubes into an ice-bath, followed by the addition of 2 ml of ice-cold 10% (W/V) TCA and thorough mixing on a Vortex mixer. Either 0.3 ml of a 2% (W/V) BSA solution or 0.2 ml of a 1% (W/V) yeast RNA solution were then added. After standing for 10 min on ice, the TCA-precipitated samples were processed as described below for the measurement of radioactivity.

(b) Measurement of Radioactivity:

This was carried out either by centrifugation or by filtration methods as described below:

(i) Centrifugation Method: TCA-precipitated samples containing 0.6 mg BSA were analyzed by this method. The samples were centrifuged for 4 min in a clinical model centrifuge. The precipitate thus collected was washed 3 times with 5 ml portions of ice-cold 5% (W/V) TCA. TCA was drained off by leaving the tubes in an inverted position for about 3 min. Finally, the precipitate was dissolved in 0.2 ml of Soluene ™-100 and with the help of a Pasteur pipet it was transferred to the counting vials containing 10 ml of the scintillation fluid.

(ii) Filtration Method: TCA-precipitated samples containing 0.2 mg of yeast RNA were analyzed by this method. The samples were filtered through the glass-fiber filters (Whatman GF/C). Each assay tube was rinsed onto the filter with 5 ml of ice-cold 5% (W/V) TCA. Every filter was then washed 5 times with 5 ml portions of cold 5% (W/V) TCA, air-dried and transferred to the counting vials. Filters were dispersed in 10 ml of scintillation fluid in the presence of 0.3 ml Soluene ™-100.

Both these methods of radioactivity measurement were used because practically there was no difference (qualitative or quantitative) in the data obtained.

The vials were cooled overnight at 4°C and the radioactivity of the samples was counted in a Nuclear-Chicago Scintillation Spectrometer, Model 6848. The counting efficiency was usually about 32%.

Unless otherwise defined, standard incubation and assay conditions were used for the determination of enzyme activity. The additions into or deletions from the standard assay system of any component in individual experiments are given in the legend for the appropriate figure or table. In order to maintain the sensitivity of the enzyme assay, the determinations were done at non-saturating concentrations of UTP, the ^{3}H-labelled precursor which was used to monitor the synthesis of RNA *in vitro*.

One unit of enzyme activity corresponds to the incorporation of one picomole of UMP into RNA in 20 min at 37°C under the standard conditions for enzyme assay.

The RNA polymerase activity is expressed as the specific activity of UMP (picomoles/mg of protein) or the specific radioactivity (counts per min/mg of protein) incorporated into RNA.

Whenever required, native CT-DNA (2 mg/ml in 0.01 M NaCl) was heat-denatured, just prior to the enzyme assay, in boiling water for 10 min and was quickly quenched in ice.

F. PREPARATION AND OTHER TREATMENTS OF CEREBRAL CHROMATIN

Chromatin was prepared at 0-4°C by a method modified from that of Clark and Felsenfeld (1971). The purified nuclei, isolated from cerebral cortex, were gently homogenized in EDTA-buffer (0.02 M EDTA-Na_2, 0.08 M NaCl and 0.005 M Tris-HCl, final pH 5.4). The

nuclear homogenate was centrifuged at 9,750 x g/10 min and the pellet so collected was washed twice with EDTA-buffer, followed by two additional washings with 0.02 M Tris-HCl, pH 8.0. The final gelatinous pellet was suspended in 0.02 M Tris-HCl, pH 8.0 and was sheared in a Branson Sonifier for 1.5 min at 0°C (9 bursts of 10 sec each at 3 amp voltage with an interval of 20 sec after every burst for cooling). The sonicated solution was centrifuged at 12,100 x g/30 min and the resulting supernate constituted the final chromatin product. The chromatin, stored at 0°C, was used to study the template activity within 24 h after preparation.

Removal of histones from cerebral chromatin was carried out at 0°C by adding 0.25 ml of ice-cold 2 N H_2SO_4/ml of chromatin solution (final concentration of H_2SO_4 being 0.4 N). The solution was thoroughly mixed on a Vortex mixer, allowed to stand at 0°C for 30 min with occasional mixing and then centrifuged at 27,000 x g/20 min. The pellet thus obtained was washed with cold distilled water using a glass rod and was suspended by gentle homogenization in 0.02 M Tris-HCl, pH 8.0. This material, prepared fresh was used as the dehistonized chromatin template.

The de-proteinization of cerebral chromatin was based on the method of Marushige and Dixon (1969).

G. CHEMICAL ANALYSIS OF RNA, DNA AND PROTEIN

The separation of RNA, DNA and protein into individual fractions was carried out according to the method of Schneider (1957). An aliquot of the test sample was precipitated at 0°C with TCA (final concentration 5% W/V), washed twice with 5% (W/V) TCA and once with 95% ethanol (5 ml portions each time). Insoluble precipitate was dissolved in 2 ml of 0.2 N KOH and left overnight at 37°C. 0.05 ml of 70% perchloric acid was added while the sample was pre-cooled to 0°C. The supernatant obtained by centrifugation in a clinical model centrifuge was used for RNA determination by orcinol reaction (Schneider, 1957). The residue was washed once with 5% (W/V) TCA and extracted in 5% (W/V) TCA at 90°C for 15 min. DNA in the extract was determined by diphenylamine reaction (Schneider, 1957). The final pellet was washed once with 95% ethanol, dissolved in dilute alkali and was used for protein determination by the method of Lowry *et al*. (1951).

Yeast RNA, CT-DNA (in 5% TCA) and bovine serum albumin were employed as the standards for the determination of RNA, DNA and protein, respectively.

For the measurement of small quantities of RNA, DNA and protein in the samples, aliquots were pipetted out without going through the above extraction procedure.

The determination of basic proteins (histones) was carried out in the following manner: An aliquot of chromatin fraction was separately treated with H_2SO_4 (0.4 N) and the acid-soluble fraction was mixed with 3 volumes of 95% ethanol and left overnight at -20°C. The precipitate obtained by centrifugation at 27,000 x g/20 min was dissolved in dilute alkali and the content of histone proteins was determined by the method of Lowry et al. (1951).

The absorbance at 260 nm for nucleic acids and 280 nm for proteins was followed for the purpose of qualitative analysis.

H. MISCELLANEOUS METHODS

(a) Analysis of Contaminating Nucleases:

The activities of ribonuclease and deoxyribonuclease enzymes were assayed by measuring the release of acid-soluble material in 0.7 N perchloric acid according to Rosenbluth and Sung (1969).

(b) Analysis of RNA Product by Sucrose-Density Gradient Centrifugation

All enzyme assays for the purpose of RNA product analysis by sucrose-density gradient centrifugation were carried out for 60 min in the presence of 3-fold higher radioactive concentration of ^{3}H-UTP (i.e., 3 µCi/assay instead of 1 µCi/assay in the standard reaction mixture).

At the end of the incubation, the assay tubes were immediately cooled in ice and 0.1 ml of carrier UTP (20 mM solution) was added. Whenever treated with SDS, 0.1 ml of a 1% (W/V) SDS solution was added, followed by incubation at 37°C for 5 min. A 0.4 ml aliquot of this assay mixture was carefully layered on top of a linear gradient of sucrose, pre-cooled to 4°C. The sucrose-gradients were prepared (using a Density-Gradient sedimentation system of Buchler Instruments Inc., New Jersey) by mixing 2.3 ml each of 5% (W/V) and 20% (W/V) sucrose solutions in 0.01 M Tris-HCl, pH 8.0 containing 0.1 M NH_4Cl. After centrifugation at 39,000 rpm/5 h in a Spinco Rotor #SW 39, fractions of 15 drops were collected by piercing with a hypodermic needle from the bottom of the cellulose nitrate tube. Fractions were made TCA-insoluble in the presence of 0.2 mg of yeast RNA as the carrier and were processed for the measurement of radioactivity according to the filtration method (see Chapter 2.2 E, b).

Unlabelled rat liver RNA was used as a reference. After the centrifuge run, fractions of 15 drops were diluted with 3 ml portions of distilled water and the absorbance at 260 nm was read in a Beckman Model DU Spectrophotometer.

CHAPTER THREE: EXPERIMENTAL RESULTS

3.1. SOLUBILIZATION AND PARTIAL PURIFICATION OF BRAIN NUCLEAR RNA POLYMERASE

A. PURITY OF ISOLATED NUCLEI

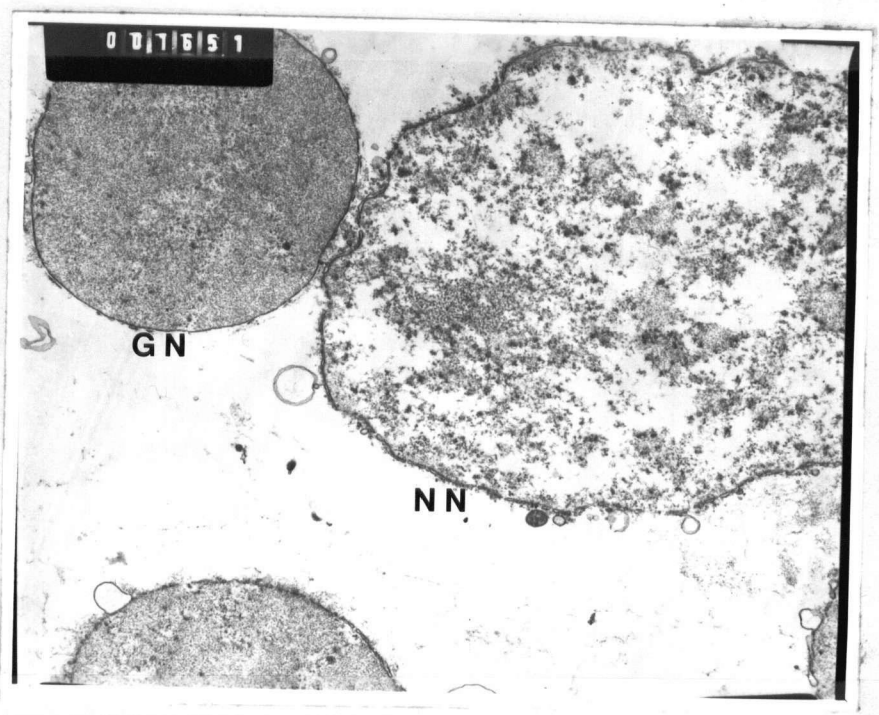
The nuclear pellet, isolated according to the method outlined in Chapter 2.2B, contained highly purified and intact nuclei from both neuronal and glial cell populations of cerebral tissue (Fig. 1A). The criteria used to define the types of nuclei from beef cerebral cortex are those described by others (Sporn et al., 1962; Lovtrup-Rein & McEwen, 1966; Kato & Kurokawa, 1967; Rappoport et al., 1969). The neuronal nuclei are considerably larger in size than the glial nuclei, and display a light background of the nucleoplasmic chromatin with a prominent nucleolus. The nuclei from glial cells have denser nucleoplasm without apparent nucleoli. Some of the important morphological features of nuclear structure, such as the double-layered nuclear envelope, nucleoplasmic diffused chromatin and a prominent nucleolus, were visualized clearly in a typical neuronal nucleus (Fig. 1B). As judged by low magnification electron microscopy, there was no contamination of whole cell or any other cytoplasmic organelles, for example, the mitochondria. As shown in Table I, the yields of nuclear DNA and RNA, on the average, were 402 µg/g wet tissue and 98 µg/g wet tissue, respectively. The RNA to DNA

FIG. 1. THE LOW MAGNIFICATION ELECTRON MICROGRAPHS OF NUCLEAR PREPARATIONS FROM BEEF CEREBRAL CORTEX

(A) Showing nuclei from two different cell populations of brain, namely, the smaller glial-cell nuclei (GN) and the larger neuronal-cell nuclei (NN). x 9,710.

(B) Showing the salient features of nuclear structure in a typical neuronal nucleus, i.e. the double-layered nuclear envelope (NE), nucleoplasmic chromatin (Np) and nucleolus (Nc). x 15,048.
Freshly prepared nuclei were embedded in 2% agar, sliced and fixed in 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.5, and were post-fixed in 1% OsO_4 in the same buffer. The sections of 60-90 mμ thickness were stained with uranyl magnesium acetate and lead citrate. The photographs were taken using a Philips 300 electron microscope.

A



B

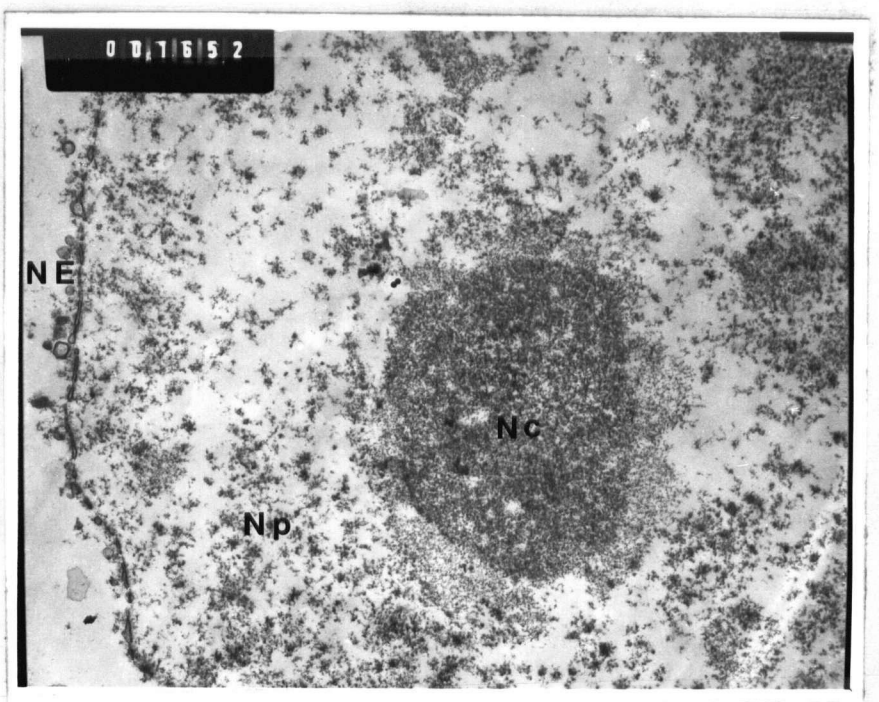


TABLE I. THE CONTENT OF DNA AND RNA IN THE NUCLEAR PREPARATIONS OF BEEF CEREBRAL CORTEX

	RNA (µg/g wet tissue)	DNA (µg/g wet tissue)	Mass Ratios $RNA/_{DNA}$
	82.4	380	0.217
	104.0	376	0.276
	98.0	370	0.265
	100.0	448	0.223
	110.0	470	0.234
	96.0	370	0.259
* Mean:	98.4	402.3	0.245

* Six independent determinations were conducted in duplicate as described in Methods (Chapter 2.2E, a)

mass ratio of approximately 0.245 is of the same order as that described by others for the purified nuclear fractions from the brain of an adult animal (Sporn et al., 1962; Lovtrup-Rein & McEwen, 1966; Balazs & Cocks, 1967). It should be noted that a lower mass ratio of RNA to DNA is biochemical evidence for freedom of isolated nuclei from contaminating cytoplasmic RNA (see Wang, 1967; Busch, 1967).

B. SOLUBILIZATION AND SEPARATION OF BRAIN NUCLEAR RNA POLYMERASES

RNA polymerase from various bacterial sources is solubilized simply by mechanical disruption of cells in the presence of low ionic strength buffer (Chamberlin & Berg, 1962). The satisfactory and consistent methods of purification of bacterial RNA polymerase, which yield significant quantities of the highly purified enzyme (i.e. the yields of 100-400 mg of enzyme per kg of wet cells and purities above 95%), have been described (Zillig et al., 1966; Burgess, 1969; Berg et al., 1971). Similar means of solubilizing the nuclear RNA polymerase from mammalian sources have not been successful, possibly because nuclear enzyme is firmly-bound to the insoluble nucleoprotein matrix (Weiss, 1960). This is one of the major reasons that the progress in the field of mammalian RNA polymerase has been very slow.

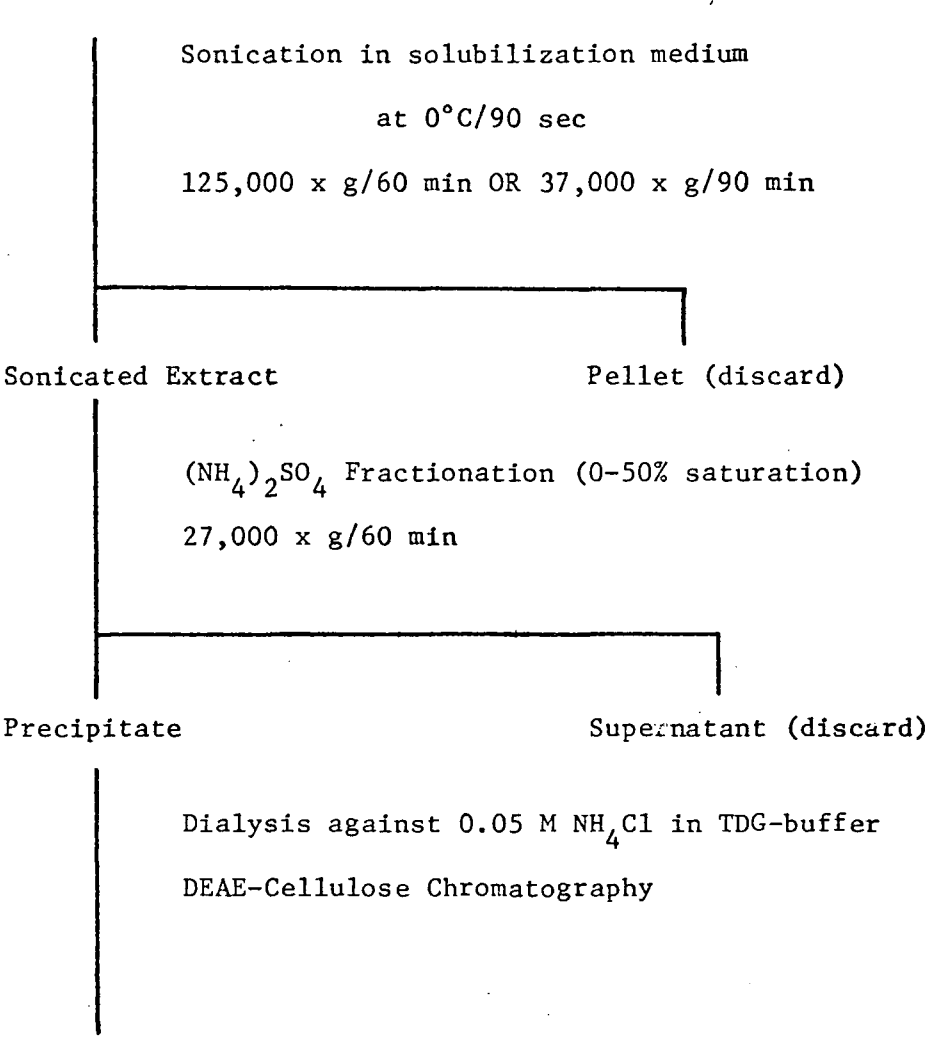
In recent years, various attempts to solubilize the nuclear RNA polymerase have included gentle homogenization, lysis or sonication either in isotonic buffer solutions around neutral pH or

in buffers of high ionic strength in slightly alkaline pH range. The necessity for a variety of conditions required to extract nuclear enzyme might be due to the difference in mammalian sources used. We failed to extract RNA polymerase from beef brain nuclei by gentle homogenization or lysis in isotonic buffer solutions. Preliminary success in solubilizing brain nuclear RNA polymerase was obtained by sonication in a buffer of low ionic strength containing higher concentrations of glycerol (Singh & Sung, 1970, 1971a). Subsequently, it was found that more enzyme can be extracted by homogenizing the residual material in Tris-OH, pH 10.4 (Singh & Sung, 1971b) or more effectively in 0.2 M KCl contained in the solubilization medium. The replacement of KCl by $(NH_4)_2SO_4$, which has been utilized for the purpose of solubilization of rat liver or sea urchin enzyme (Roeder & Rutter, 1969) or calf thymus enzyme (Kedinger et al., 1970), did not solubilize the RNA polymerase activity from brain nuclei. Approximately, 80% of the nuclear RNA polymerase was solubilized by sonication in the presence of 0.2 M KCl dissolved in the solubilization medium as given in Chapter 2.2. From the point of view of calculating the recoveries of solubilized enzyme, it should be noted that the extracted enzyme is almost completely dependent on the addition of exogenous DNA whereas the activity of nuclear suspension is not, because the endogenous nucleo-protein complex serves the function of template. The difference in template function may influence the

yield of soluble enzyme markedly. Therefore, the expression of recovery of RNA polymerase activity solubilized from isolated brain nuclei is in relative terms only.

The solubilized RNA polymerase was partially purified by ammonium sulphate fractionation and DEAE-cellulose chromatography, as outlined in Figure 2. Two peaks of RNA polymerase activity were resolved on a DEAE-cellulose column. The first peak of enzymic activity was eluted in the flow-through (at 0.05 M NH_4Cl concentration) at the end of protein peak followed by a second peak of enzymic activity eluted at 0.3 M NH_4Cl concentration (Fig. 3). These peaks of polymerase activity, which were characterized by certain catalytic properties (to be described in the next section of this Chapter), were termed RNA polymerase I and polymerase II, respectively, in conformation of the terminology developed for mammalian RNA polymerases (Roeder & Rutter, 1969). The data obtained from a typical experiment on the partial purification of brain nuclear RNA polymerases are summarized in Table II. Although there was no significant increase in the specific activity of sonicated extract on ammonium sulphate fractionation, this step has the merit of concentrating the relatively large volumes of soluble enzyme. The chromatography on a DEAE-cellulose column resulted in an approximately 12-fold increase in the specific activity of RNA polymerase II while the specific activity of RNA polymerase I remained without

FIG. 2. AN OUTLINE FOR THE PARTIAL PURIFICATION OF BRAIN NUCLEAR RNA POLYMERASE



(The details of each step are given in Chapter 2.2)

FIG. 3. CHROMATOGRAPHIC RESOLUTION OF BRAIN NUCLEAR RNA POLYMERASES ON A DEAE-CELLULOSE COLUMN

About 56 mg protein of the ammonium sulphate fraction (0-50% saturation), which has been dialyzed against the equilibrating buffer, was loaded on a DEAE-cellulose column (1.2 x 18 cm). After washing with one column volume of equilibrating buffer, the elution was accomplished by a linear gradient of NH_4Cl (0.05 - 0.6 M) dissolved in TDG-buffer, the total volume being 200 ml. Fractions of 3.5 ml each were collected. Aliquots of 0.1 ml were assayed for RNA polymerase activity with native CT-DNA as template under the conditions given in Methods (Chapter 2.2E, a).

(o ——— o) Absorbance at 280 nm; (● - - - - ●) RNA polymerase activity expressed as the radioactivity (cpm) of ^{3}H-UMP incorporated; (.....) NH_4Cl concentration gradient.

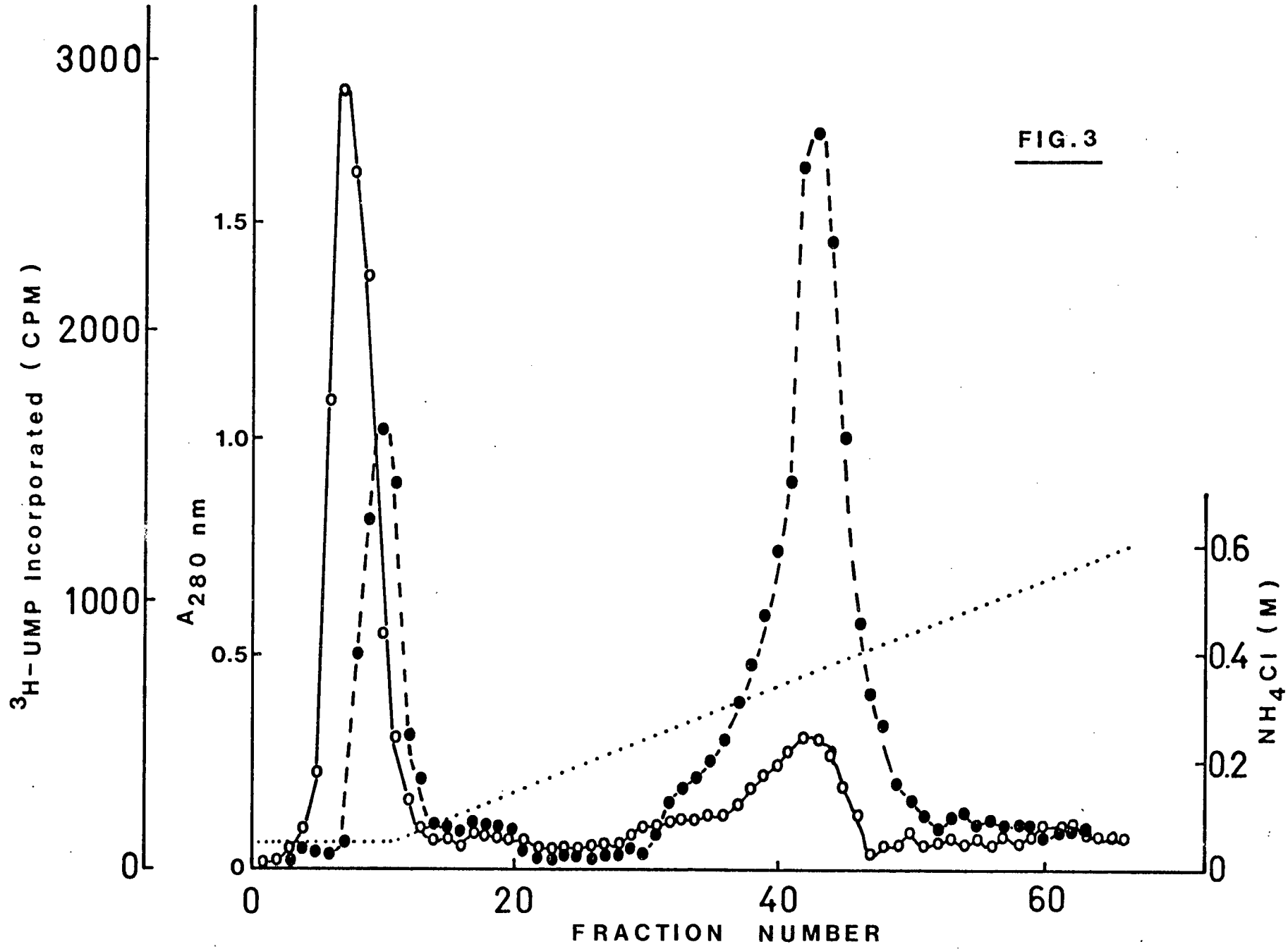


FIG. 3
^{3}H-UMP Incorporated (CPM)
3000
2000
1000
0
A_{280} nm
1.5
1.0
0.5
0
NH_4Cl (M)
0.6
0.4
0.2
0
0
20
40
60
FRACTION NUMBER

TABLE II. PARTIAL PURIFICATION OF MULTIPLE RNA POLYMERASES FROM BEEF BRAIN NUCLEI

Fractions	Total Protein (mg)	Total Activity (Units)	Yield of Activity (%)	Specific Activity (Units/mg)	Fold Purification
Sonicated Extract*	141.75	124.34	100	0.875	1
Ammonium Sulphate	56.78	99.45	80	1.75	2
DEAE-Cellulose** Chromatography					
(a) Polymerase I	6.07	11.08	8.9	1.82	2.08
(b) Polymerase II	3.40	36.06	29	10.59	12.1

* The high-speed supernatant, obtained from the nuclei isolated from 120 g wet brain tissue, constituted the sonicated extract.

** Most active fractions from a DEAE-cellulose column (1.2 x 19 cm) are included in this Table.

any significant purification. It should be considered that the specific activity of sonicated extract is predominantly due to polymerase II and hence the DEAE-cellulose chromatography does not bring about any significant increase in the specific activity of polymerase I. The deterioration of polymerase I activity during the purification procedure may also affect the specific activity of this enzyme. Usually, the yield of total enzyme activity recovered from the DEAE-cellulose column was about 90% of that of the fraction charged on to it.

The RNA polymerase activity of sonicated extract as well as that of chromatographed fractions was very unstable and thus could not be preserved for longer periods. The addition of substrates (ribonucleoside triphosphates) did not prevent loss of enzyme activity. Native calf thymus DNA (0.1 mg/ml) and bovine serum albumin (0.1 mg/ml) were somewhat effective in the stabilization of the enzyme activity. The deterioration of the enzyme activity was prevented most effectively by high concentrations of glycerol (higher than 25%) if the enzyme fractions are stored at -65°C. Under these conditions, the sonicated extract could be stored for about 2 weeks without any significant loss of enzyme activity, about 65-75% of RNA polymerase II activity could be preserved for about 10 days and only 50% of RNA polymerase I was detectable after about 2 days of storage.

The ratio of absorbance at 280 nm to 260 nm in the enzymatically most active fractions was usually around 1.0, indicating the presence of some nucleic acid material as the contamination.

On the other hand, there was no detectable contaminating activity of ribonuclease or deoxyribonuclease as determined at pH 8.0, the pH value at which the brain nuclear RNA polymerase activity is maximally assayed.

3.2. CATALYTIC PROPERTIES OF BRAIN NUCLEAR RNA POLYMERASES

A. GENERAL CHARACTERISTICS OF RNA POLYMERASE REACTION

The results summarized in Tables III and IV establish that the assay for RNA polymerase activity, which has been routinely used in this work, measures the synthesis of RNA as directed by DNA template. This conclusion is supported by the fact that the solubilized RNA polymerase from beef brain nuclei requires the presence of buffer, a divalent cation (Mn^{++} or Mg^{++}), a sulfhydryl reagent (β-mercaptoethanol), all four ribonucleoside triphosphates (ATP, UTP, CTP and GTP) and DNA. The omission of any of these components from the complete reaction mixture significantly decreased the incorporation of ^{3}H-UTP into TCA-insoluble precipitate. Moreover, this incorporation was considerably inhibited (up to the extent of 65%) by the presence of added pancreatic DNase and actinomycin D (Table III). The inhibition by deoxyribonuclease is related with the hydrolysis of the DNA template whereas actinomycin D has been shown to bind to certain regions on the DNA template and thereby prevent the RNA synthesis (see Goldberg & Friedman, 1971). These data clearly establish that the RNA polymerase activity

TABLE III. REQUIREMENTS OF RNA POLYMERASE SOLUBILIZED FROM BEEF BRAIN NUCLEI

Reaction mixture	UMP incorporated into RNA	
	pmoles/mg protein	% of control
Complete*	1.560	100
- Mn^{++}, Mg^{++}	0.575	37
- ATP	0.448	28
- ATP, CTP, GTP	0.264	17
- DNA	0.268	17
- enzyme	0.043	<3
- β-mercaptoethanol	1.220	78
+ DNase (25 µg/ml)	0.645	41
+ Actinomycin D (2.5 µg/ml)	0.545	35

* The complete reaction mixture was the same as described in Methods (Chapter 2.2E, a). Incubation was carried out at 37°C for 20 min.

TABLE IV. TREATMENT OF THE REACTION PRODUCT BY RNase, DNase AND ALKALI

Additions*	Acid-insoluble cpm	% of control
Control	2042	100
Control + None	2758	136
Control + RNase (25 µg/ml)	1381	68
Control + DNase (25 µg/ml)	2071	101
Control + RNase (25 µg/ml) + DNase (25 µg/ml)	1057	51
Control + KOH (0.2 N, overnight at 37°C)	132	6

* Additions were made at the end of 20 min incubation at 37°C (Control) and the reaction mixture was further incubated for 30 min at 37°C.

of brain nuclear sonicated extract is DNA-dependent. The RNA nature of the reaction product was demonstrated by its sensitivity either to ribonuclease or to alkaline digestion, while DNase was without any effect (Table IV). These experiments were conducted using a crude soluble preparation of brain nuclear RNA polymerase. Subsequent work on the purification of this enzyme led to the resolution of two RNA polymerase activities on a DEAE-cellulose column (as discussed earlier in this Chapter), and, therefore, it was essential to re-examine the requirements for RNA synthesis by the separated enzymic fractions. As shown in Table V, the activity of both RNA polymerases I and II were dependent on DNA and required the presence of a divalent cation, and sulfhydryl reagent and all four ribonucleoside triphosphates.

B. EFFECT OF ENZYME CONCENTRATION

The incorporation of labelled UTP into acid-insoluble material, as catalyzed by RNA polymerases I and II from beef brain nuclei, was found to be directly proportional to the amount of enzyme in the range of 0-200 µg (Fig. 4). In all the experiments reported in this thesis, the amount of enzyme used was within this range. From Figure 4 it is clear that polymerase I elicited almost half the activity of polymerase II at around similar concentrations of enzymic proteins.

TABLE V. REACTION REQUIREMENTS OF BRAIN NUCLEAR RNA POLYMERASES I AND II

Reaction Mixture	Radioactivity of UTP incorporated			
	RNA Polymerase I		RNA Polymerase II	
	cpm/assay	% of Control	cpm/assay	% of Control
* Complete (Control)	560	100	1850	100
- Mn^{++}, Mg^{++}	32	5.9	23	1.2
- β-mercaptoethanol	356	63.5	1130	61
- ATP	122	21.8	172	9.3
- ATP, CTP, GTP	135	24	178	9.6
- DNA	28	5	55	2.9
- Enzyme	18	3.2	23	1.2

* The complete reaction mixture was the same as described in Methods (Chapter 2.2E, a) containing 156 µg and 110 µg protein of the most active fractions of polymerases I and II from DEAE-cellulose column, respectively. Incubation was for 20 min at 37°C.

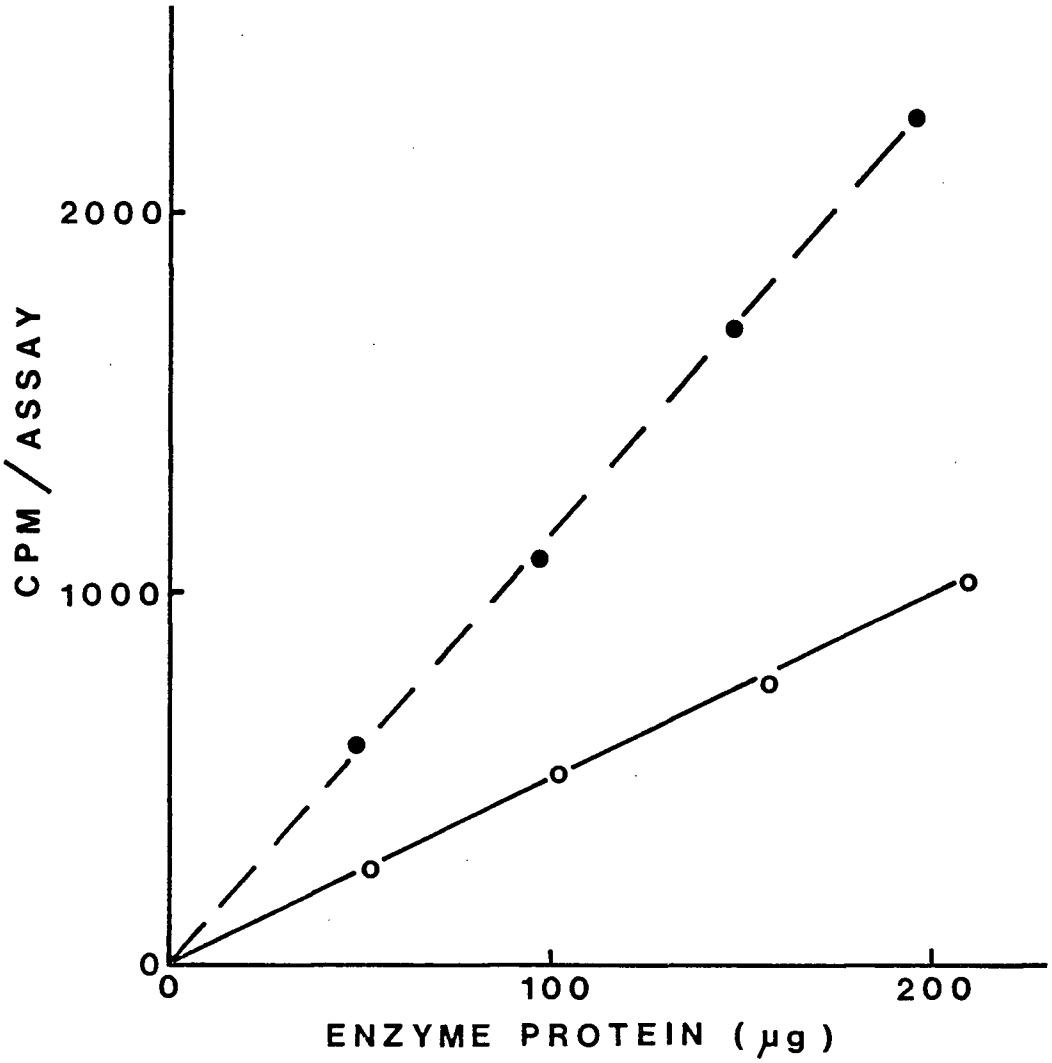


FIG. 4. THE EFFECT OF INCREASING AMOUNTS OF ENZYME PROTEIN ON RNA SYNTHESIS

The RNA synthesis was carried out at 37°C for 20 min using various amounts of polymerase I (o ——— o) or polymerase II (• - - - •) contained in the complete reaction mixture as given in Chapter 2.2E, a.

C. EFFECT OF pH

The enzyme activity-pH relationship of two RNA polymerases isolated from beef brain nuclei is depicted in Figure 5. As seen in this Figure, the activity of both the polymerases was maximally assayed near pH 8.0.

D. EFFECT OF DIVALENT CATIONS

The requirements for divalent cations (Mn^{++} or Mg^{++}) of brain nuclear RNA polymerases I and II are shown in Figure 6. The activity of RNA polymerase I was stimulated more by Mg^{++} than Mn^{++} reaching an optimum around 8 mM for Mg^{++} and about 3 mM for Mn^{++} (Fig. 6A). The ratio of Mn^{++}/Mg^{++}-activities for this enzyme (at their optimum concentrations) was lower than one. On the contrary, RNA polymerase II preferred Mn^{++} to Mg^{++} with optimum concentrations of about 3 mM and 6-8 mM, respectively (Fig. 6B). At these optimum concentrations, the ratio of Mn^{++}/Mg^{++}-activities for polymerase II was approximately 3-4. Moreover, both the enzymes exhibited highest activity in the presence of Mn^{++} and Mg^{++} together. This effect was not additive because the enzyme activity observed in the presence of Mn^{++} plus Mg^{++} was never equal to the sum of the enzymic activities measured with Mn^{++} or Mg^{++} alone.

E. EFFECT OF KCl

Figure 7 depicts the effect of various concentrations of KCl on the activity of polymerase I and polymerase II with native DNA

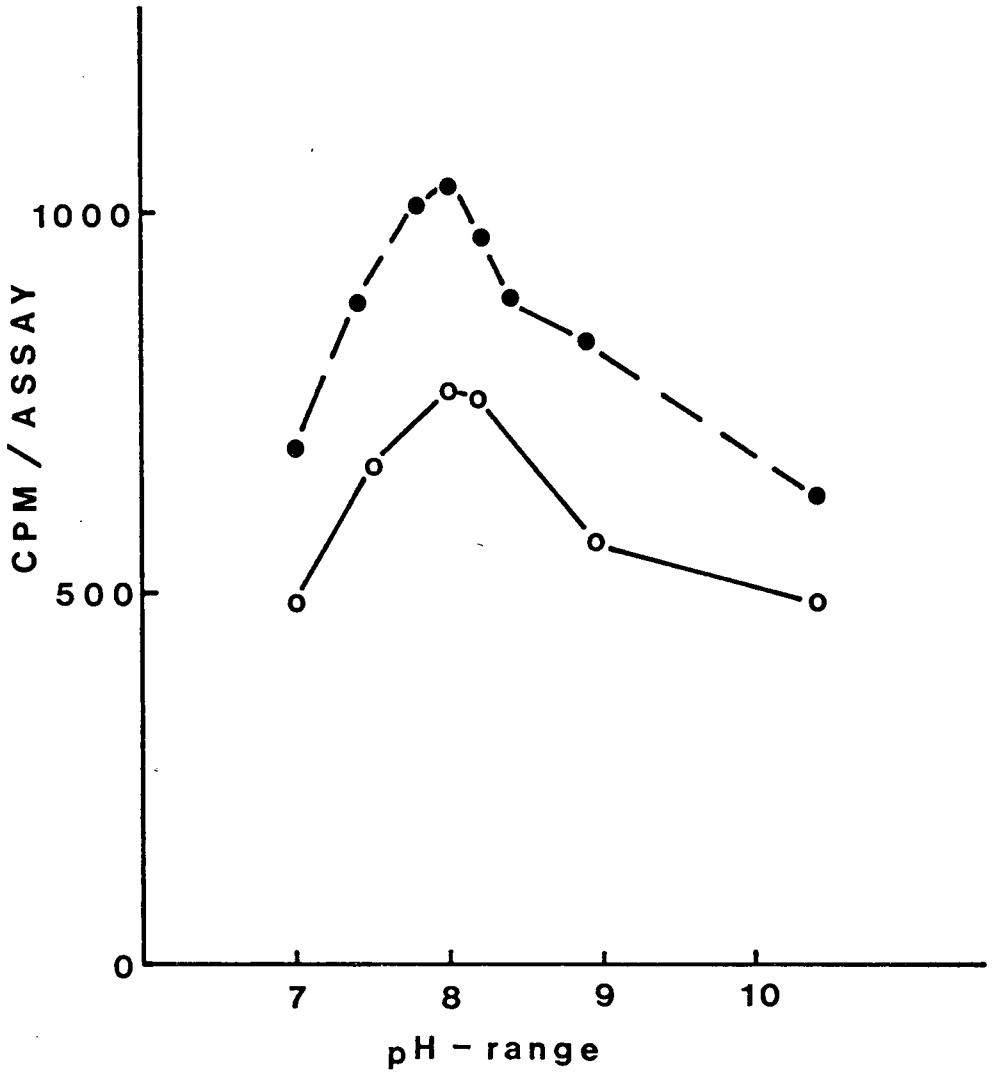


FIG. 5. THE ENZYME ACTIVITY-pH RELATIONSHIP

The activity of polymerase I (o ——— o) and polymerase II (● - - - - ●) was assayed in the presence of 0.03 M Tris-buffers of different pH values. Other assay conditions were the same as described in Chapter 2.2E, a.

FIG. 6. THE EFFECT OF DIVALENT CATIONS ON BRAIN NUCLEAR RNA POLYMERASES

The activities of RNA polymerase I (6A) and II (6B) as determined in the presence of various concentrations of Mn^{++} (o.....o), Mg^{++} (●— — —●) and Mn^{++} plus Mg^{++} (o————o). The amount of enzymic protein per assay was 146 µg and 99 µg for polymerases I and II, respectively. Incubation was carried out at 37°C for 20 min.

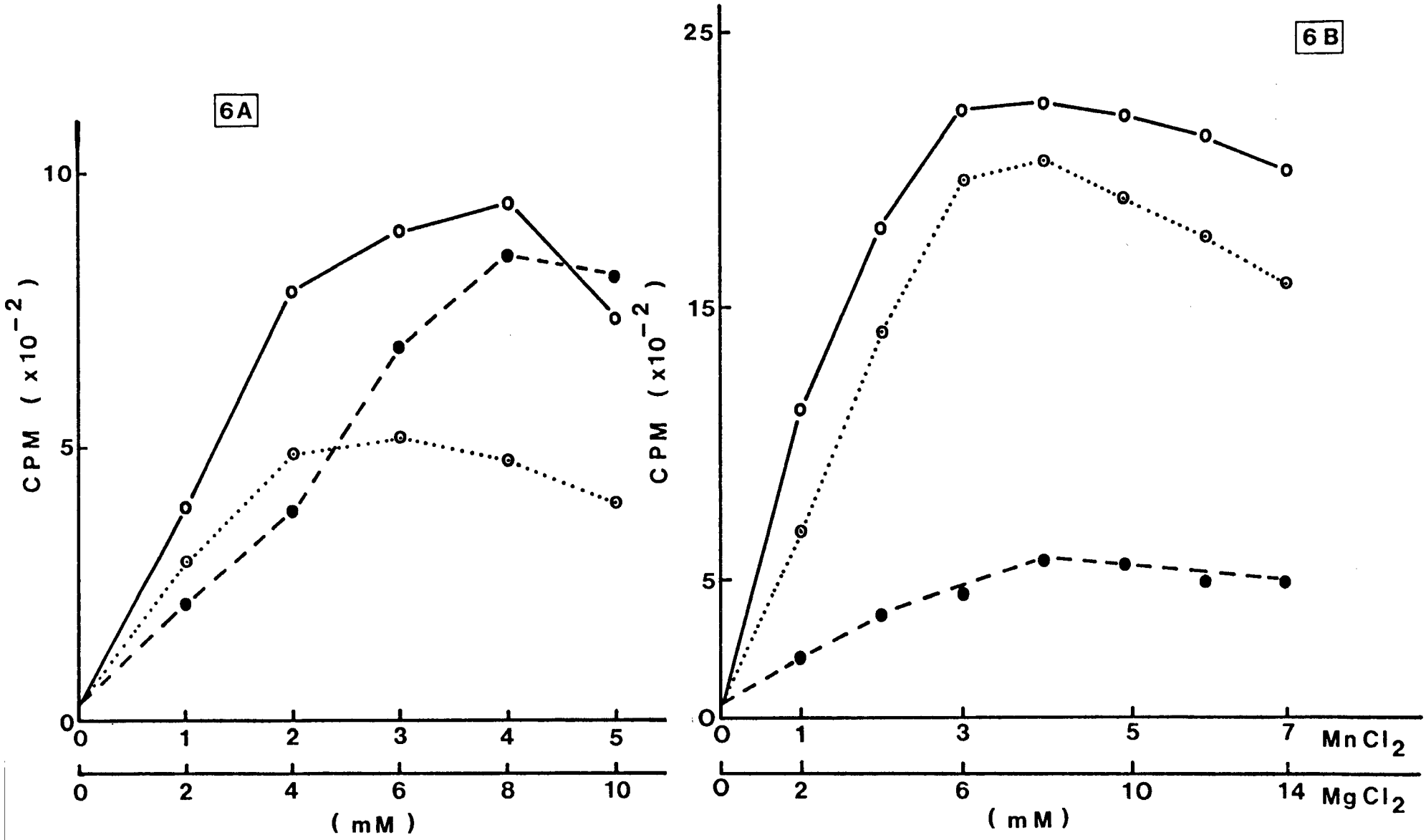


FIG. 6

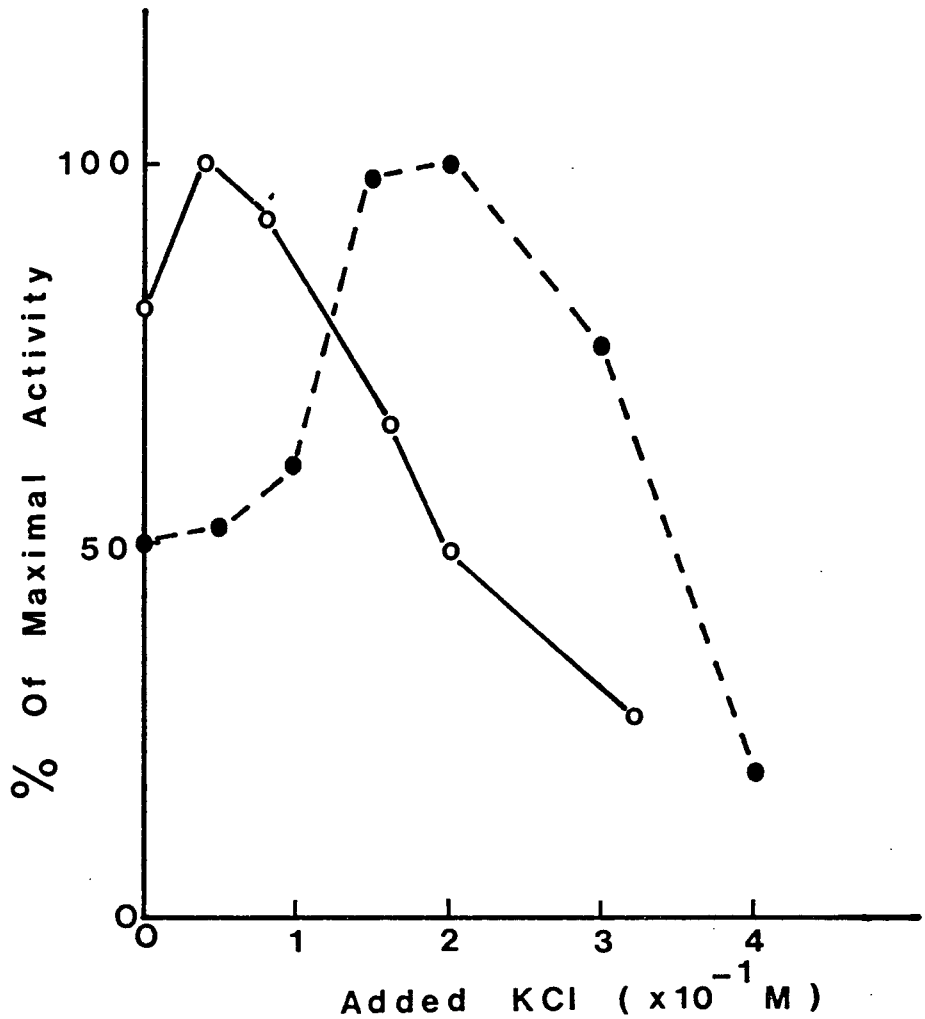


FIG. 7. THE EFFECT OF KCl ON BRAIN NUCLEAR RNA POLYMERASES

Polymerase I (o————o) and Polymerase II (●– – –●) were assayed in the presence of various amounts of KCl under the standard conditions (see Chapter 2.2E, a).

as the template. The two enzymes responded to KCl profiles differentially, i.e. polymerase I exhibited maximal activity around 0.05 M salt while polymerase II exhibited a rather sharp optimum at about 0.2 M KCl. Conceivably, at 0.2 M concentration of KCl, polymerase II was approximately 50% greater than the control (without KCl) whereas polymerase I was inhibited by about 51%. In addition, the stimulation of polymerase II by KCl (near 0.2 M) was found to be much more pronounced in the presence of Mn^{++} or Mn^{++} plus Mg^{++} than in the presence of Mg^{++} alone (Fig. 8). Furthermore, the addition of KCl at 0.16 M, but not at 0.04 M, resulted in a significant increase in the rate of RNA synthesis by polymerase II (Fig. 9). Thus the stimulatory effect of KCl appears to be selective for polymerase II only.

F. TEMPLATE REQUIREMENTS

Earlier it was shown that RNA polymerase I and polymerase II, isolated from the nuclei of beef cerebral cortex, required the presence of exogenously added native DNA as the template (Table V). In this regard, it was thought that these two RNA polymerases might display some preference towards native or heat-denatured DNA. Kinetic data summarized in Table VI show that polymerase I is more active with native DNA as template than with denatured DNA (the ratio of denatured/native DNA activity being less than one).

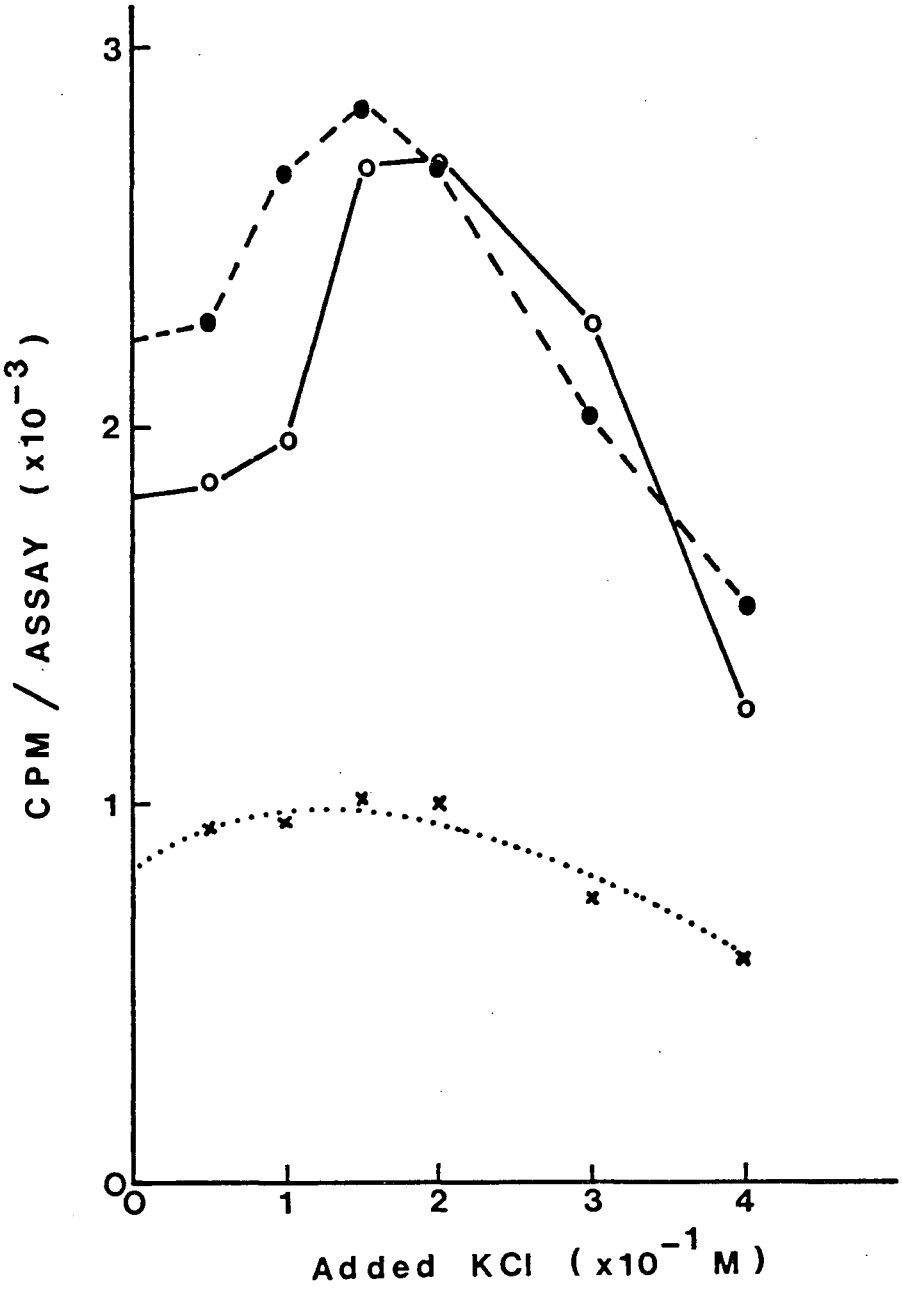


FIG. 8. THE EFFECT OF KCl ON BRAIN NUCLEAR RNA POLYMERASE II ACTIVITY WITH RESPECT TO DIVALENT CATIONS

RNA polymerase II (81 µg protein) was assayed in the presence of different concentrations of KCl with Mn^{++} alone (•— — —•), Mg^{++} alone (x·····x), and Mn^{++} plus Mg^{++} (o————o). Other assay conditions were the same as those given in Chapter 2.2E, a.

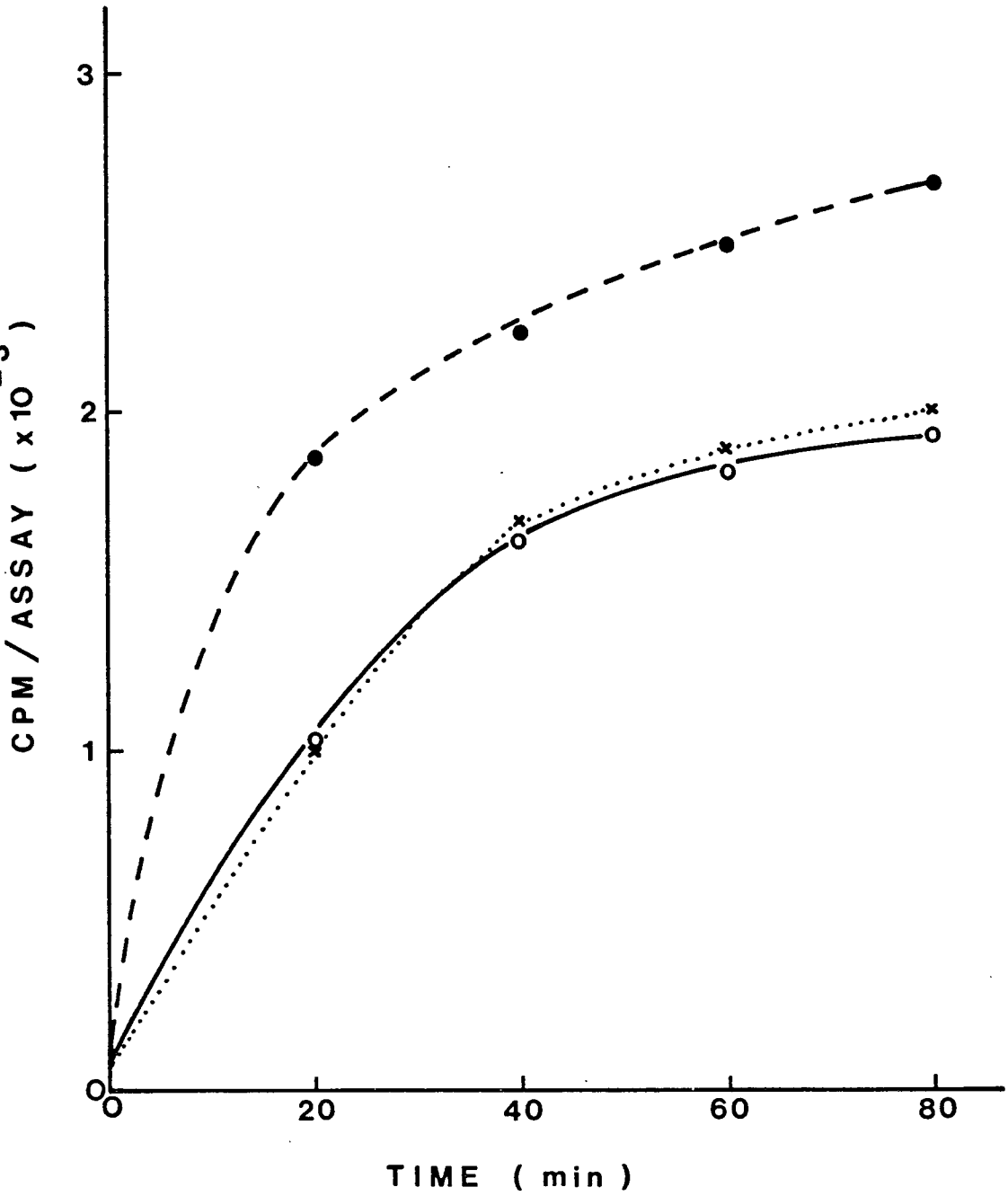


FIG. 9. THE INFLUENCE OF KCl ON THE RATE OF REACTION OF RNA POLYMERASE II OF BRAIN NUCLEI

RNA polymerase II (81 µg protein) was assayed using standard reaction mixture for different periods of incubation in the absence of KCl (o————o) or in the presence of 0.04 M KCl (x.....x) or 0.16 M KCl (•— — —•).

TABLE VI. THE RELATIVE RATES OF TRANSCRIPTION OF NATIVE AND HEAT-DENATURED DNA TEMPLATES BY BRAIN NUCLEAR RNA POLYMERASES

Enzyme*	Time of Incubation (min)	DNA Template		Ratio of Heat-denatured/Native Activity
		Native	Heat-denatured	
		cpm/assay	cpm/assay	
Polymerase I	15	215	136	0.63
	30	383	141	0.37
	45	456	141	0.31
Polymerase II	10	508	1620	3.19
	20	815	2591	3.18
	40	1204	4263	3.54
	60	1702	6087	3.51

* The most active fractions from a DEAE-Cellulose column of RNA polymerase I (135 µg protein) and RNA polymerase II (73 µg protein) were assayed under standard reaction conditions (see Chapter 2.2E, a).

Conversely, the activity of polymerase II was about 3-fold greater with denatured DNA than with native DNA as template. Moreover, the saturation of polymerase II activity occurs at much lower quantities of denatured DNA than native DNA (Fig. 10), pointing to its preference for denatured DNA as template.

G. RELATIVE INCORPORATION OF RIBONUCLEOSIDE TRIPHOSPHATES

As shown in Table VII, polymerase I as well as polymerase II catalyzes the incorporation of all four bases into TCA-insoluble products (RNA). The ratios of A + U/G + C incorporated under the direction of native CT-DNA by polymerase I and II were 0.65 and 0.96 respectively. Although these values might indicate that polymerase I synthesizes "GC-rich" RNA and polymerase II produces "AU-rich" RNA, more sophisticated data such as those obtained from hybridization-competition assays will be required to justify the validity of this statement. It should be pointed out that no attempt was made to exclude the formation of homo-polymers, if any, in these experiments and thus these base ratios might not represent the true function of two enzymic activities.

H. EFFECT OF α-AMANITIN TOXIN

α-Amanitin is a highly toxic small cyclic peptide and was initially shown to inhibit drastically the Mn^{++}/ammonium sulphate-stimulated RNA polymerase activity of rat liver intact nuclei

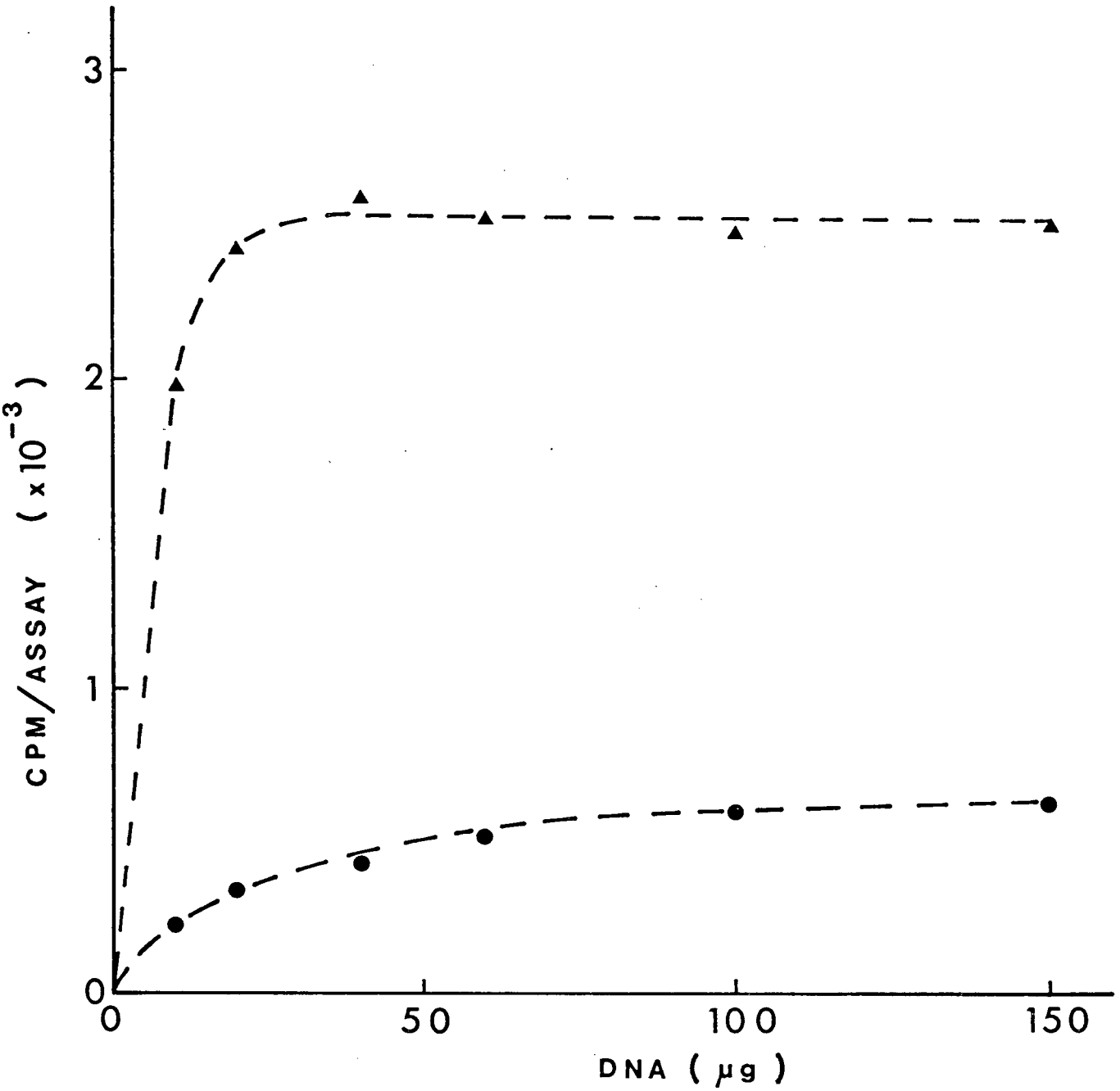


FIG. 10. RELATIVE TRANSCRIPTION OF HEAT-DENATURED AND NATIVE DNA BY BRAIN NUCLEAR RNA POLYMERASE II

RNA polymerase (92 µg protein) was assayed in the presence of various amounts of heat-denatured CT-DNA (▲— — —▲) or native CT-DNA (●— — —●). Other enzyme assay conditions were the same as described elsewhere (see Chapter 2.2E, a).

TABLE VII. RELATIVE INCORPORATION OF NUCLEOTIDES BY BRAIN NUCLEAR RNA POLYMERASES

Nucleoside Triphosphate* (^{3}H-Labelled)	Radioactivity Incorporated (cpm)	
	Polymerase I	Polymerase II
ATP	1780	1757
UTP	900	903
CTP	3042	1389
GTP	1404	1373
$\frac{A + U}{G + C}$	0.65	0.96

Separate incubations were carried out as described in Methods (Chapter 2.2E, a) with native CT-DNA as template. The amounts of enzymic protein were 147 µg and 73 µg for polymerase I and polymerase II respectively.

* The final concentration of each labelled nucleoside triphosphate (1 µCi/assay) during enzyme assays was the same (0.1 mM).

(Stirpe & Fiume, 1967). The high specificity of action of α-amanitin makes this toxin an invaluable tool for the study of RNA polymerase in animal cells (Novello & Stirpe, 1970; Sekeris et al., 1970; Shaaya & Sekeris, 1970). In view of the high selectivity of α-amanitin action, its effect on brain nuclear RNA polymerases was tested. Figure 11 illustrates that α-amanitin inhibited polymerase II almost completely while the activity of polymerase I is not influenced by this toxin. Although the mechanism of differential response of two polymerases for α-amanitin is unknown, it might indicate the existence of some structural difference in different polymerases. It is important to note that α-amanitin action is apparently related to polymerase protein rather than DNA template (Kedinger et al., 1970; Meihlac et al., 1970; Jacob et al., 1970; Lindell et al., 1970).

I. EFFECT OF CERTAIN ANTIBIOTICS

The effect of two antibiotics, namely, actinomycin D and rifampicin was investigated with brain nuclear RNA polymerases I and II. Figure 12 shows that various concentrations of actinomycin D inhibited both the enzymic activities up to the extent of 70%. The antibiotic rifamycin is a very potent inhibitor of E. coli RNA polymerase. It has been used as a very important tool for the elucidation of subunit function of bacterial RNA polymerase because rifampicin interacts with the polymerase rather than DNA template

FIG. 11. THE EFFECT OF α-AMANITIN ON BRAIN NUCLEAR RNA POLYMERASES I AND II

RNA polymerase I (•— — —•) and RNA polymerase II (o————o) were separately assayed in the presence of various amounts of α-amanitin. The enzyme assay conditions were the same as described in Methods, Chapter Two.

FIG. 12. THE EFFECT OF CERTAIN ANTIBIOTICS ON TWO RNA POLYMERASES OF BRAIN NUCLEI

Polymerase I was assayed in the presence of various qualities of actinomycin D (o— — —o) and rifampicin (•.....•). Other assay conditions were the same as mentioned in Chapter Two. Under similar conditions, polymerase II was separately assayed in the presence of actinomycin D (o————o) and rifampicin (x-------x).

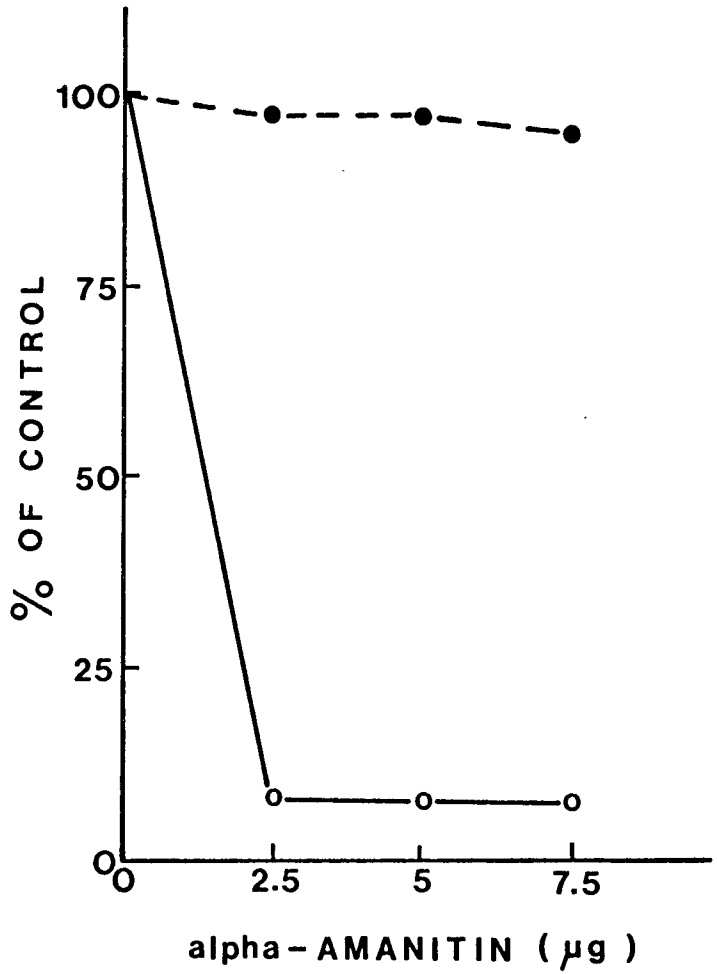

100
75
50
25
0
% OF CONTROL
0
2.5
5
7.5
alpha-AMANITIN (µg)

FIG.11

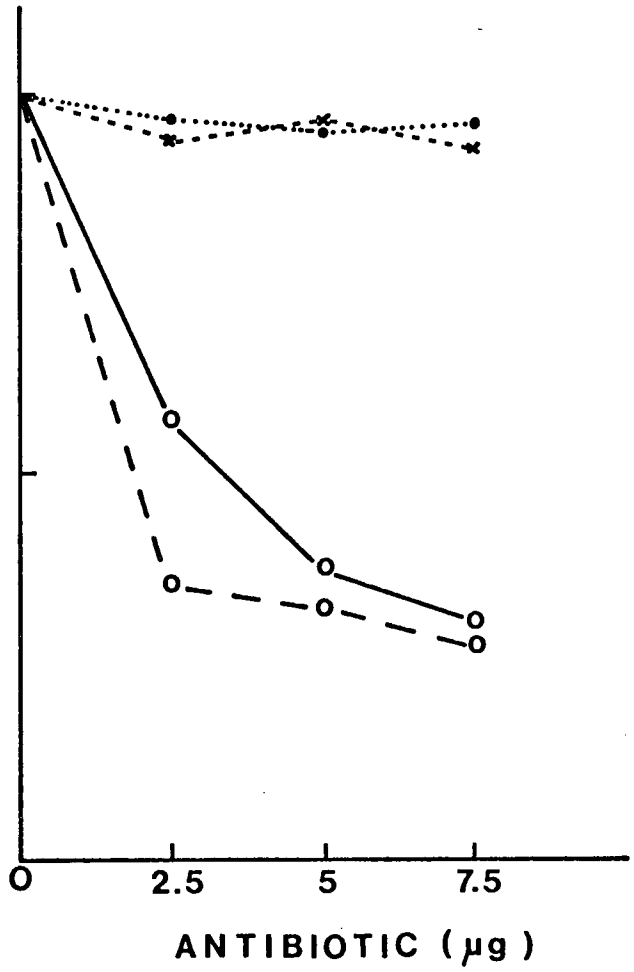

0
2.5
5
7.5
ANTIBIOTIC (µg)

FIG.12

(see Lill et al., 1970; Wehrli & Staehelin, 1970; Zillig et al., 1970). It was therefore interesting to test the effect of rifampicin on brain nuclear RNA polymerases. The data also depicted in Figure 12 demonstrate that rifampicin is without any detectable influence on either of the two polymerases from brain nuclei. This finding is in accordance with that of others who have observed that bacterial RNA polymerase but not mammalian RNA polymerase is inhibited by the rifamycins (Hartmann et al., 1967; Wehrli et al., 1968).

3.3. POLYAMINES AND THE ACTIVITY OF MULTIPLE FORMS OF RNA POLYMERASE FROM BRAIN NUCLEI

As discussed in the introductory chapter, polyamines influence the synthesis of RNA in a variety of cells and at their low concentrations they stimulate DNA-dependent RNA polymerase activity assayed in intact nuclei of mammalian cells. The fact that polyamines have been shown to preserve the nuclear morphology (MacGregor & Anderson, 1960) and that brain cell nuclei contain, at least, two RNA polymerase activities (as demonstrated in previous sections of this chapter), it was very interesting to investigate the effect of polyamines on the isolated and partially purified RNA polymerases. The data presented in Table VIII reveal that the activity of RNA polymerase II was increased up to 186% of the control (100%) by spermidine and up to 140% of control (100%) by spermine with their

TABLE VIII. EFFECT OF POLYAMINES ON RNA POLYMERASE II OF BRAIN CELL NUCLEI

Spermidine (mM)	Enzyme Activity cpm/assay	Enzyme Activity % of control	Spermine (mM)	Enzyme Activity cpm/assay	Enzyme Activity % of control
0	1451	100	0	1451	100
1	1738	120	1	1490	103
2	1940	134	2	1746	120
3	2229	154	3	1955	135
4	2602	179	4	2028	140
6	2700	186	6	1545	107
10	2008	138	10	1281	89

RNA polymerase II (125 µg protein) was assayed under standard assay conditions (see Chapter 2.2E, a) in the presence of various concentrations of spermidine or spermine.

optimum concentrations around 4-6 mM and 3-4 mM, respectively. Since spermidine elicited a much more pronounced stimulation than did spermine, the former polyamine was chosen for further study. It should be mentioned that spermidine could not be substituted for Mn^{++} or Mg^{++} as the cation in the reaction mixture.

A. EFFECT OF SPERMIDINE ON MULTIPLE ACTIVITIES OF BRAIN NUCLEAR RNA POLYMERASE

The effect of spermidine on the activity of polymerase I and polymerase II is shown in Figure 13. This Figure illustrates that the addition of spermidine (5-6 mM) resulted in an increase of 65-80% over the control (without spermidine) in polymerase II activity while the activity of polymerase I was only 30-50% stimulated. This observation might indicate that the two enzymes responded to spermidine somewhat differently. In order to delineate this effect further, the activities of polymerases I and II were determined in the presence of their preferred divalent cation. The results of these experiments are depicted in Figure 14, which clearly demonstrates that the magnitude of stimulation by spermidine of polymerase II activity was much higher (about 250% of the control) in the presence of Mn^{++} than in the presence of Mg^{++} alone (Mn^{++} being the preferred divalent cation for polymerase II as described before in Figure 6B). However, polymerase I was only 40-50% stimulated by spermidine in the presence of Mg^{++} only and

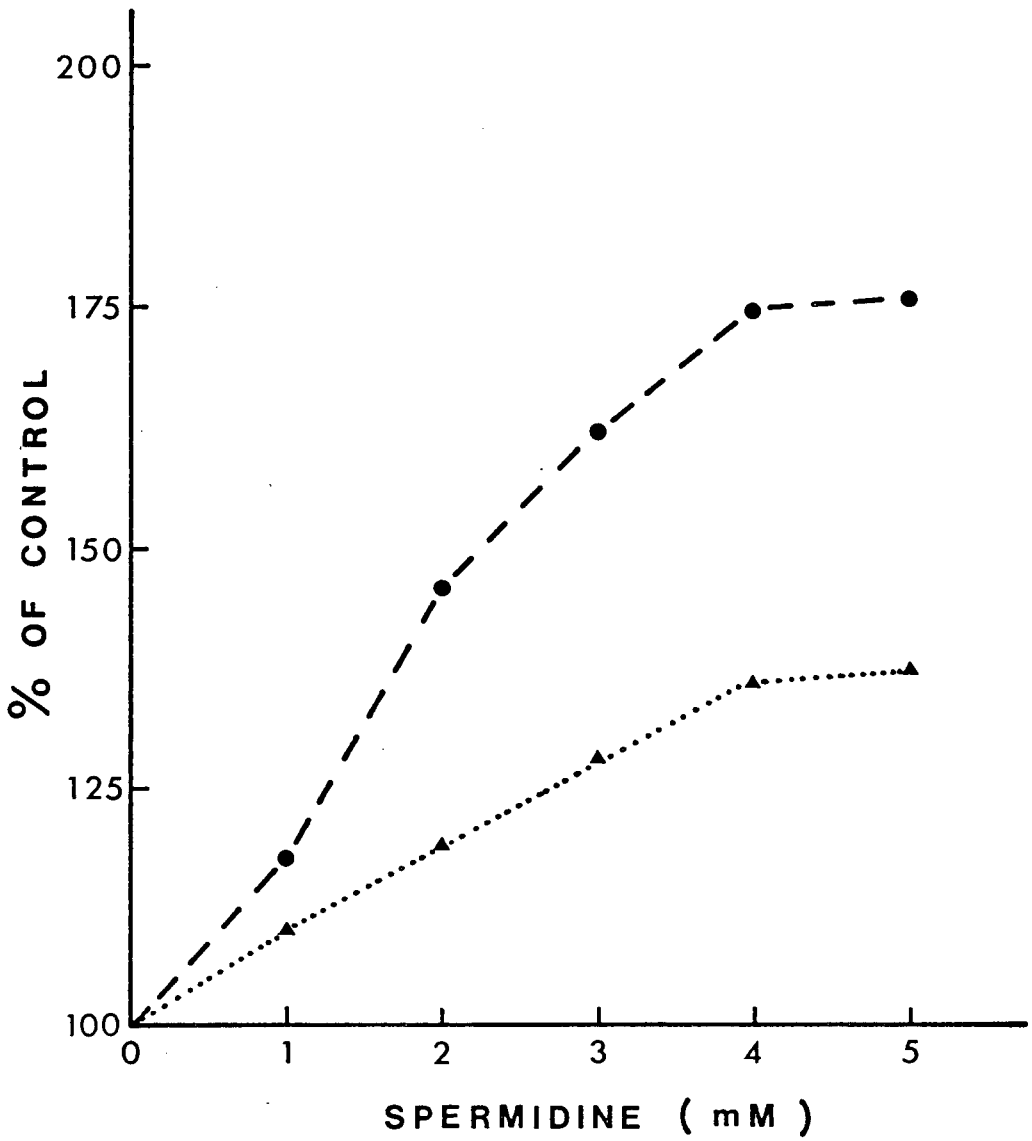
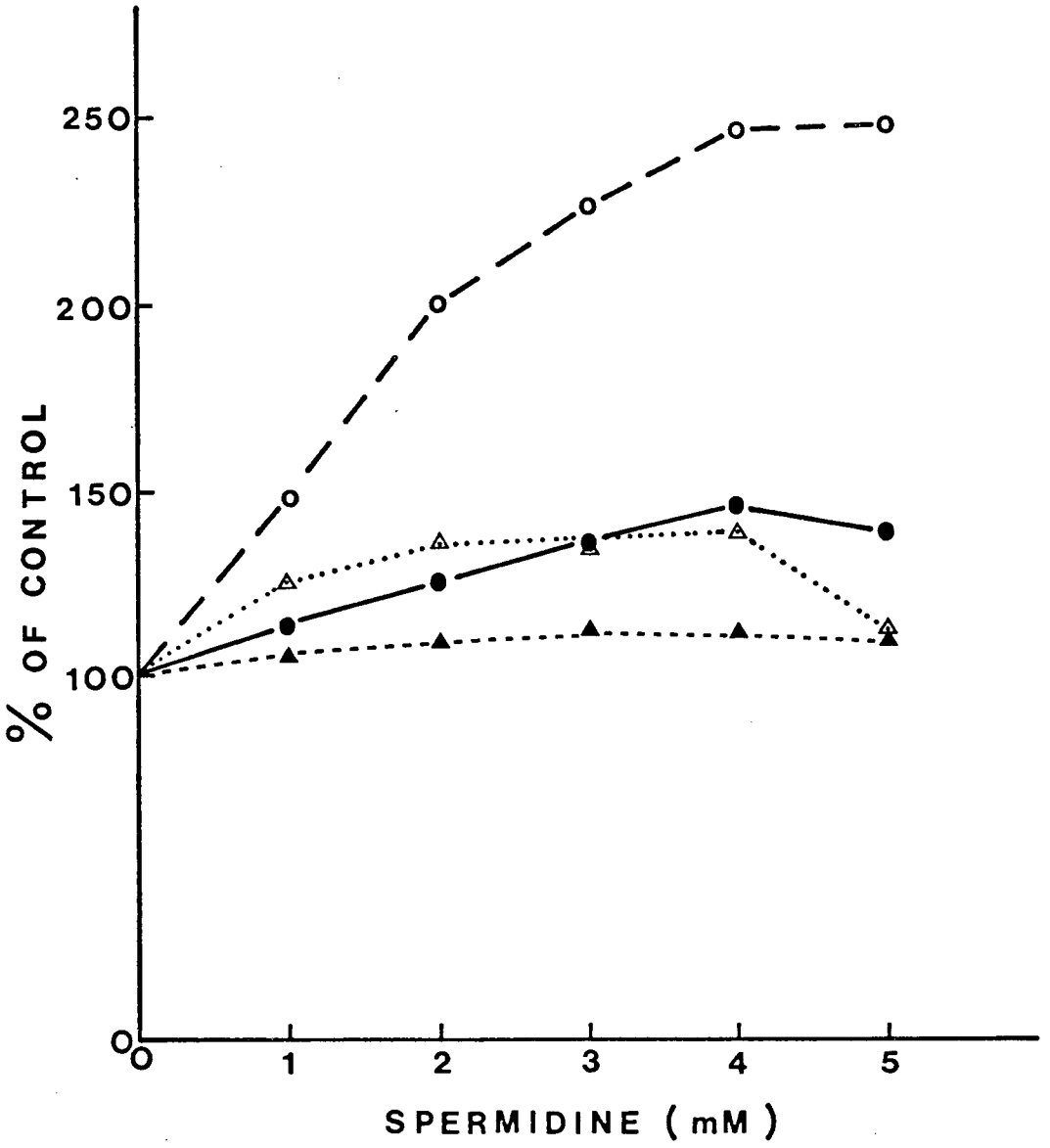


FIG. 13. THE INFLUENCE OF SPERMIDINE ON TWO RNA POLYMERASES OF BRAIN NUCLEI

Independent assays were carried out in the presence of various concentrations of spermidine for RNA polymerase I (▲ ..... ▲) and RNA polymerase II (● — — — ●). Other assay conditions were those described in Chapter 2.2E, a.

FIG. 14. THE EFFECT OF SPERMIDINE ON BRAIN NUCLEAR RNA POLYMERASES WITH REGARD TO DIVALENT CATION

Polymerase I was assayed in the presence of Mn^{++} (3 mM) only (▲-------▲) or Mg^{++} (6 mM) only (●———●). The activity of polymerase II was determined in the presence of Mn^{++} (o— — —o) or Mg^{++} (Δ.....Δ). Other assay conditions were the same as given in Chapter 2.2E, a. The concentrations of spermidine present during the enzyme assays were those as denoted in the figure.


250
200
150
100
0
% OF CONTROL
0
1
2
3
4
5
SPERMIDINE (mM)

this stimulatory effect is suppressed if Mn^{++} is the divalent cation in the assay mixture (Mg^{++} being the preferred divalent cation for polymerase I as shown previously in Figure 6A). If spermidine stimulated polymerase II preferentially, as revealed by the above studies, then there should be some specificity of spermidine stimulation with respect to native and denatured DNA as template because this enzyme prefers denatured DNA (as shown earlier in Table VI). This was investigated by following the kinetics and the data are summarized in Figures 15, 16, and 17. The kinetic data obtained in the presence of both Mn^{++} and Mg^{++} showed that the stimulatory effect of spermidine (4 mM) required the presence of native DNA as template for polymerase II activity (Fig. 15). Under these conditions, spermidine had little or no effect in enhancing the enzyme activity which was primed with heat-denatured DNA as template. This effect, however, varied depending upon the divalent cation present in the reaction mixture. As depicted in Figure 16, the activity of polymerase II assayed in the presence of Mn^{++} only was found to be stimulated by spermidine (4 mM) with either native or heat-denatured DNA as template. In the presence of Mg^{++} alone, spermidine (4 mM) was somewhat stimulatory with native DNA template only but it was relatively inhibitory with heat-denatured DNA template (Fig. 17). Thus spermidine stimulated the activity of polymerase II with both native as well

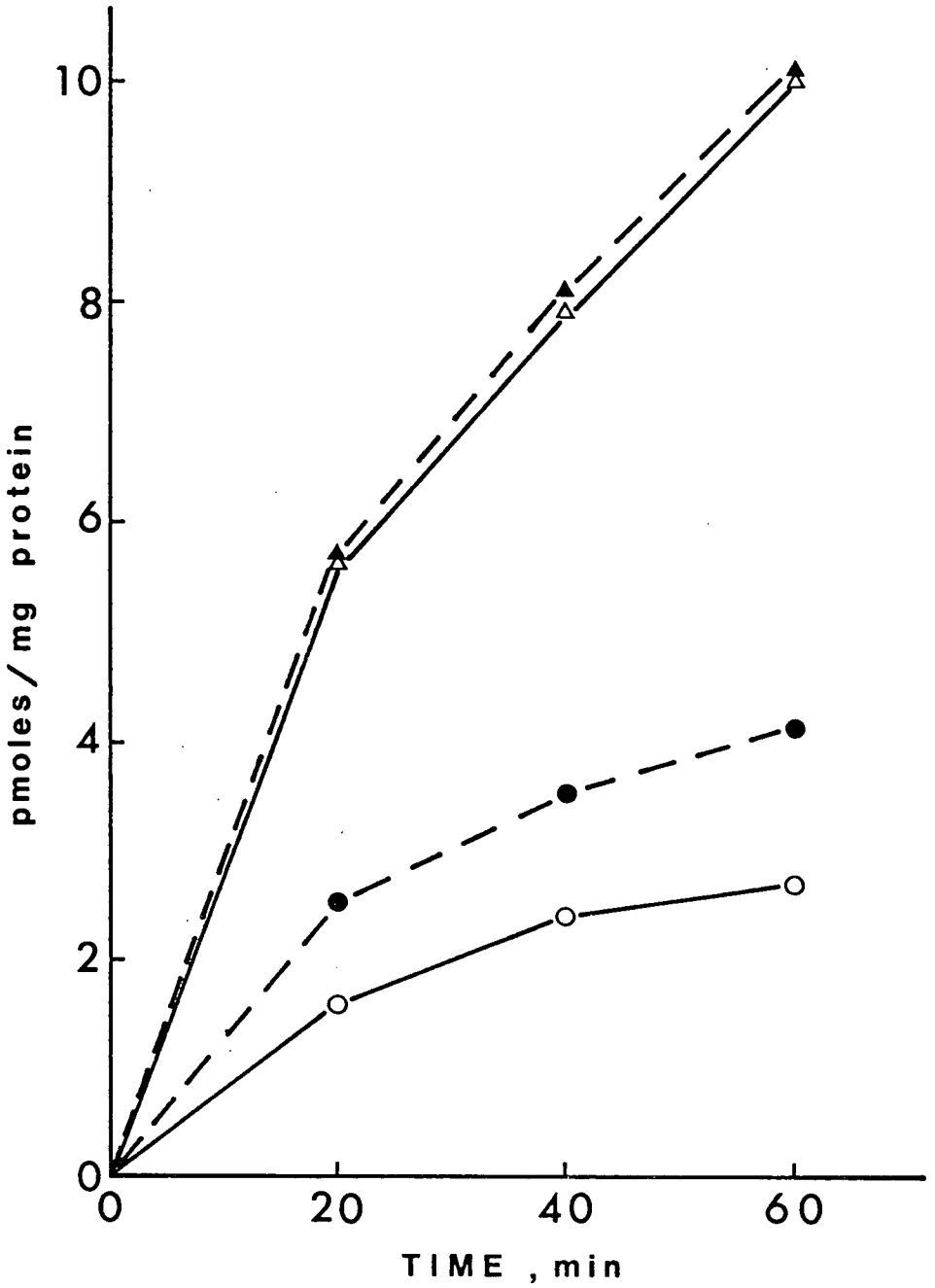


FIG. 15. THE KINETICS OF SPERMIDINE STIMULATION OF BRAIN NUCLEAR RNA POLYMERASE II WITH REGARD TO TEMPLATE

The enzyme was assayed in the presence of Mn^{++} (3 mM) and Mg^{++} (6 mM) with native DNA template (without spermidine, o————o, and with 4 mM spermidine •— — —•), and with heat-denatured DNA template (without spermidine, Δ————Δ, with 4 mM spermidine, ▲— — —▲).

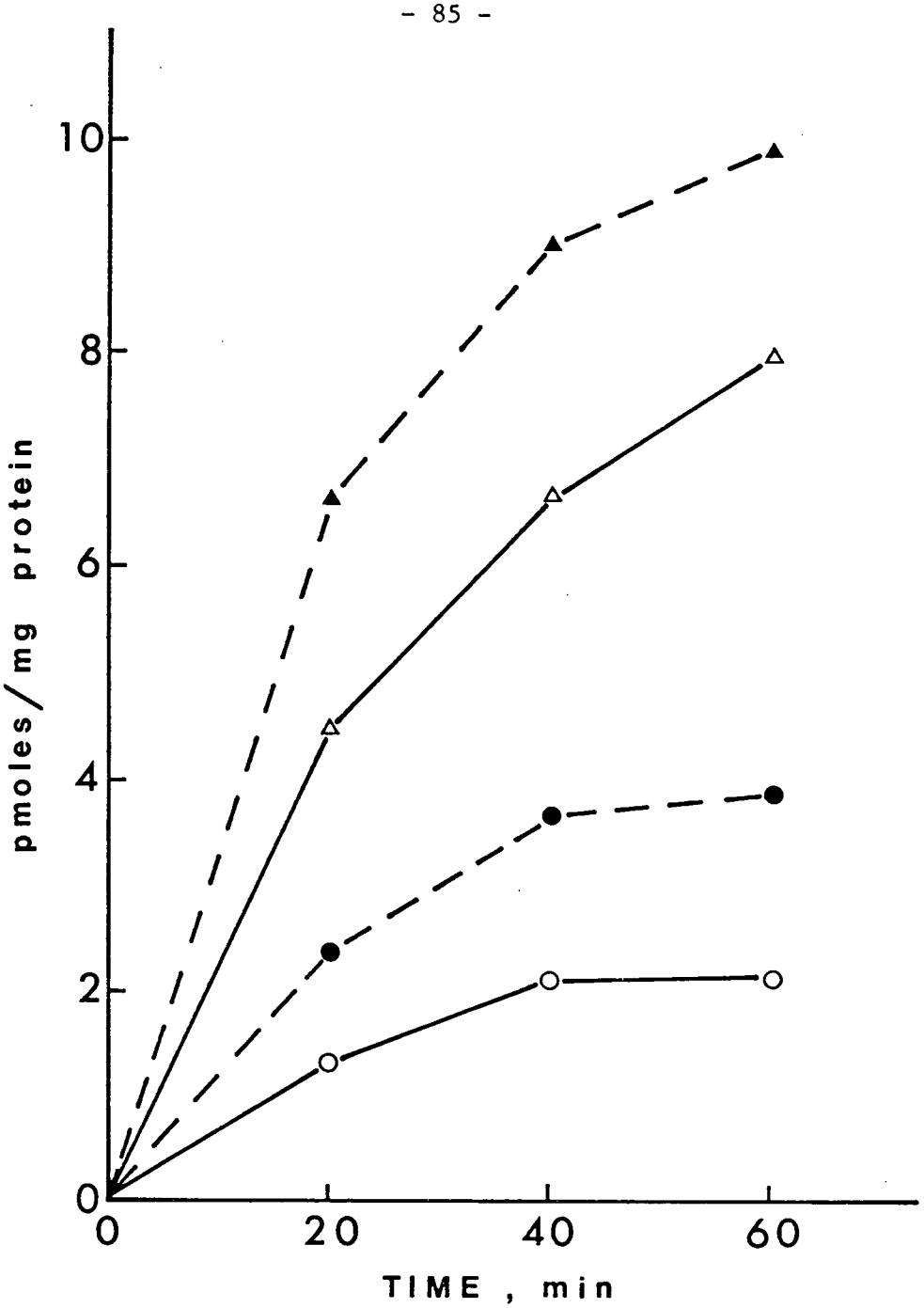


FIG. 16. THE KINETICS OF SPERMIDINE STIMULATION OF BRAIN NUCLEAR RNA POLYMERASE II WITH REGARD TO TEMPLATE

The enzyme was assayed in the presence of Mn^{++} (3 mM) only with native DNA template (without spermidine, o————o, and with 4 mM spermidine •— — —•), and with heat-denatured DNA template (without spermidine, Δ——— Δ, with 4 mM spermidine, ▲— — —▲).

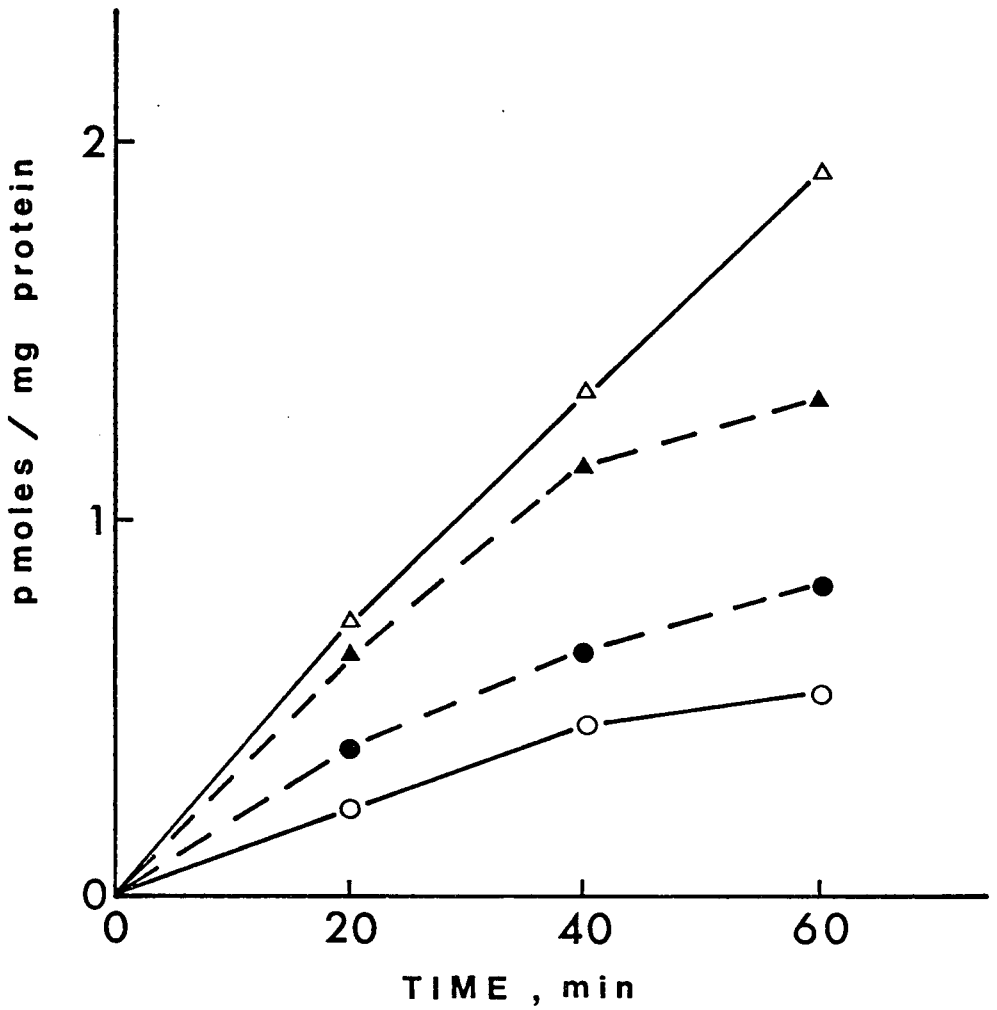


FIG. 17. THE KINETICS OF SPERMIDINE STIMULATION OF BRAIN NUCLEAR RNA POLYMERASE II WITH REGARD TO TEMPLATE

The enzyme was assayed in the presence of Mg^{++} (6 mM) only with native DNA template (without spermidine, o————o, and with 4 mM spermidine •— — —•), and with heat-denatured DNA template (without spermidine, Δ——— Δ, with 4 mM spermidine, ▲— — —▲).

as denatured DNA as template in the presence of Mn^{++} only. With denatured DNA, this effect was suppressed if Mg^{++} was supplemented to the Mn^{++}-containing assay system, and spermidine was inhibitory rather than stimulatory if Mg^{++} was the only divalent cation. These observations tend to suggest that the Mn^{++}-primed RNA polymerase activity may be preferentially stimulated by spermidine.

B. INHIBITION OF POLYMERASE II ACTIVITY BY CALF THYMUS HISTONE AND YEAST RNA AND EFFECT OF SPERMIDINE ON THIS INHIBITION

Histones and RNA are found in mammalian cell nucleus and that the molecules of a similar nature, if added to the assay mixture, significantly inhibited the activity of RNA polymerase II from beef brain nuclei (Fig. 18), it was tempting to see if polyamines could either prevent or overcome this inhibition. In order to perform such a study, reconstituted systems were employed and the results of these experiments are given in Table IX. The addition of 100 µg yeast RNA or 200 µg calf thymus histone fraction into the reaction mixture resulted in a considerable degree of inhibition (greater than 60%) of polymerase II activity. Spermidine (5 mM) added at the end of the twentieth minute of incubation stimulated the yeast RNA inhibited enzyme activity (over 2 fold increase), but it has almost no effect on the enzyme activity which was inhibited by calf thymus histone. Figure 19 illustrates that the inability of

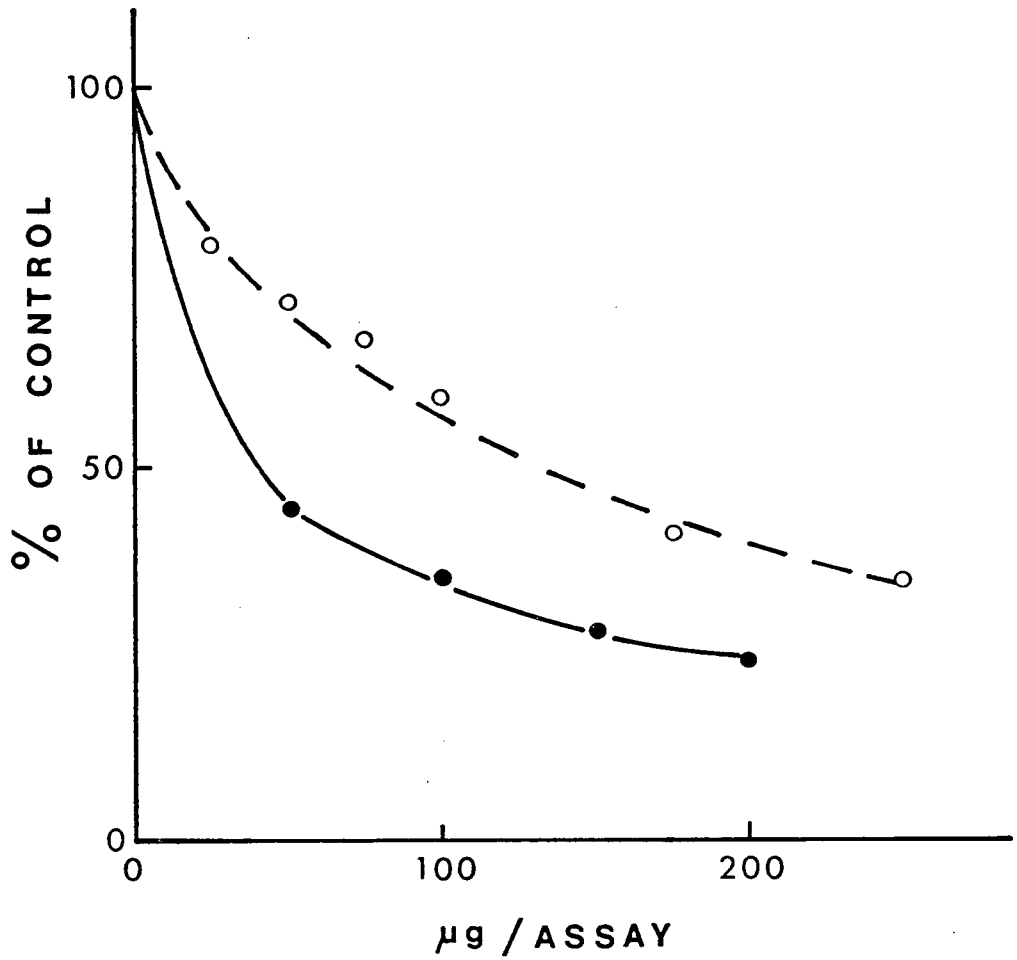


FIG. 18. THE INHIBITION OF BRAIN NUCLEAR RNA POLYMERASE II ACTIVITY BY CALF THYMUS HISTONE AND YEAST RNA

The enzyme activity was measured in the presence of various amounts of calf thymus histone (o— — —o) and yeast RNA (•————•).

TABLE IX. THE EFFECT OF SPERMIDINE ON BRAIN NUCLEAR RNA POLYMERASE II ACTIVITY WHICH WAS INHIBITED BY YEAST RNA AND CALF THYMUS HISTONE

Assay Mixture	Time of incubation		
	20 min	60 min	
	Specific activity (pmoles/mg protein)	Specific activity (pmoles/mg protein)	Increase by spermidine† (%)
EXPERIMENT 1			
Complete	3.10	4.06	-
Complete + spermidine*	3.10	5.65	40
Complete + yeast RNA	1.14	1.54	-
Complete + yeast RNA + spermidine*	1.14	3.20	108
EXPERIMENT 2			
Complete	3.99	6.45	-
Complete + spermidine*	3.99	8.00	24
Complete + calf thymus histone	0.695	1.34	-
Complete + calf thymus histone + spermidine*	0.695	1.42	6

The enzyme activity was assayed in the absence or in the presence of yeast RNA (0.1 mg/assay) or calf thymus histone (0.2 mg/assay) using complete reaction mixture as given elsewhere (Chapter 2.2E, a).

* Spermidine (5 mM) was added at the end of the twentieth minute of incubation period and the reaction was continued until 60 min at 37°C.

† The increase in enzyme activity by spermidine was calculated based on the control value (the enzyme activity without spermidine).

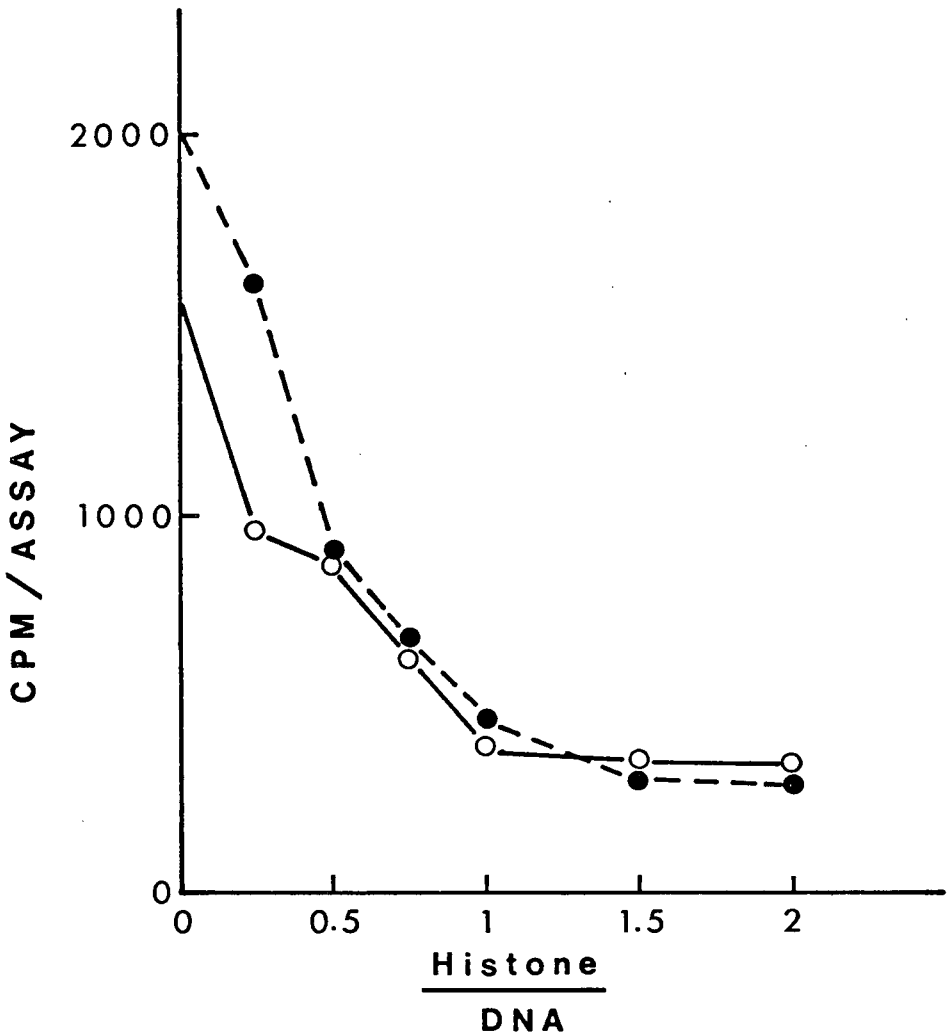


FIG. 19. THE INFLUENCE OF SPERMIDINE ON THE HISTONE-INHIBITED RNA POLYMERASE II ACTIVITY

The enzyme activity was determined in the presence of various amounts of calf thymus histone (without spermidine, o————o, and with 5 mM spermidine, •— — — •) using standard conditions of enzyme assay.

spermidine to overcome the histone-inhibition of polymerase II activity is not dependent upon the amount of histone present during the assay. Moreover, the counteracting effect of spermidine (5 mM) of yeast RNA-inhibition was also found to be independent of quantities of yeast RNA (50-250 μg) present in the assay system (Fig. 20).

C. THE NATURE OF RNA SYNTHESIZED BY POLYMERASE II UNDER THE INFLUENCE OF SPERMIDINE

The data presented in the foregoing sections clearly demonstrated that the activity of RNA polymerase II is profoundly stimulated by spermidine. Therefore, it was very alluring to investigate the nature of RNA produced by polymerase II under the action of spermidine. The experiments to conduct this type of study included the sucrose-density gradient centrifugation analysis of RNA synthesized *in vitro* by polymerase II in the absence or presence of spermidine. Figure 21 shows that the RNA synthesized in 60 min with or without spermidine sediments as a single peak. Since the radioactivity of ^{3}H-UTP incorporated into RNA under the influence of spermidine is much higher than in the presence of spermidine, indicating that the total amount of RNA synthesized *in vitro* is increased by spermidine. This would suggest that perhaps polymerase II under the action of spermidine transcribes the same segments of DNA because the nature of newly-synthesized RNA was indistinguishable by sucrose-density gradient centrifugation.

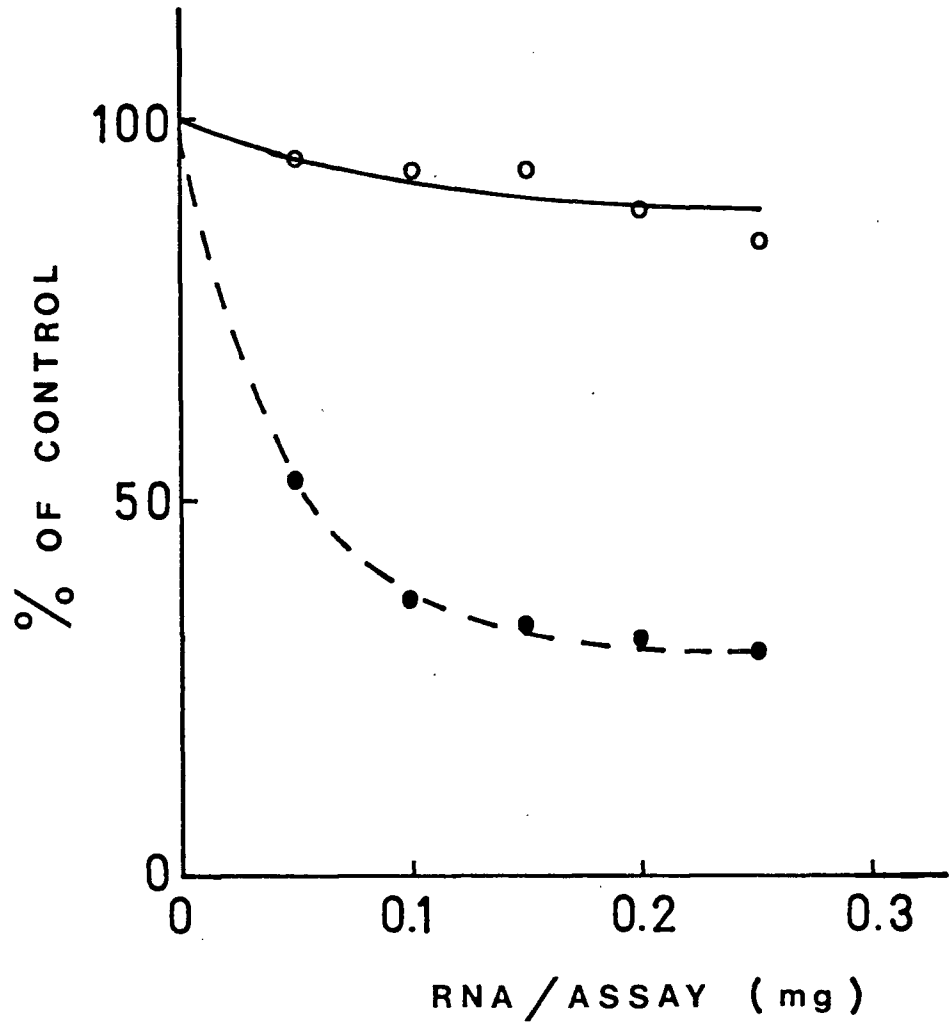


FIG. 20. THE COUNTERACTING EFFECT OF SPERMIDINE ON RNA-INHIBITED RNA POLYMERASE II ACTIVITY

The enzyme activity was measured in the presence of various quantities of yeast RNA (without spermidine, • - - - - •, and with 5 mM spermidine, o ——— o) using standard reaction mixture. Incubation was carried out at 37°C for 20 min.

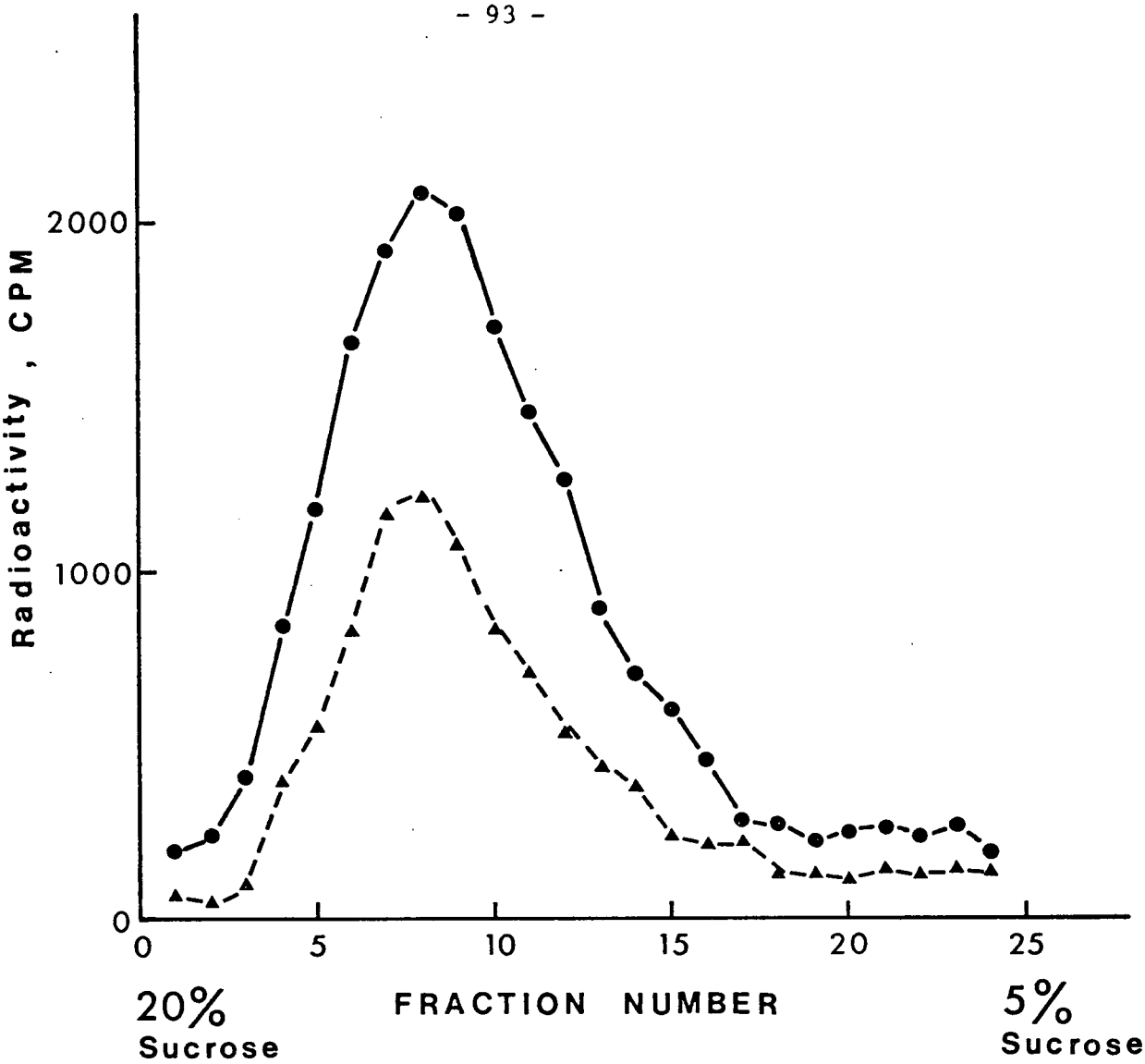


FIG. 21. SUCROSE-DENSITY GRADIENT PROFILE OF RNA SYNTHESIZED BY POLYMERASE II UNDER THE ACTION OF SPERMIDINE

The enzyme was assayed under the conditions described in Chapter Two with spermidine (5 mM) or without spermidine. At the end of 60 min incubation, the reaction product was separated by sucrose-density gradient centrifugation according to the procedure outlined in Methods (see Chapter 2.2H, b). The radioactivity of ^{3}H-UMP incorporated into RNA, which was synthesized with spermidine (●————●) or without spermidine (▲- - - - -▲), is plotted.

Subsequently, it was found that the product of polymerase II activity may be a complex of the type Enzyme-DNA-RNA rather than free RNA. This was substantiated by the finding that the radioactivity peak of RNA shifted towards a lighter region if the reaction mixture had been treated with SDS prior to its analysis by sucrose-density gradient centrifugation (Fig. 22). Treatment of reaction mixture with SDS denatures the enzyme protein and dissociates it from the Enzyme-DNA-RNA complex, and thus resulted in a shift of radioactivity peak of RNA towards a lighter region of the sucrose-density gradient. This finding is compatible with the fact that the product of RNA polymerase reaction is an Enzyme-DNA-RNA complex (Bremer & Konrad, 1964). It should be pointed out that the pattern of RNA synthesized in the presence or absence of spermidine was found to be similar no matter whether the reaction mixture was treated or not with SDS before analysis by sucrose-density gradient centrifugation (Fig. 23). This Figure also illustrates the fact that the product of the reaction is somewhat smaller in size than 18 S ribosomal RNA of the rat liver used as a reference.

3.4. TRANSCRIPTION OF BRAIN CHROMATIN BY BRAIN NUCLEAR RNA POLYMERASE II

In experiments, reported in earlier sections of this Chapter, a commercial grade of purified calf thymus native DNA has been

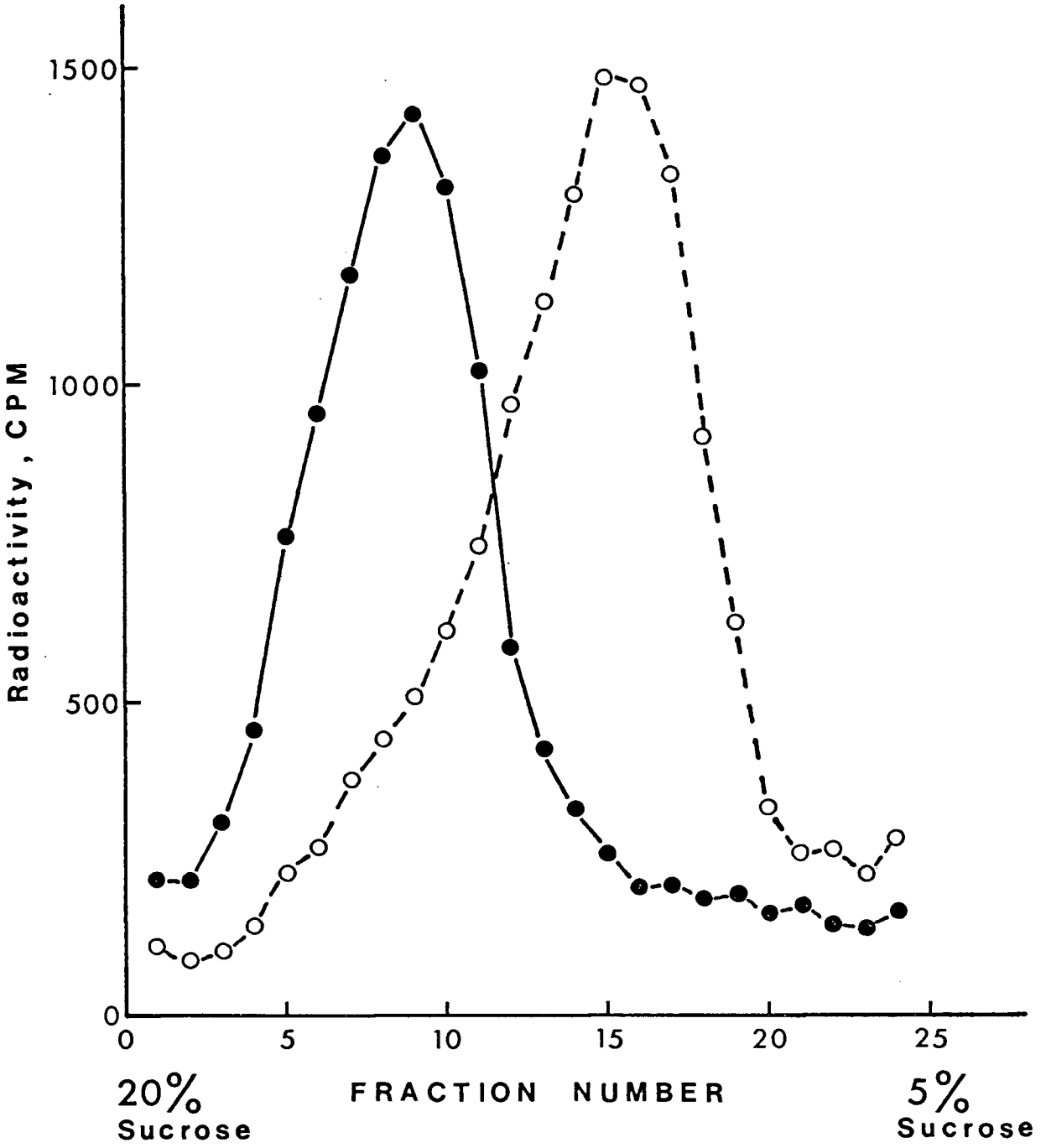
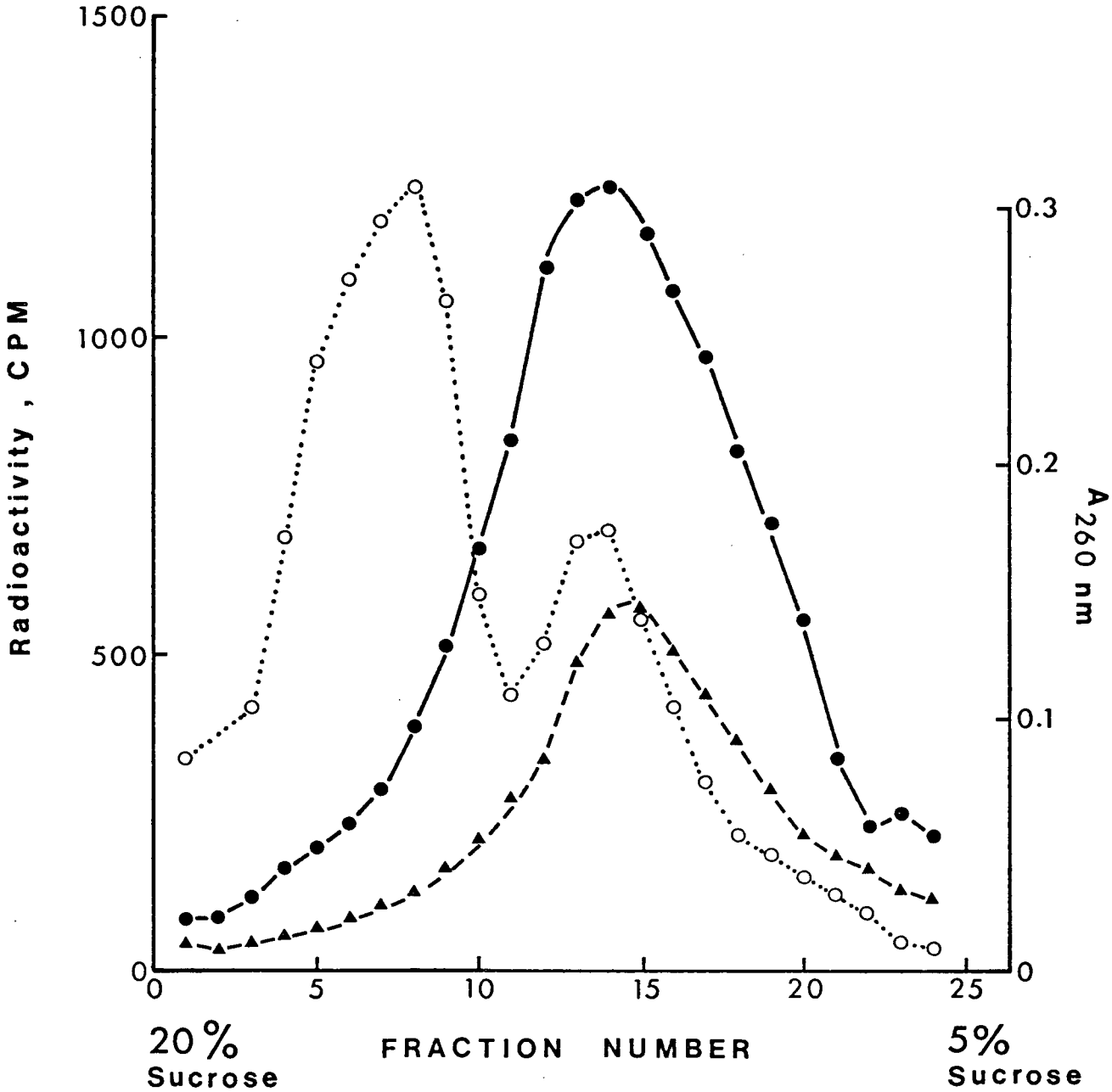


FIG. 22. SUCROSE-DENSITY GRADIENT PROFILE OF RNA SYNTHESIZED BY BRAIN NUCLEAR RNA POLYMERASE II

The enzyme assay conditions were the same as those given in Chapter Two. After 60 min incubation, the reaction product was treated with SDS for 5 min at 37°C prior to the analysis by sucrose-density gradient centrifugation (see Chapter 2.2H, b). The radioactivity of RNA in the untreated (•————•) and SDS-treated (o— — — o) reaction product,is diagrammed.

FIG. 23. THE NATURE OF RNA SYNTHESIZED BY BRAIN NUCLEAR RNA POLYMERASE II UNDER THE INFLUENCE OF SPERMIDINE

The enzyme assay and the separation of RNA were carried out according to the procedure outlined in the legend to Figure 21, except that the reaction product was treated with SDS prior to its analysis by sucrose-density gradient centrifugation. The radioactivity of RNA, which was synthesized in the presence of 5 mM spermidine (●————●) or in the absence of spermidine (▲- - - - -▲), is plotted. Absorbance at 260 nm (o·····o) represent the rat liver ribosomal RNA which was simultaneously run as a reference.

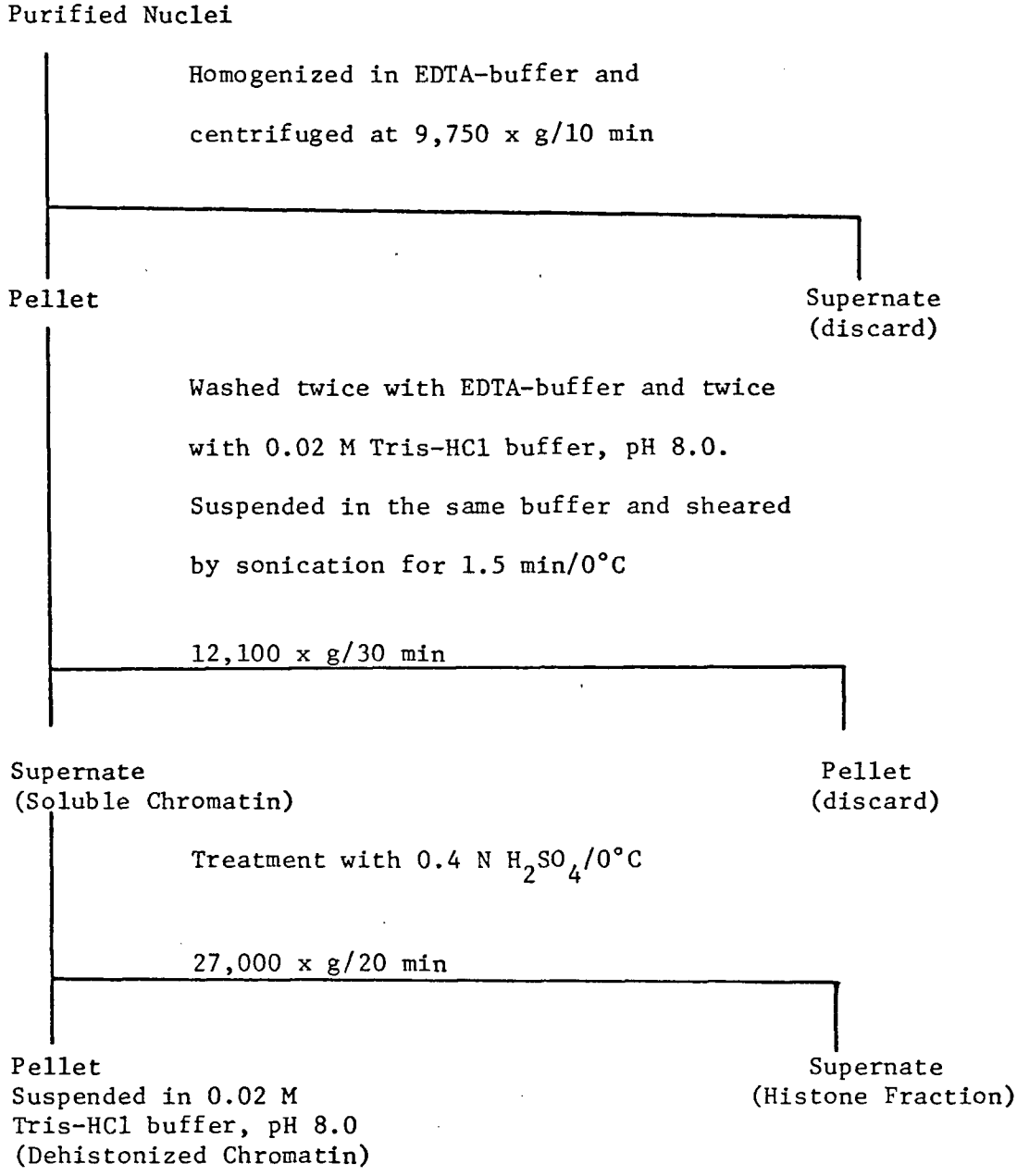

1500
1000
500
0
Radioactivity , CPM
0.3
0.2
0.1
0
A 260 nm
0
5
10
15
20
25
20 %
Sucrose
FRACTION NUMBER
5%
Sucrose

utilized as template for brain nuclear RNA polymerases. However, the genetic material in the differentiated cells is represented by chromatin, a matrix of DNA, RNA and protein molecules. Moreover, the RNA polymerase activity in mammalian cells is confined primarily in the nucleus, being firmly associated with the chromatin matrix. Therefore, it was of interest to determine the ability of isolated enzymes to transcribe RNA from chromatin, a template of more physiological nature. In order to do so, chromatin was isolated from cerebral cortex and RNA polymerase II from the same tissue was used to transcribe RNA off the DNA in cerebral chromatin (reconstituting a homologous system). The observations made on this type of study are described in this Section. It should be pointed out that because of the greater instability of polymerase I, polymerase II was utilized to investigate the transcriptive properties of the isolated chromatin.

A. CERTAIN CHARACTERISTICS OF BRAIN CHROMATIN

The soluble chromatin prepared by a method, as outlined in Figure 24, exhibited a sharp peak of absorption-maximum at 260 nm, which also coincided with the peak of native calf thymus DNA (Fig. 25). The ratio of 280 nm/260 nm absorbance in chromatin preparations was usually around 0.6. The absorbance at 320 nm was practically negligible indicating the absence of turbidity in the soluble chromatin, which was found to contain DNA, RNA and proteins in the mass ratios of 1:0.09: 1.92 (Table X). All these features of soluble brain chromatin are compatible with those which have been described for the chromatin preparations from various other tissues (see Bonner et al., 1968a; see Hearst & Botchan, 1970).

FIG. 24. AN OUTLINE FOR THE PREPARATION OF CHROMATIN AND DEHISTONIZED CHROMATIN FROM BEEF CEREBRAL CORTEX



(The details of each step are given in Chapter 2.2)

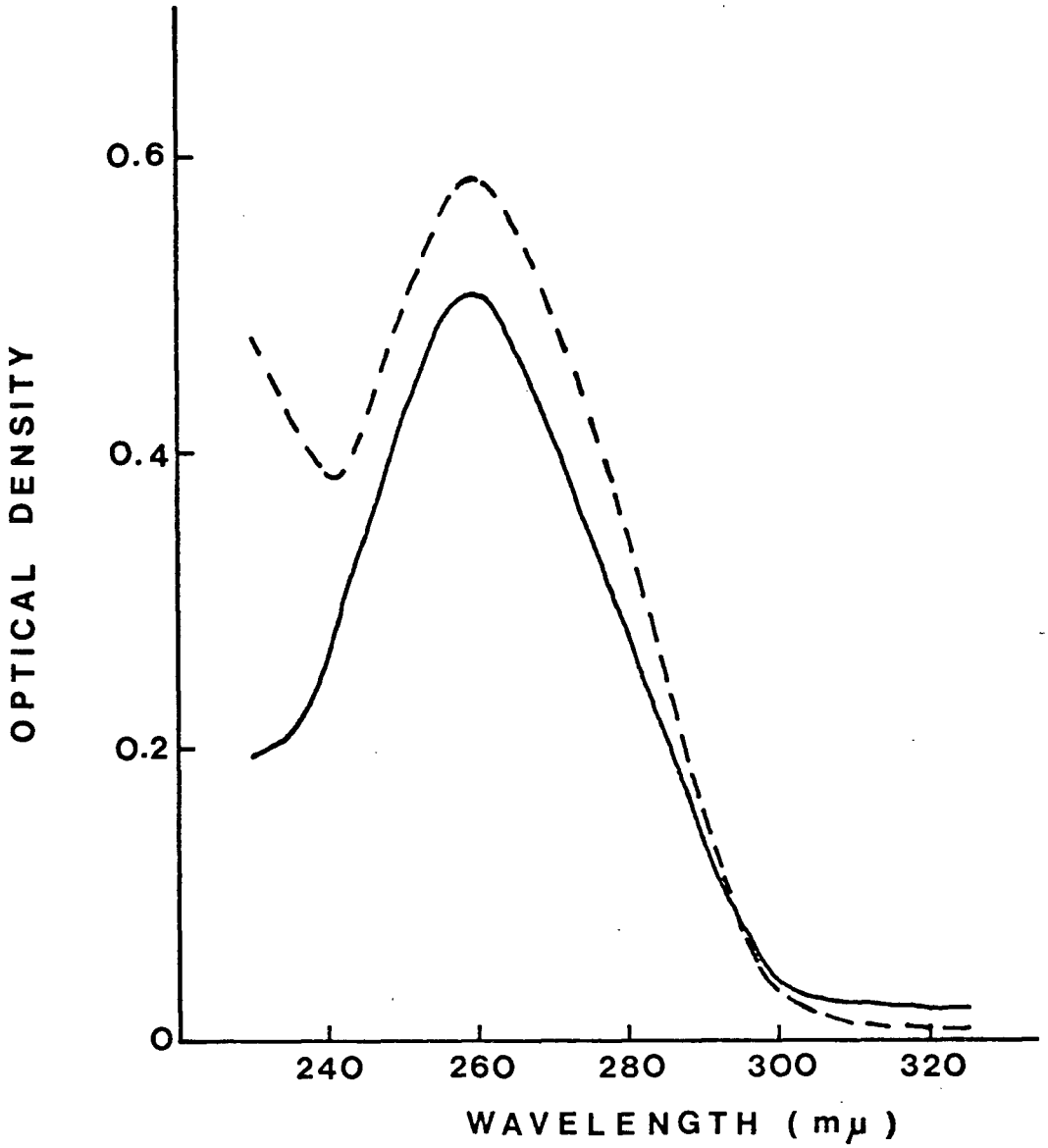


FIG. 25. THE UV-ABSORPTION SPECTRA OF BEEF BRAIN CHROMATIN AND CT-DNA

The absorbance spectrum of a diluted sample of brain chromatin (- - - -) or calf thymus DNA (———) was recorded using a Spectronic, Model 505 (Bausch & Lomb Inc., N.Y.).

TABLE X. CHEMICAL COMPOSITION OF BEEF BRAIN CHROMATIN

Component	Mass Ratio*
DNA	1.0
RNA	0.09
Histone protein	1.02
Non-histone protein	0.9

* Average values of three determinations.
The determinations were carried out in duplicate after extraction of each component as described in Methods (Chapter 2.2E, a).

B. TEMPLATE ACTIVITY OF BRAIN CHROMATIN FOR POLYMERASE II OF BRAIN NUCLEI

In order to manipulate the correct values for the transcription of brain chromatin by brain nuclear polymerase II, it was essential to check the endogenous RNA polymerase activity in the soluble chromatin preparations. Figure 26 depicts that such chromatin preparations contained almost negligible amounts of endogenous RNA polymerase activity, assayed even in the presence of as high as 0.4 M KCl. It should be pointed out that the conditions of preparing chromatin from purified nuclei are those which are very detrimental to the nuclear RNA polymerase activity and perhaps do not solubilize it as some polymerase activity is detectable in the discarded chromatin pellet. This figure also shows that soluble chromatin served as a template for added enzyme (polymerase II), whose activity is further stimulated by the presence of KCl, reaching an optimum around 0.25 M.

When compared with calf thymus native DNA as template, the isolated chromatin from beef brain supported RNA synthesis in a homologous system by brain nuclear RNA polymerase II up to less than 25% (Fig. 27). Moreover, the synthesis of RNA by polymerase II on deproteinized chromatin (brain DNA) was of an equal magnitude to that obtained with calf thymus DNA, indicating that the source of DNA used as a reference for comparison does not matter. These re-

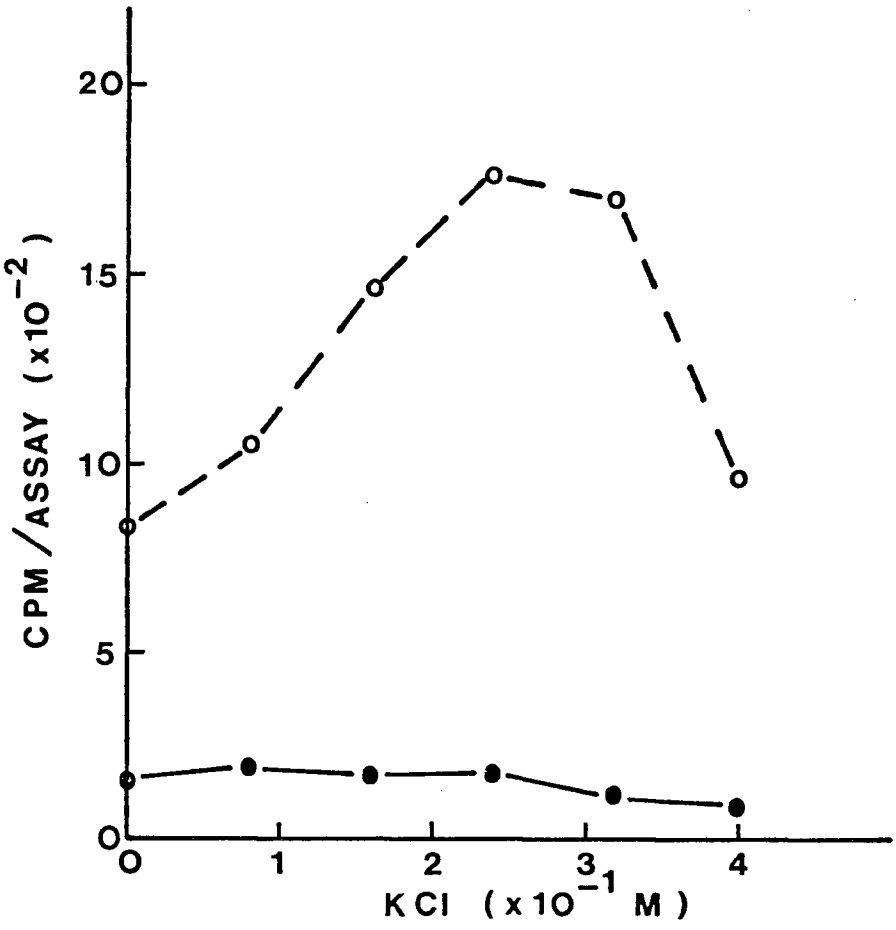


FIG. 26. THE EFFECT OF KCl ON THE CHROMATIN-TEMPLATED RNA POLYMERASE II ACTIVITY

Brain nuclear RNA polymerase II (76 µg protein) was assayed in the presence of various concentrations of KCl using soluble chromatin (containing 60 µg DNA) from beef brain as the template (o— — —o). Other assay conditions were the same as described in Methods. The endogenous RNA polymerase activity of the soluble chromatin (without added enzyme) was simultaneously determined (•————•).

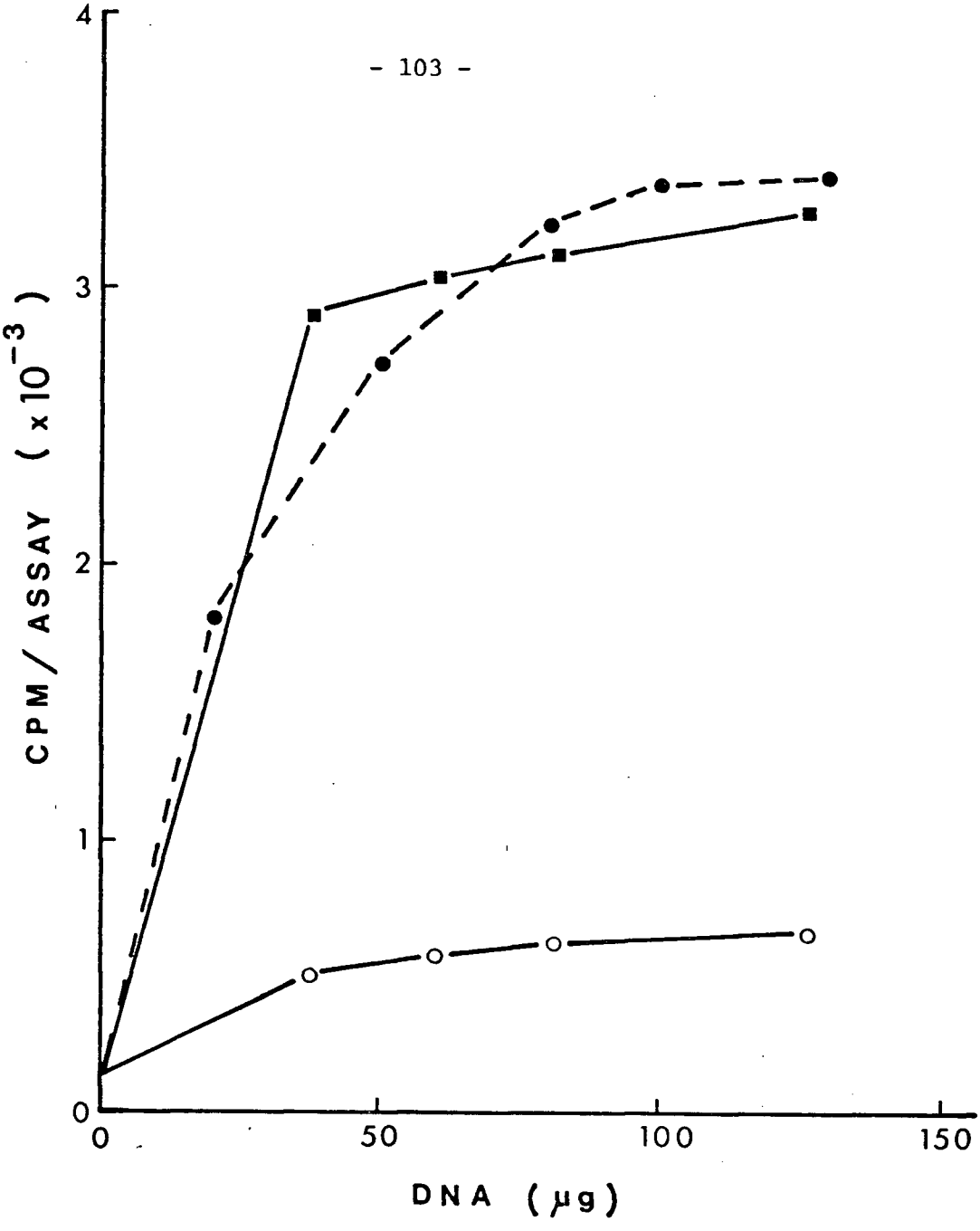


FIG. 27. TEMPLATE ACTIVITY OF BRAIN CHROMATIN

Brain nuclear RNA polymerase II (0.051 mg protein) was assayed in the presence of various amounts of calf thymus DNA, obtained from Worthington Biochemical Corporation (●— — —●), deproteinized chromatin (■————■) and equivalent amounts of DNA in native chromatin (o————o).

sults suggest that most of the genomic activity in differentiated brain cells is somehow repressed.

Figure 28 demonstrates that if dehistonized chromatin from brain (the chromatin which had been treated with 0.4 N H_2SO_4 to remove histone proteins, see Figure 24) was utilized as the template for polymerase II, the resulting enzyme activity was found to be about 3-fold greater than that obtained with pure DNA. However, similarly prepared dehistonized chromatin from cerebral tissue was as active as pure DNA as a template for E. coli RNA polymerase (Fig. 29). Also the rate of activity of polymerase II (Fig. 30), but not E. coli enzyme (Fig. 31), was about three times greater with dehistonized chromatin than pure DNA as template (both the enzymes being assayed under similar conditions in the presence of equivalent amounts of DNA in dehistonized chromatin). These observations imply that an activating mechanism of DNA transcription exists in the genome of brain cells which is only detectable by using an homologus enzyme (i.e. brain nuclear RNA polymerase II) but not by using an heterologous enzyme (i.e. E. coli RNA polymerase).

RNA polymerase II of brain nuclei and E. coli enzyme exhibited a selective response towards α-amanitin toxin and the antibiotic rifampicin, i.e. polymerase II of brain nuclei primed with CT-DNA, chromatin and dehistonized chromatin is almost com-

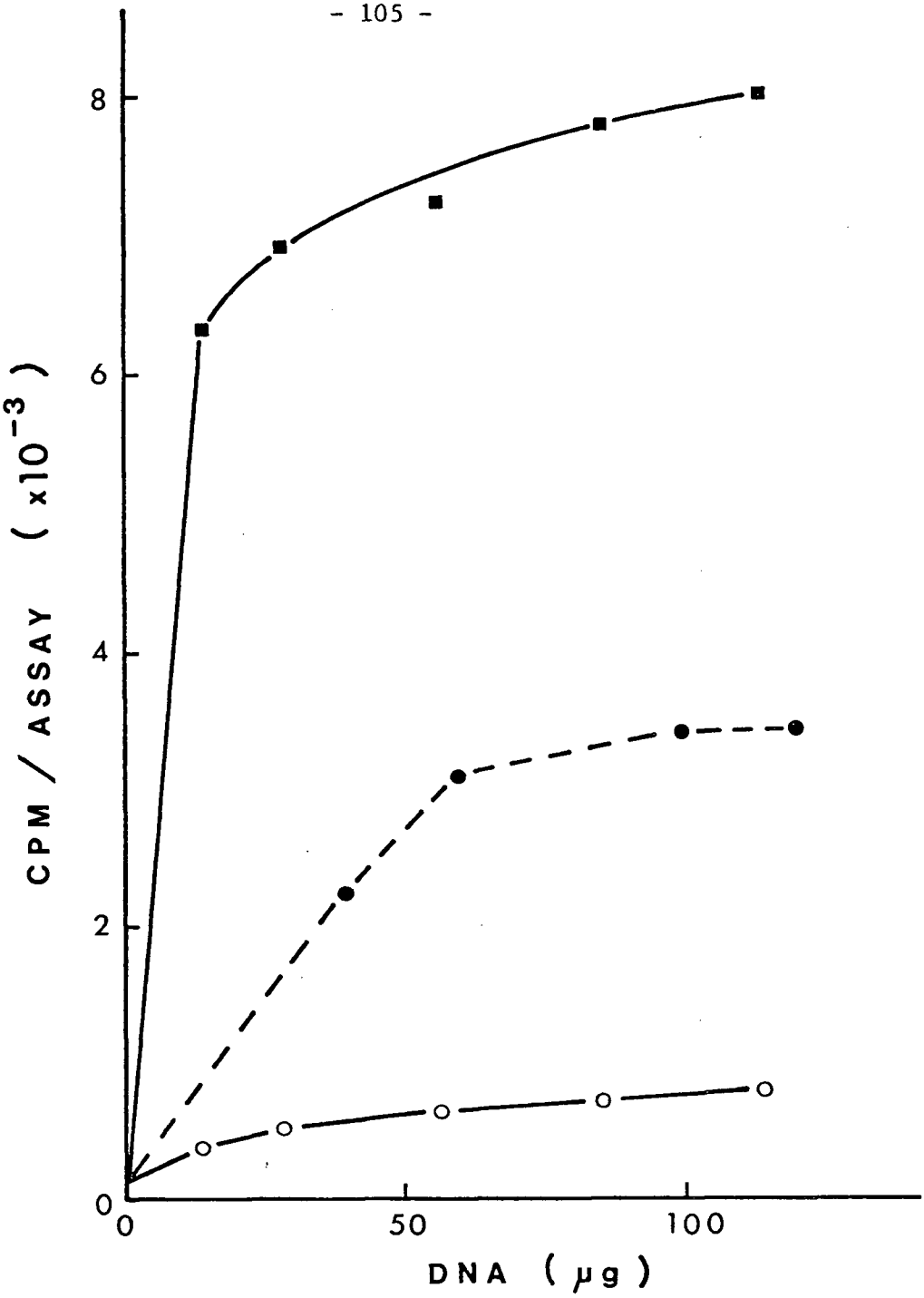


FIG. 28. TEMPLATE ACTIVITY OF BRAIN DEHISTONIZED CHROMATIN FOR BRAIN NUCLEAR RNA POLYMERASE II

Brain nuclear RNA polymerase II (0.051 mg protein) was assayed in the presence of calf thymus DNA (●— — —●) and equivalent amounts of DNA in dehistonized chromatin (■ ——— ■) and native chromatin (o ——— o).

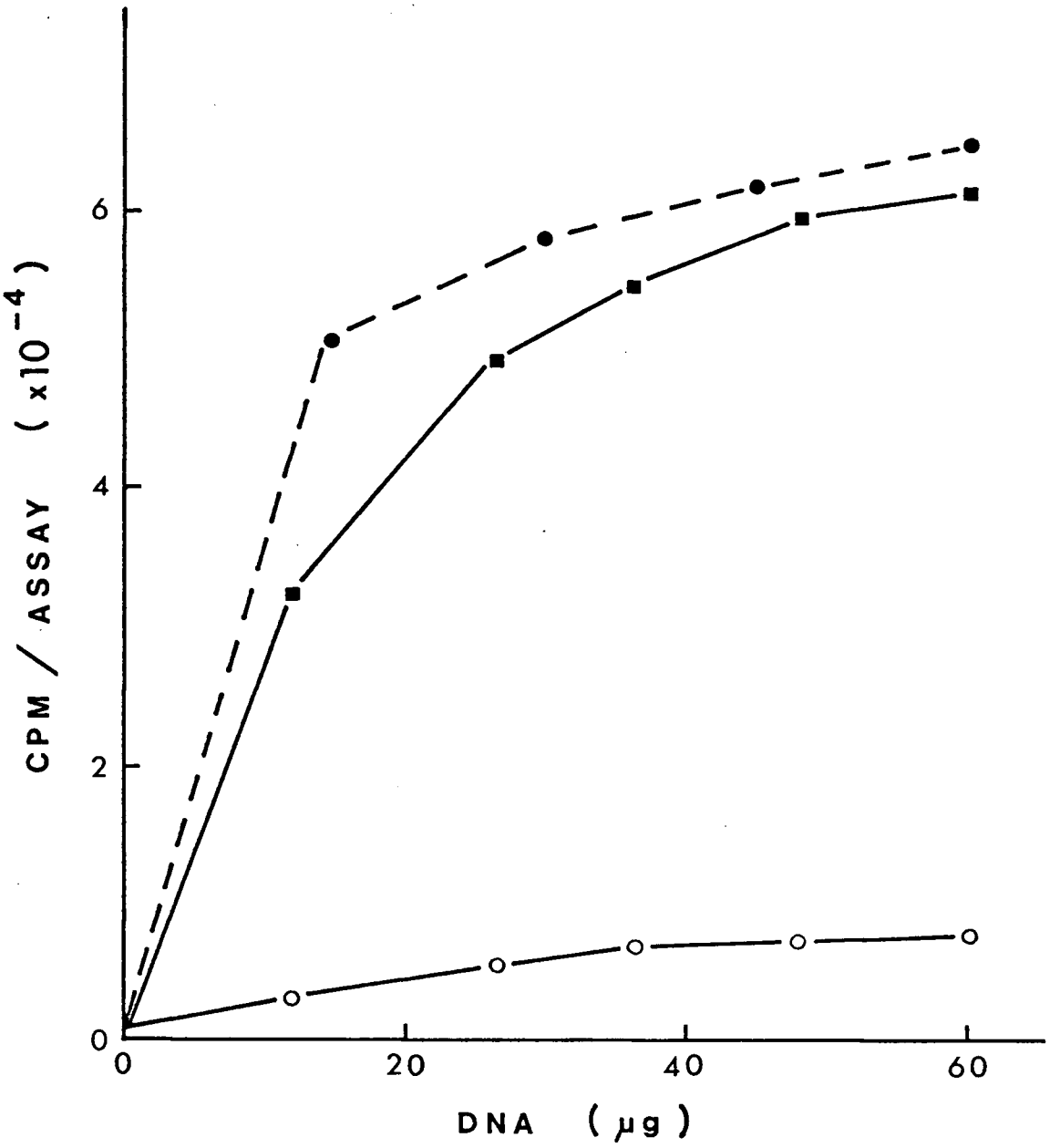


FIG. 29. TEMPLATE ACTIVITY OF BRAIN DEHISTONIZED CHROMATIN FOR E. COLI RNA POLYMERASE

E. coli RNA polymerase (0.0225 mg protein) was assayed in the presence of calf thymus DNA (●— — —●) and equivalent amounts of DNA in dehistonized chromatin (■————■) and native chromatin (o————o).

FIG. 30. THE RELATIVE RATES OF TRANSCRIPTION OF CHROMATIN, DEHISTONIZED CHROMATIN AND CT-DNA BY BRAIN NUCLEAR RNA POLYMERASE II

The enzyme (76 µg protein) was assayed in the presence of chromatin (60 µg DNA, o ——— o) or dehistonized chromatin (60 µg DNA, ■ ——— ■) or CT-DNA (60 µg, ● — — — ●) as template. Other conditions of enzyme assay were the same as given in Chapter 2.2E, a.

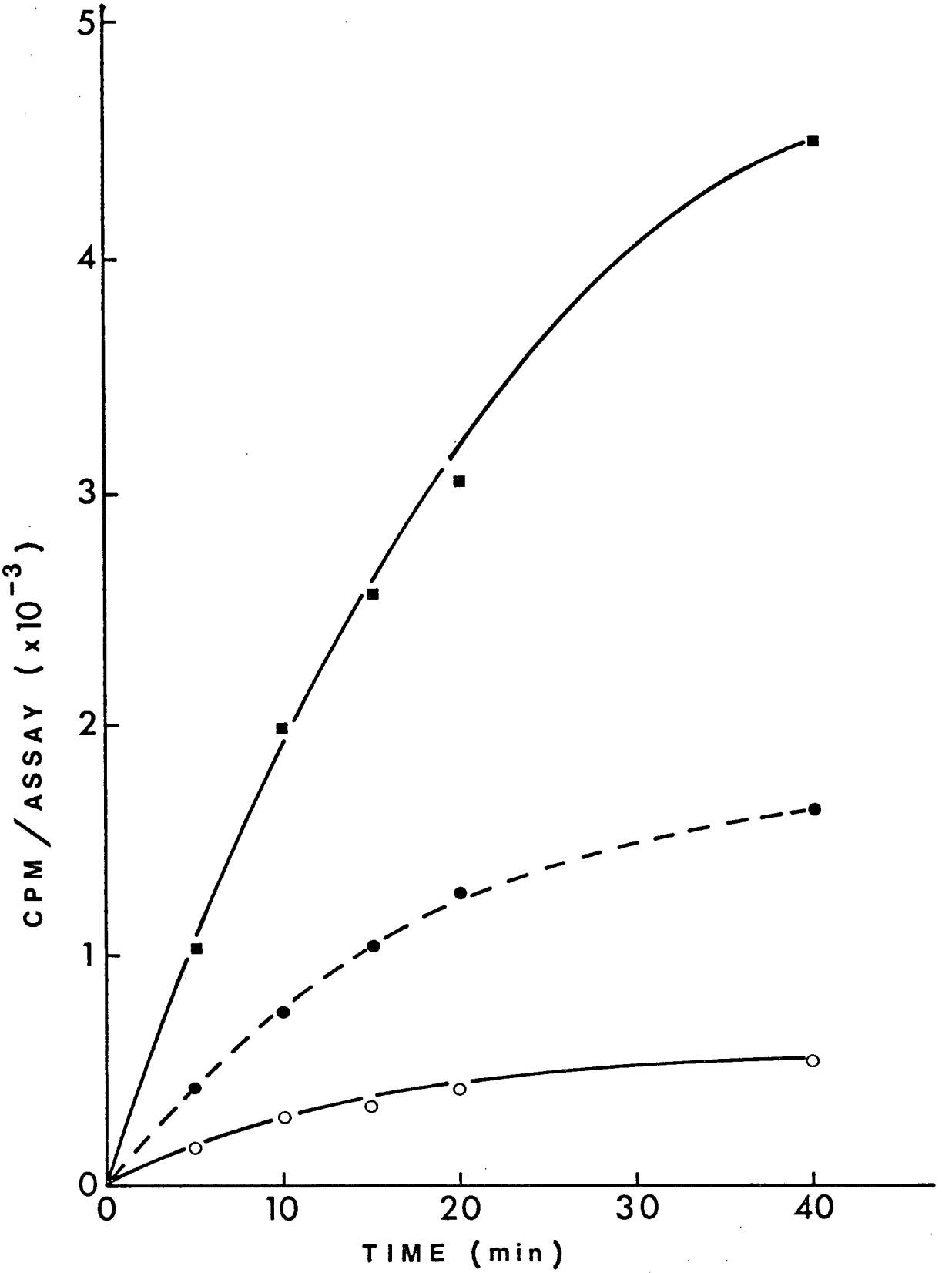
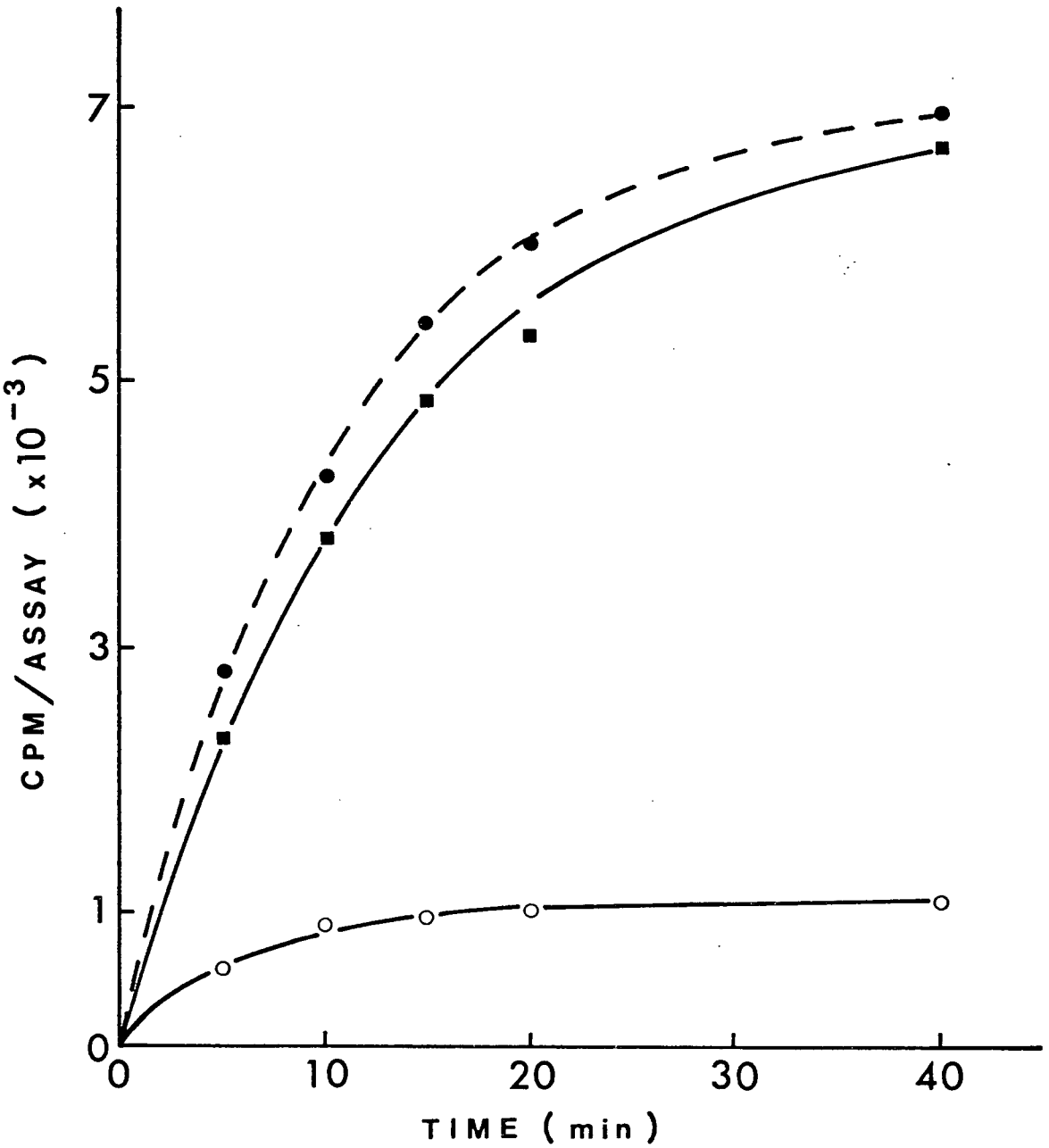

5
4
3
2
1
0
CPM / ASSAY (x10⁻³)
0
10
20
30
40
TIME (min)

FIG. 31. THE RELATIVE RATES OF TRANSCRIPTION OF CHROMATIN, DEHISTONIZED CHROMATIN AND CT-DNA BY E. COLI RNA POLYMERASE

The enzyme (22.5 µg protein) was assayed in the presence of chromatin (60 µg DNA, o——————o) or dehistonized chromatin (60 µg DNA, ■——————■) or CT-DNA (60 µg, ●— — —●) as template. Other conditions of enzyme assay were the same as for brain nuclear polymerase II (see Fig. 30), except that carrier UTP (0.1 mM) was included in the reaction mixture.


7
5
3
1
0
CPM/ASSAY (x10⁻³)
0
10
20
30
40
TIME (min)

pletely inhibited by α-amanitin but not by rifampicin whereas E. coli enzyme primed with the same templates is inhibited by rifampicin but not by α-amanitin (Table XI). Since the pattern of sensitivity in each case was unaltered by the presence of three different types of templates, indicating that some portion (which is required for the interaction with α-amanitin or rifampicin in respective cases) may be structurally different in the molecule of RNA polymerases obtained from two organisms of widely distinct evolutionary origin. In this regard, it is important to note that the mechanism of action of α-amanitin and rifampicin is related to the polymerase protein and not to the DNA template (for discussion see Chapter 1 and also Goldberg & Friedman, 1971).

As described in the foregoing section, the removal of histones from cerebral chromatin accentuated RNA synthesis in vitro by brain nuclear RNA polymerase II to levels even higher than those obtained with pure DNA, it was very enticing to look for the nature of RNA transcribed from the DNA in chromatin and in dehistonized chromatin. The nature of RNA synthesized by polymerase II with pure CT-DNA, chromatin and dehistonized chromatin as templates was analyzed by sucrose-density gradient centrifugation. Figure 32 shows that RNA transcribed on chromatin and dehistonized chromatin as templates sediments as a single peak in slightly lighter regions than the

TABLE XI. THE EFFECT OF α-AMANITIN AND RIFAMPICIN ON BRAIN NUCLEAR RNA POLYMERASE II AND *E. COLI* RNA POLYMERASE AS DIRECTED BY CT-DNA, CHROMATIN AND DEHISTONIZED CHROMATIN TEMPLATES

Enzyme	Template	α-Amanitin		Rifampicin	
		(µg/assay)	(% of control)	(µg/assay)	(% of control
Brain Nuclear Polymerase II	CT-DNA	0	100	0	100
		2.5	9.5	2.5	98
		5.0	8.5	5.0	97
	Chromatin	0	100	0	100
		2.5	7.4	2.5	96
		5.0	8.0	5.0	97
	Dehistonized chromatin	0	100	0	100
		2.5	5.5	2.5	95.8
		5.0	5.6	5.0	96
E. coli Polymerase	CT-DNA	0	100	0	100
		2.5	98.5	2.5	3.5
		5.0	99.6	5.0	3.4

/Continued....

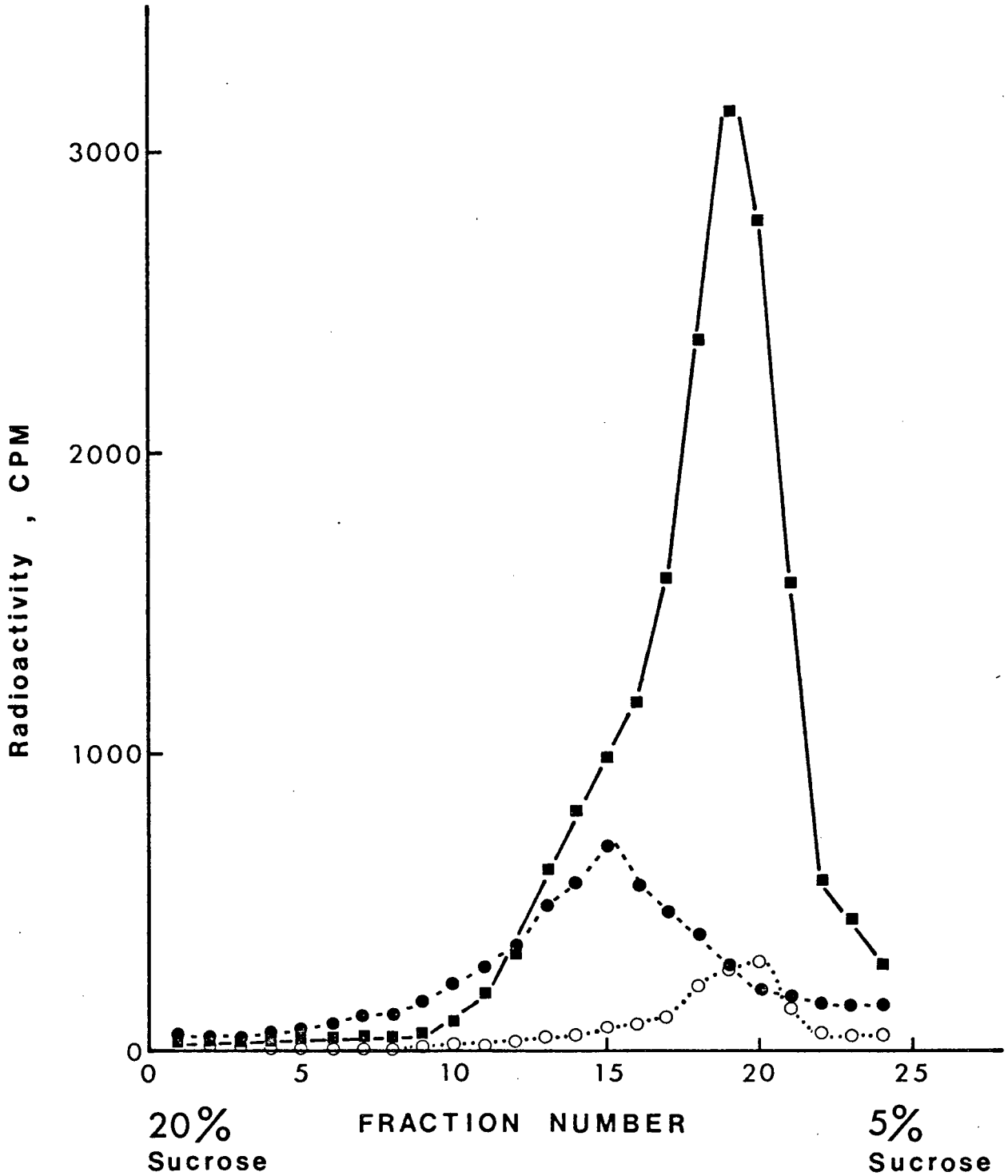
TABLE XI. THE EFFECT OF α-AMANITIN AND RIFAMPICIN ON BRAIN NUCLEAR RNA POLYMERASE II AND E. COLI RNA POLYMERASE AS DIRECTED BY CT-DNA, CHROMATIN AND DEHISTONIZED CHROMATIN TEMPLATES Continued

Enzyme	Template	α-Amanitin		Rifampicin	
		(μg/assay)	(% of control)	(μg/assay)	(% of control)
E. coli Polymerase	Chromatin	0	100	0	100
		2.5	101	2.5	4.2
		5.0	99	5.0	3.6
	Dehistonized chromatin	0	100	0	100
		2.5	100	2.5	3.2
		5.0	99.5	5.0	3.4

Brain nuclear polymerase II (56 μg protein/assay) and E. coli polymerase (22.5 μg protein/assay) were assayed in the absence (control) or presence of various amounts of either α-amanitin or rifampicin. The amount of DNA template in each case was 60 μg/assay. Other conditions of enzyme assay were the same as described in Methods (Chapter 2.2E, a).

FIG. 32. SUCROSE-DENSITY GRADIENT PROFILE OF RNA TRANSCRIBED FROM CHROMATIN, DEHISTONIZED CHROMATIN AND CT-DNA BY BRAIN NUCLEAR RNA POLYMERASE II

Polymerase II (112 µg protein) was separately assayed using 60 µg each of chromatin DNA, dehistonized chromatin DNA and CT-DNA. After 60 min incubation, the reaction mixtures were treated with SDS and the RNA product was analyzed by sucrose density gradient centrifugation. The radioactivity of ^{3}H-UMP incorporated into RNA, which was transcribed from chromatin (o·····o) or dehistonized chromatin (■ ——— ■) or CT-DNA (● - - - - - - ●), is diagrammed.


Radioactivity , CPM
3000
2000
1000
0
0
5
10
15
20
25
20%
Sucrose
FRACTION NUMBER
5%
Sucrose

RNA which is made on CT-DNA. This finding illustrates two points: (1) RNA chains transcribed from the naked DNA (free of chromosomal protein and RNA molecules) are longer in length than those synthesized with chromatin or dehistonized chromatin, and (2) no unique species of RNA is made on chromatin from which most of the histone proteins have been selectively removed. Although a definitive interpretation will require an extensive analysis by more specific assays such as hybridization-competition measurements, nevertheless, it appears as if the association of chromosomal macromolecules with the genome brings about reduction in size of RNA transcribed in vitro.

CHAPTER FOUR. DISCUSSION AND CONCLUSIONS

4.1. MULTIPLE FORMS OF DNA-DEPENDENT RNA POLYMERASE

Although DNA-dependent RNA polymerase enzyme, which catalyzes the incorporation of ribonucleotides into RNA as instructed by DNA, was first identified in nuclear preparations from rat liver (Weiss & Gladstone, 1959), the study of RNA polymerase from various eukaryotic organisms has proceeded very slowly. This slow progress could be attributed to the fact that the intrinsic enzyme activity of intact nuclei is low and that many difficulties are encountered in obtaining a soluble enzyme fraction which will depend on exogenous DNA for its catalytic function. Recent studies on RNA polymerase from nuclei of eukaryotic cells have testified to all these difficulties (see Chapter One).

Despite numerous initial difficulties, various trial experiments involving a variety of conditions eventually led to the solubilization of RNA polymerase in high yields from beef brain nuclei. The experimental data, as presented in the preceding Chapter of this thesis, demonstrate the existence of at least two functional species of DNA-dependent RNA polymerase in the nuclei of brain cells which are designated as RNA polymerase I and polymerase II. These enzymes are readily separated from each other by chromatographic procedures and appear to be distinct entities. There is no evidence to rule out the possibility of the interconversion of one form to the other.

In addition to chromatographic resolution, the separated RNA polymerase species are distinguishable based on some of their differing catalytic properties; for example, the profiles of manganese, magnesium and potassium chloride, the relative transcriptive activity with native and denatured DNA templates and the sensitivity to α-amanitin toxin are very different for each polymerase. The Mn^{++}/Mg^{++} activity ratio is about 3-4 times greater for brain nuclear polymerase II than for polymerase I, whose activities are maximal at high and low ionic strengths, respectively. Moreover, polymerase I prefers native DNA to denatured DNA as template whereas polymerase II is about 3-4 times more active with denatured DNA than with native DNA as the template. At very low concentrations, α-amanitin selectively inhibits RNA polymerase II almost completely while RNA polymerase I is resistant to α-amanitin. These properties of brain nuclear polymerase I and polymerase II are similar to those which have been reported recently for two species of nuclear DNA-dependent RNA polymerase of other eukaryotes (Roeder & Rutter, 1969; Chambon et al., 1970; Goldberg & Moon, 1970; Roeder et al., 1970; Tocchini-Valentini & Crippa, 1970; Ponta et al., 1971; Strain et al., 1971). An additional species of nuclear RNA polymerase (called polymerase III) has been described in sea urchin (Roeder & Rutter, 1969) and in yeast (Blatti et al., 1970; Adman & Hall, 1971; Ponta et al., 1971). This enzyme is not detected in the nuclei of brain cells. However, it should be mentioned that polymerase III in the

aquatic fungus, Blastocladiella emersonii, has been shown to be derived from the contaminating mitochondria (Horgen & Griffin, 1971) and thus this enzyme activity may be of mitochondrial origin instead of being a nuclear component.

As discussed in the Introductory Chapter, the concept of multiple RNA polymerases is not new. The existence of, at least, two RNA polymerase activities was initially detected by Windell and Tata (1964) in isolated nuclei of rat liver. Subsequent work led these workers to propose that the nuclei of eukaryotic cells contain two activities of DNA-dependent RNA polymerase, namely, the Mg^{++}-stimulated activity and the Mn^{++}/ammonium sulphate-stimulated activity (Windell & Tata, 1966). This view was further strengthened by the high-resolution autoradiographic studies on the intranuclear localization of these two RNA polymerase activities (Pogo et al., 1967; Maul & Hamilton, 1967). The isolation and separation of two RNA polymerases from the nuclear extracts of beef brain (as reported in this thesis) and of other eukaryotic tissues (Roeder & Rutter, 1969; Chambon et al., 1970; Goldberg & Moon, 1970; Roeder et al., 1970; Tocchini-Valentini & Crippa, 1970; Ponta et al., 1971; Strain et al., 1971) constitutes one of the most convincing pieces of evidence to confirm the earlier proposal of Windell and Tata (1966). Moreover, the Mg^{++}-stimulated activity (designated RNA polymerase I) and the Mn^{++}/ammonium sulphate-stimulated activity (designated RNA polymerase II) has been localized in the nucleolar

and nucleoplasmic regions respectively (Roeder & Rutter, 1970).

From the viewpoint of regulation of RNA synthesis in eukaryotic organisms, the occurrence of selectively localized DNA-dependent RNA polymerases merits important attention. The synthesis of ribosomal RNA takes place in the nucleolus (Brown & Gurdon, 1964; Penman et al., 1966; Perry, 1967; Reeder & Brown, 1971) whereas DNA-like RNA synthesis occurs predominantly in the nucleoplasm (Perry et al., 1964; Georgiev, 1967), the extranucleolar region of the nucleus which contains the bulk of the nuclear DNA. Since polymerase I is of the nucleolar origin while polymerase II is localized in the nucleoplasm (Roeder & Rutter, 1970), these enzymes may be implicated in the specific transcription of nucleolar genes and extranucleolar genes, respectively. Rutter and his collaborators (1970) have substantiated this hypothesis based on their finding that the RNA synthesized by isolated nuclei in the presence of α-amanitin is predominantly ribosomal RNA while in the absence of this toxin it is non-ribosomal nuclear RNA, as determined by hybridization-competition assays. Thus it is likely that polymerase I functions in the transcription of nucleolar DNA (transcripts being primarily ribosomal RNA) whereas polymerase II is involved in the transcription of a greater range of nucleoplasmic genes (producing more of DNA-like RNA or messenger RNA). In addition,

if polymerase III is a nuclear component then it may have a unique transcriptive role in the extranucleolar regions (perhaps transfer RNA?) (Blatti et al., 1970).

In earlier work with isolated nuclei, the Mg^{++}-primed and Mn^{++}/ammonium sulphate-stimulated RNA polymerase activities were found to make GC-rich RNA (ribosomal type) and AU-rich (DNA-like), respectively (Windell & Tata, 1966). In these studies it was also shown that the effect of ammonium sulphate on Mn^{++}-primed polymerase activity is perhaps related to the dissocation of certain chromosomal proteins with the resultant activated transcription of those genes which code for DNA-like RNA. Liau et al. (1965) have demonstrated that partial removal of acid-soluble proteins from the isolated nucleoli shifts the base composition of the RNA synthesized in vitro (from GC-rich to AU-rich). These observations imply that the chromosomal proteins associated with the endogenous templates (nucleolar and extranucleolar DNA) may restrict, in part, the involvement of separate RNA polymerases in the selective synthesis of ribosomal or messenger RNA even though these enzymes are found in association with their respective templates. Thus protein factors, perhaps similar to those necessary for specific transcription during bacteriophage infection (see Travers, 1971; Bautz et al., 1970; Summers & Siegel, 1970; Hager et al., 1970), provide a good means of regulating the transcription of specific genes by separate RNA

polymerases. In this regard, it is important to point out that certain protein factors have been described very recently which are capable of modulating the activity of RNA polymerases from the nuclei of green coconuts, Cocus nucifera (Mondal et al., 1972).

Furthermore, many hormones have been shown to activate gene transcription (see Tata, 1966). Although the mechanism of action is not yet clear it appears as if the Mg^{++}-primed RNA polymerase activity is selectively modulated in response to several hormones (Pegg & Korner, 1965; Tata, 1966; Sereni & Barnabei, 1967; Lukacs & Sekeris, 1967; Hamilton et al., 1968; Jacob et al., 1969; Yu & Feigelson, 1971). Recently hydrocortisone was shown to increase the activity of RNA polymerase I isolated from rat liver nuclei after the in vivo administration of the hormone (Sajdel et al., 1971). These workers have presented evidence to show that hydrocortisone stimulates the polymerase I activity by an allosteric mechanism. Smuckler and Tata (1971) have observed significant increases in the levels of polymerase I isolated from the hepatic nuclei of rats treated with growth hormone or triiodothyronine. These findings do suggest that the transcriptive role of RNA is profoundly under the control of certain hormones. However, the precision of this control mechanism remains to be established.

The present knowledge about the eukaryotic RNA polymerase I and II is inadequate to permit a definite interpretation

of their functioning in the selective synthesis of ribosomal and messenger RNA, respectively. Nevertheless, the existence of multiple forms of RNA polymerase could reasonably be implicated in the synthesis of distinct species of cellular RNA in higher organisms. Although there is great potential in mediating a regulation of gene transcription by different RNA polymerases, the mechanism of such a control system is far from being understood. This is possibly due to the fact that many additional control mechanisms, such as those involving chromosomal macromolecules and hormones, operate in eukaryotes and thus complicate any fundamental system of regulation. Therefore, the transcriptive specificity does not appear to be solely due to distinct species of RNA polymerase localized specifically within the various nuclear structures (e.g. nucleolus and nucleoplasm), but the transcriptional transitions could still be brought about by other factors.

4.2. MODULATION OF RNA POLYMERASE ACTIVITY BY POLYAMINES

As discussed in Chapter One, polyamines have been postulated to play an important role in the control of RNA metabolism, possibly by modulating some phase in RNA synthesis. In addition, polyamines have been shown to enhance the RNA synthesizing capacity of isolated nuclei (MacGregor & Mahler, 1967; Dutton & Mahler, 1968; Caldarera *et al.*, 1968; Barbiroli *et al.*, 1971a). The data reported in this

thesis demonstrate that the RNA polymerase isolated from beef brain nuclei is significantly stimulated by spermine or spermidine. The effect of spermidine is much more pronounced than spermine, and it stimulates both the RNA polymerases separated from brain nuclei. The magnitude of stimulation by spermidine of brain nuclear polymerase II is much greater than that of polymerase I, when these enzymes are assayed in the presence of their preferred divalent cation. Moreover, the influence of spermidine on the polymerase II activity is found to be selective with respect to its preferred DNA template. Spermidine stimulated Mn^{++}-primed enzyme activity (i.e. polymerase II) with both native and heat-denatured DNA as the templates. However, the stimulatory effect of spermidine with heat-denatured DNA was either lost or suppressed if Mg^{++} was added to the Mn^{++}-containing assay system, and spermidine was inhibitory rather than stimulatory if Mg^{++} was the only divalent cation. These observations are interpreted to mean that nucleoplasmic RNA polymerase (or polymerase II) and consequently, the synthesis of DNA-like RNA may be preferentially stimulated by polyamines such as spermidine.

In a preliminary report, Russell et al. (1971) have shown that polyamines elevate RNA polymerase activity of rat liver nucleoli above that level found with other stimulating cations (e.g. Mg^{++}, Mn^{++}, NH_4^+, etc.). The same workers also observed that anucleolate

mutants of Xenopus laevis, which lack the capacity to synthesize ribosomal RNA, do not synthesize normal amounts of putrescine and spermidine (Russell, 1971). They interpreted their data to indicate an activation of ribosomal RNA synthesis by polyamines. This, in fact, contrasts with the observations of polyamine effects on brain nuclear RNA polymerase I and polymerase II, as recorded in this thesis. On the other hand, the data presented here on the spermidine stimulation of separated enzymes are similar to those obtained by others (Stirpe & Novello, 1970; Barbiroli et al., 1971a). These investigators have observed a greater stimulation of Mn^{++}/ammonium sulphate-primed activity than the Mg^{++}-primed activity by polyamines. Moreover, based upon the analysis on methylated albumin kieselguhr columns of RNA synthesized in spermine-treated and untreated chick embryos, it has been resolved that the magnitude of increase by spermine in the synthesis of DNA-like RNA is much greater than that of ribosomal RNA (Barbiroli et al., 1971b).

The mechanism of stimulation of DNA-dependent RNA polymerase activity by spermidine is very far from being understood. A possible action of spermidine in vivo might be displacement of histones from the DNA template (endogenous template in interphase nucleus being chromatin) with resultant activation of the genetic transcription. This does not appear to be the case, at least, as resolved by reconstitution experiments which indicated that spermidine could not

overcome the histone-inhibition. However, such a possibility for spermidine action cannot be excluded based on these experiments.

Using E. coli RNA polymerase it has been shown that more RNA chains are initiated in the presence of spermidine (Peterson et al., 1968). The results reported in this thesis show that under the influence of spermidine more RNA chains are synthesized by brain nuclear RNA polymerase II with calf thymus DNA as template. It, therefore, may be reasonable to assume that, at least in vitro, spermidine may act by causing the release of the RNA product from the Enzyme-DNA-RNA complex which is formed during the course of the reaction. This view gains some support from the finding that spermidine could counteract the inhibitory effect of yeast RNA on brain nuclear RNA polymerase II. Moreover, the available evidence suggests that the polyanions such as tRNA inhibit E. coli RNA polymerase by preventing the binding of the enzyme to DNA template, and thus inhibit the initiation of RNA chains (Richardson, 1966). Since spermidine could circumvent the inhibition of RNA polymerase II activity by yeast RNA, it is quite possible that spermidine stimulates the initiation of RNA chains, not necessarily from different segments of DNA. In doing so, spermidine could act either by accelerating the binding of the enzyme to DNA template (perhaps specifically at the initiation signals) or by stabilizing the Enzyme-DNA complex.

4.3. TRANSCRIPTION OF BRAIN CHROMATIN BY MAMMALIAN RNA POLYMERASE

Based on the strategy, as discussed in the Introductory Chapter, the template activity of brain chromatin was studied using a homologous or a heterologous RNA polymerase. The data presented in this thesis demonstrate that the capacity of the isolated chromatin from beef brain to function as a template for RNA synthesis *in vitro* by brain nuclear RNA polymerase II is markedly repressed. In accord with earlier reports (Huang & Bonner, 1962; Allfrey *et al.*, 1963; Paul & Gilmour, 1966; Georgiev *et al.*, 1966) only 20-25% of the normal transcription, such as that occurring on pure DNA, is observed by brain polymerase II and also by *E. coli* enzyme. These observations suggest that most of the genome in nerve cells is somehow repressed, reinforcing the support for the "masking" hypothesis of gene transcription in higher organisms (see Bonner *et al.*, 1968b; Paul *et al.*, 1970).

The data, such as those referred to above, on the template activity of the isolated chromatin preparations by RNA polymerase are difficult to reconcile with the recent observations made on the structure of mammalian chromatin (Clark & Felsenfeld, 1971; Itzahki, 1971). These workers, based on their physico-chemical studies involving the titration of chromatin with the polymers of basic amino acids and the digestion by nucleases, have suggested that nearly 35-50% of the DNA in chromatin is "naked" and not covered with

proteins. However, it is noteworthy that the measurement of RNA synthesis on chromatin template by exogenously added RNA polymerase is a function of biological activity which, in turn, is dependent on a variety of parameters. Thus, the small discrepancy in these results is most likely reflected by the different nature of the experiments.

A group of DNA-bound basic proteins, the histones, have long been suggested as the repressors of gene expression (Stedman & Stedman, 1950). The repressor function of histones is indicated by the fact that the selective removal of histones by acid- or detergent-treatment raises the template capacity of the isolated chromatin to the same level as that obtained with pure DNA as template for bacterial RNA polymerase (Marushige & Bonner, 1966; Paul & Gilmour, 1966; Georgiev et al., 1966; Smart & Bonner, 1971). Moreover, the activity of DNA-dependent RNA polymerase is greatly suppressed by histones in vitro (see Hnilica, 1967; Georgiev, 1969; Spelsberg et al., 1969; and Figure 18 of this thesis). A similar pattern of the template activity of dehistonized chromatin from brain chromatin (the chromatin which had been treated with 0.4 N H_2SO_4 to remove histones) is observed using E. coli RNA polymerase. However, this pattern varies with brain nuclear RNA polymerase II which, in fact, is about 3-4 times more active with dehistonized chromatin than with pure DNA as the template. This observation implies that an activating mechanism of the DNA transcription exists

in the genetic material (chromatin) of brain cells which is only detectable in a reconstituted homologous system but not in a heterologous system.

Since RNA polymerase II from brain nuclei prefers heat-denatured DNA rather than native DNA (see Table VI of this thesis), it may be argued that the treatment of native chromatin with acid might have caused the denaturation of chromatin DNA and that is why dehistonized chromatin is the preferred template to pure DNA for this enzyme. However, it has been documented that the treatment of chromatin with 0.4 N H_2SO_4 at 0°C (the conditions utilized in the experiments described in this thesis) does not bring about the denaturation of chromatin DNA (for discussion see Bonner et al., 1968a; Hnilica, 1967; Murray, 1969). The above mentioned argument may also be excluded based on the finding that E. coli RNA polymerase utilizes dehistonized chromatin as a template as good as pure DNA. Such a result would not be expected if the DNA in dehistonized chromatin had undergone denaturation because with denatured DNA as the template E. coli enzyme is very poorly active (Chamberlin & Berg, 1962; Hurwitz et al., 1962; Furth & Loh, 1964; Stevens & Henry, 1964).

Furthermore, it has been shown that 'nicks' in DNA template (produced by limited digestion with DNase) stimulate transcription

by bacterial RNA polymerase (Vogt, 1969). Therefore, it may be questioned whether the activated transcription of brain dehistonized chromatin by brain RNA polymerase II is a consequence of some nuclease action. In view of the observation that the activated transcription of brain dehistonized chromatin is catalyzed by brain enzyme only and not by E. coli RNA polymerase, the possibility of nuclease effect appears very unlikely.

Although the mechanism of activated transcription, such as observed herein, is not clear, chromosomally-bound macromolecules (e.g. chromosomal RNA or acidic proteins) may be implicated in this phenomenon. There is no evidence for deciding if chromosomal RNA or acidic proteins are solely responsible for this effect because dehistonized chromatin contains both of them. In this connection, it is of particular importance that non-histone proteins have been shown to activate the chromatin-templated bacterial RNA polymerase (Teng & Hamilton, 1969; Kamiyama & Wang, 1971; Teng et al., 1971; Kostraba & Wang, 1972). Moreover, it has been reported that non-histone proteins which stimulate chromatin-templated transcription have protein kinase activity (Kamiyama & Dastugue, 1971). It is possible that non-histone proteins can activate the chromatin transcription either by neutralizing the ionic-charge effects or by counteracting the histone-repression. Therefore, the possibility of involvement of chromosomal RNA as a gene activator, as advanced by Bonner and his collaborators (Bekhor et al., 1969; Mayfield & Bonner, 1971) cannot be excluded.

If the non-histone proteins or the chromosomal RNA function as gene activators in the process of genetic transcription, as discussed above, then RNA synthesis on dehistonized chromatin should be greater than when pure DNA is used as the template. But this is not so because bacterial RNA polymerase transcribes dehistonized chromatin or pure DNA without any preference (Marushige & Bonner, 1966; Paul & Gilmour, 1966; Smart & Bonner, 1971; also this thesis). This anticipated function is, in fact, resolved by utilizing a homologous enzyme system. Thus, in the former case, the failure to detect the anticipated template activity of the dehistonized chromatin might be due to the use of a heterologous enzyme. At present, it is difficult to visualize a definite explanation for this kind of discrepancy but two comments are worth making: (i) polymerases of different origins might be highly selective in their action, perhaps in the step of chain-initiation, and (ii) the molecular orientation of macromolecules in the dehistonized chromatin may enforce the specificity with homologous RNA polymerase but not with heterologous enzyme. Therefore, it is considered very likely that the transcriptional control mechanisms which operate at the level of RNA polymerase are highly specific in mammalian cells.

4.4. CONCLUDING REMARKS

The observations recorded in this thesis on the transcription of genes by RNA polymerase in brain cells, are summarized as follows:

(1) The conditions are developed which solubilized in high yields the enzyme RNA polymerase from the nuclei of brain cells. By chromatographic analysis, the solubilized enzyme is separated into two peaks of RNA polymerase activity, designated as RNA polymerase I and polymerase II.

(2) These two polymerases are completely dependent upon exogenously added DNA template. RNA polymerase I prefers native DNA to heat-denatured DNA as template while RNA polymerase II utilizes heat-denatured DNA much better than native DNA as template.

(3) Both the polymerases exhibit an optimum pH around pH 8.0. Their activities are greatly sensitized by actinomycin D.

(4) The polymerase I requires Mg^{++} in preference to Mn^{++} as the divalent cation whereas polymerase II is about four times as active with Mn^{++} as with Mg^{++}. Both the enzymes exhibit maximal activity in the presence of Mn^{++} plus Mg^{++} (at their optimum concentrations).

(5) Two RNA polymerases from brain cell nuclei respond to different KCl profiles. The activity of polymerase I is somewhat enhanced by 0.05 M KCl, but it is significantly inhibited as the

concentration of KCl is raised to 0.2 M and above. KCl markedly stimulates the polymerase II activity with an optimum concentration around 0.2 M. The stimulation by KCl of polymerase II is much more pronounced in the presence of Mn^{++} or Mn^{++} plus Mg^{++} than in the presence of Mg^{++} alone.

(6) RNA polymerase II is almost completely inhibited by the toxin α-amanitin while the activity of polymerase I is not affected by this toxin.

(7) The polyamines, such as spermidine or spermine, sharply enhanced the activity of both the polymerases. Spermidine exerts a much more pronounced stimulatory effect on polymerase II than polymerase I, particularly, when assayed in the presence of their preferred divalent cation. The enhancement of polymerase II activity by spermidine is also characteristic of the preferred template for this enzyme.

(8) The addition of yeast RNA and calf thymus histone results in a considerable degree of inhibition of polymerase II activity. Spermidine appears to counteract the inhibition due to yeast RNA, but not that due to histone.

(9) The product of the reaction of RNA polymerase II sediments around 18 S in a sucrose-density gradient and apparently is a complex of the kind Enzyme-DNA-RNA.

(10) The capacity of the isolated cerebral chromatin to serve as a template for RNA synthesis by a homologous or a heterologous RNA polymerase is very much lower than that of the pure DNA. Most probably, the acid-soluble chromosomal proteins are responsible for this greatly reduced template activity of chromatin.

(11) The brain nuclear RNA polymerase II is 3-4 times more active with acid-treated chromatin than pure DNA as the template, but E. coli RNA polymerase uses these two templates without any preference.

(12) The RNA synthesized on chromatin templates (native or acid-treated) by brain polymerase II is somewhat smaller in size than that made on pure DNA.

In the light of accumulating evidence that mammalian RNA polymerase displays vast multiplicity and that the template activity of a more physiological template can be modulated invariably, a dual nature of the transcriptional control is anticipated. The multiple nature of RNA polymerase may primarily be implicated in the synthesis of different types of cellular RNA. On the other hand, the template characteristics are likely to be of the utmost importance in enunciating the secrets of cellular differentiation. The brain cells are probably no exception to the above statement.

In spite of the recent advances that have been made in the field of mammalian RNA polymerase, a great deal still remains to be understood, e.g. how multiple RNA polymerases recognize the signals for RNA chain initiation and termination? What is the structure-functional relationship in different RNA polymerases? It is hoped that future research, particularly in these areas, will lead to major break-throughs in elucidating the mechanism of control of RNA synthesis in mammalian cells.

The enhancement of the RNA polymerase activity by polyamines in vitro occurs in those concentrations of polyamines which are within the physiological range. Although such an effect is well documented, yet the physiological involvement of polyamines in the process of gene transcription remains to be established. The preferential stimulation of polymerase II by spermidine may suggest that the synthesis of DNA-like RNA (i.e. messenger RNA) is markedly influenced by polyamines.

The data presented here on the transcription of chromatin DNA by a homologous RNA polymerase reveal certain important characteristics which are quantitatively distinct from those observed using a heterologous enzyme. This might plausibly suggest that the transcriptional control mechanisms are probably highly specific in mammalian cells.

Thus one of the primary contributions of the experimental work described in this thesis is that it demonstrates the existence of at least two DNA-dependent RNA polymerases in the nuclei of brain cells. In addition, it explores the hypothesis that the transcriptional control may be mediated not only by the transcriptional enzyme but also by the physiological state of the template.

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