

CHAPTER II

ALTERATION IN TRANSCRIPTION BY BENZO(A)PYRENE

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2.1. INTRODUCTION

2.1.1. BACKGROUND INFORMATION

The biological effects of BP and other carcinogenic substances are thought to arise due to their interaction with cellular macromolecules. The adduct formation with DNA is believed to be a crucial determinant in carcinogenesis by these chemicals (Swenberg et al. 1985). The lesion or damage done to DNA by the carcinogen has to express itself in the cell for the subsequent carcinogenic events to occur. The initial response of the cell is to repair this damage, but the repair process may be error-free or error-prone. In the earlier case removal of adduct is accomplished without any subsequent effect, while in the latter case removal of adduct is accompanied by a mutational event. Thus the damage will be fixed genetically which may be expressed later in the life of the cell or its progeny. Another possible way the lesion is fixed is during DNA-replication without repair of the damage. As discussed in Chapter I, DNA replication and DNA synthesis are affected by BP.

Holiday and Jeggo (1985) suggested relationship between changing gene expression and carcinogenesis. The variety of ways in which gene expression is altered as

suggested by them are: mutation, chromosomal rearrangement, crossing over and gene conversion, gene amplification and DNA-methylation. Since hypomethylation of DNA is observed in general in tumor cells, they suggested altered methylation as one of the heritable ways of expressing altered gene. Thus, study of altered expression of the gene - an important way to fix the lesion, may be of significance in understanding the basic mechanism of carcinogenesis.

Transcription, one of the measures of gene expression, when probed after the treatment with carcinogen may offer a biochemical means of monitoring the changes occurring in the cell and which may have consequences for the future cancerous course that the cell undergoes. The process of transcription has been observed to be altered in tumor cells. In Taper hepatoma cells, Church et al. (1969) observed alteration in gene expression. Turkington (1971) described changes in hybridizable nuclear RNA in mouse mammary tumors. Supowit and Rosen (1980; 1981) noted changed gene expression in neoplastic mammary tissue, which they concluded was due to a distinct type of poly A⁺ messenger RNA. In a fast growing hepatoma cell line total poly A⁺-RNA population was observed to be increased and this was due to enhanced stability of larger size messenger RNA (Mattei et al. 1982). Petropoulos et al. (1985) observed lack of expression for certain RNA population in Morris hepatoma cell. Excretion of RNA

catabolites in urine by MC-induced tumor bearing mice indicated alteration in RNA metabolism (Thomale and Nass 1982). Alteration in metabolism of double-stranded RNA sequences in liver of rats was observed in response to a variety of carcinogens (Clawson and Smuckler 1982). One of the possible functions of the oncogene, as suggested by Bishop (1985), was regulation of transcription from other genes. From tumor cell, transcription altering (mainly stimulating)-factors have been isolated e.g. proteins (Nakanishi et al. 1981; Natori 1982) and small nuclear RNA (Ringuette et al. 1982). Misumi et al. (1982) noted unusual ability of RNA polymerase from mouse ascites sarcoma cells to transcribe tRNA. Alteration in different components of chromatin has been observed in cancerous cells. Alteration in DNA has already been discussed in detail in Chapter I. Stein et al. (1978) have exhaustively reviewed the role of chromosomal proteins in onset and maintenance of neoplastic state of the cell by controlling gene structure and expression. Tan et al (1982) observed different H₁-histone composition in neoplastic cells. Thus, it appears that all the components of transcription machinery i.e. the template-chromatin consisting of DNA and proteins, and the enzymes are altered in the tumor cell and as a consequence even the RNA products and their metabolism is altered. Therefore, study of transcription during early stages of experimental carcinogenesis may prove to be a useful biochemical observation in understanding the mechanism by which carcinogen alters the gene expression.

A wide range of chemical carcinogens and other toxic chemicals were shown to alter the process of transcription in animals during early stages after administration of the compound. Inhibition in transcription was observed in vivo in different organs or organ slices or in nuclei isolated from these organs in response to following compounds: dimethylnitrosamine; DMN (Stewart and Magee 1971; Herzog and Farber 1976; Winicov 1981; Garyfallides et al. 1984), 4'-fluoro-4-dimethylaminoazobenzene; FDAB (Kidson and Kirby 1965), N-hydroxy-acetaminofluorene; N-OH-AAF or 2-acetamidofluorene; 2-AAF (Troll et al. 1968; Zieve 1972; Herzog et al. 1975; Grunberger et al. 1973; Grunberger and Weinstein 1979a), ethionine (Farber et al. 1974; Swann et al. 1975; Yamano et al. 1982), aflatoxin B₁; AFB₁ (Clifford and Rees 1966; Gelboin et al. 1966; Pong and Wogan 1970; Saunders et al. 1972; Neal 1972; 1973; Yu and Grunberger 1976; Yu 1977; Emeh et al. 1981) and dimethylbenzanthracene; DMBA (Flamm et al. 1966; Alexandrov et al. 1970; deAngelo et al. 1978). Nuclear transcription was shown to be inhibited after administration of toxic and carcinogenic chemicals like methylmethane sulfonate; MMS (Herzog and Farber 1976), tetrachlorodibenzo-p-dioxin; TCDD (Kurl et al. 1982) and methylazoxymethanol acetate; MAMA, a carcinogenic cycasin derivative from seeds and roots of cycad (Yu et al. 1983). Inhibition in transcription was displayed by nuclei even after in vitro treatment with compounds like captan (Vinocour and Lewis 1985) and benzene

quinone metabolites (Post et al. 1984). MC, an inducer of xenobiotic metabolizing enzymes, generally induced stimulation in transcription (Loeb and Gelboin 1964; Hishizawa et al. 1964; Bresnick 1966; Madix and Bresnick 1967; Gelboin 1967; Liberator and Bresnick 1981b; Kleeberg et al. 1982; Szeberenyi et al. 1982). Studies have been carried out to understand the mechanism of such modulation in transcription by these compounds. Inhibitory effects of compounds like N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Yoda et al. 1978) and AAF (Troll et al. 1968; Austin and Moyer 1979) were observed even when template or enzymes were reacted with the compound before being used for transcription.

2.1.2. AIMS AND OBJECTIVES

BP, the carcinogenic PAH of our interest has been shown to bind to macromolecules of a variety of organs in different animals. The target organ for tumorigenesis varies with different routes of administration. Binding of BP to DNA has been detailed in Chapter I. Effects of BP on DNA replication and synthesis and on protein synthesis have also been described in Chapter I. However, as Grunberger and Weinstein (1979a) have pointed out, there is lack of knowledge about transcriptional effects of BP in different organs of the body. The only notable study by the French group showed transcription-modulating effect of topically applied BP (Alexandrov et al. 1970) or BP-containing fraction

of cigarette smoke (Alexandrov and Vendrely 1972) on mouse skin. Weinstein's group studied template activity of BPDE-modified DNA in vitro and observed inhibitory effect on transcription (Leffler et al. 1977; Grunberger and Weinstein 1979a).

This led us to study the effect of BP on transcription machinery in rat liver as a model system since liver responds to i.p. administration of BP by eliciting induction of metabolizing enzymes (Thakker et al. 1985) and binding of BP to macromolecules especially DNA of liver in vivo and in vitro has been established (Tables 1.4 and 1.5). BP also induces hepatoma in mice (Roe and Waters 1967) and hepatocellular carcinoma in rats (Kitagawa et al. 1980). Knowledge of transcriptional effects of BP observed in this model system can then be applied for understanding effect of BP on other target organs like skin, mammary gland, lung, forestomach, oesophagus, brain etc.

Eukaryotic transcription is a process by which chromatin, the native template, is transcribed by one of the three independent RNA polymerases to produce RNA. The template chromatin consists of DNA and proteins (mainly histones and nonhistone chromosomal proteins: NHCP). Enzymes can also transcribe native or denatured DNA and also synthetic polynucleotides. Enzymes RNA polymerases were earlier partially purified as "aggregate" enzyme. However sonication at high salt solubilized the enzymes from the

aggregate and they were then separated by column chromatography (ion-exchange) to resolve into three forms. They were termed as RNA polymerases I, II and III or A, B and C respectively by different workers. For the sake of uniformity they will be referred to as I, II and III throughout the thesis. RNA polymerase I is nucleolar, α -amanitin-resistant, rRNA synthesizing enzyme, while II and III are nucleoplasmic, α -amanitin-sensitive, mRNA and tRNA synthesizing enzymes respectively. Several excellent reviews have been published detailing various aspects of eukaryotic RNA polymerases (Blatti et al. 1970; Jacob et al. 1970; Jacob 1973; Biswas et al. 1975; Chambon 1975; Roeder 1976; Sentenac 1985).

Several observations made during experimental BP-carcinogenesis in animals point to the fact that transcription must be altered in response to BP. Binding of BP to DNA has been well established (Chapter I). It has also been observed that BP binds to specific locations of the genome viz. areas of chromatin containing rapidly labeled nascent RNA (Spelsberg et al. 1977), linker DNA of chromatin (Kurian et al. 1985), and actively transcribing (DNase I sensitive) region of genome (Arrand and Murray 1982). BPDE I was shown to bind (more than BPDE II) to internucleosomal region as compared to nucleosomes (Kootstra and Slaga 1980). BP was shown by many authors to bind differentially to chromatin proteins which may in turn control gene

expression. Pezzuto et al. (1976) showed binding of BP more to RNA and NHCP than to DNA and histones. Kootstra (1982b) observed binding of BPDE to core histones even when the latter were acetylated. Kurokawa et al. (1982) indicated that BP reacted predominantly with chromatin proteins of 44,000 and 65,000 daltons and selectively with low MW NHCP and H₁ histones. MacLeod et al. (1981) noted that H₃ and H_{2A} were major adduct forming histones while H_{2B} and H₄ did not form much of adduct with BP. However, H₁ like proteins also formed adduct with BP. Observations that binding of BPDE to DNA brought about unwinding of DNA (Gamper et al. 1980) or inhibited action of DNA methyltransferases (Wojciechowsky and Meehan 1984) also raise the possibility of effect of BP on gene expression as one of the mechanisms of fixation of lesion by the carcinogen.

Hence, the goal of our study was to identify any effect of BP on transcription machinery in rat liver and to understand the mechanism of this effect (if observed) at the molecular level, so that the knowledge can be applied to understand the initial events in the life of the cell or organ after administration of the carcinogen-BP.



2.2. EXPERIMENTAL PROCEDURES

2.2.1. MATERIALS

2.2.1.1. Chemicals:

BP, dimethylsulfoxide (DMSO), sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), DL-dithiothreitol (DTT), calf thymus DNA, 2-mercaptoethanol, poly[d(I-C)], ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$, Tris, PPO, dimethyl POPOP, Brilliant Blue-G, EDTA, ribonucleoside triphosphates (ATP, UTP, GTP and CTP), actinomycin-D, α -amanitin and yeast soluble RNA were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Sucrose (AnalaR) was obtained from BDH Chemicals Ltd., Poole, England. BPDE I was a generous gift from Prof. R.G. Harvey of Ben May Laboratory for Cancer Research, Chicago, IL, USA. Naphthalene was obtained from Fluka AG, Switzerland. DEAE-Sephadex A-25 was from Pharmacia Fine Chemicals, Uppsala, Sweden., 8-Hydroxyquinoline (AR) was obtained from "Reanal" Finomvegyszergyar, Budapest, Hungary. Folin and Ciocalteu's phenol reagent was from BDH, Glaxo Laboratories (India) Ltd., Bombay. All the other chemicals used were procured locally and were of analytical grade.

2.2.1.2. Radiochemicals:

[6-¹⁴C]Orotic acid (sp.act. 11.8 mCi mmol⁻¹) was a product of Isotope Group of this Research Centre. [α -³²P]-Uridine 5'-triphosphate (sp.act. 3000 Ci mmol⁻¹), [5,6-³H]-uridine 5'-triphosphate (sp.act. 40 Ci mmol⁻¹) and [8-³H]-guanosine 5'-triphosphate (sp.act. 10.6 Ci mmol⁻¹) were procured from Amersham International plc, Amersham, UK.

2.2.1.3. Animals:

Male Wistar albino rats (100 - 150 g) maintained on laboratory stock diet in animal house of this Research Centre were used for the experiments.

2.2.2. METHODS

2.2.2.1. Treatment of animals:

BP, dissolved in DMSO (4 mg ml⁻¹), was administered intraperitoneally (i.p) to animals at the dose of 20 mg Kg⁻¹ body weight. Appropriate vehicle control received 0.5 ml DMSO (i.p) per 100 g body weight. Animals were sacrificed by cervical dislocation before removal of organs. Sacrifice was generally made two hours after treatment unless specified otherwise.

2.2.2.2. Isolation and purification of nuclei and nucleoli from liver:

(a) Osmotonic method: Livers obtained from animals were

immersed in cold wash solution (0.32 M sucrose), blot dried and weighed. Livers from two animals were pooled together for each observation. All subsequent procedures were carried out at 4°. Liver was homogenized in 3 vol of 0.32 M sucrose-3 mM MgCl_2 , using a Potter Elvehjem glass-teflon homogenizer. Homogenate was filtered through two layers of muslin cloth and crude nuclei were pelleted out at 600 x g for 10 min in SS-34 rotor of a Sorvall RC-5B centrifuge. Nuclei were purified according to Zieve's (1972) modification of the method of Blobel and Potter (1966). Crude nuclear pellet was suspended in 0.32 M sucrose-3 mM MgCl_2 (1 ml g^{-1} liver) and was mixed with 2 vol of 2.1 M sucrose. The mixture was layered over 2.1 M sucrose and centrifuged at 60,000 x g_{av} for 1 h in a Beckman L2 65-B ultracentrifuge using type 30 rotor or in an 8 x 25 rotor of MSE Superspeed-50 centrifuge. Translucent nuclear pellet was suspended in 0.25 M sucrose-1 mM MgCl_2 (0.5 ml/10 g liver equivalent).

(b) Hypertonic method: Nuclei were also purified by modified method of Yu (1975) to prevent any loss of enzyme activity. Liver was collected in 0.25 M sucrose-3.3 mM CaCl_2 and was homogenized in 2 vol of 2.3 M sucrose-3.3 mM CaCl_2 -0.5 mM PMSF. Homogenate was filtered through two layers of muslin cloth and filtrate was diluted with equal vol of the same solution. Clean nuclear pellet was obtained by centrifugation as above. Nuclear pellet suspended in 0.32 M sucrose-

1 mM MgCl_2 could be stored at -70° for upto a week without any apparent damage to its transcriptive ability.

Isolation of nucleoli was essentially according to Yu (1977). Nuclei purified by hypertonic sucrose method were suspended in 0.34 M sucrose (12 ml/5 g liver equivalent). Nuclear structure was disrupted by sonication with an MSE microprobe (speed 6, 16 bursts of 10 seconds each) and the suspension was centrifuged at $3000 \times g$ for 20 min over a cushion of 0.88 M sucrose. Nucleolar pellet was suspended in 0.32 M sucrose.

2.2.2.3. Separation of free and engaged RNA polymerase fractions of nuclei:

The two forms of RNA polymerase activities, free and engaged, were separated generally according to the method of Yu (1975). Purified nuclei obtained by hypertonic sucrose method were suspended in 0.25 vol of 0.34 M sucrose - 0.5 mM PMSF using Potter-Elvehjem homogenizer at low speed. On centrifugation at $3000 \times g$ for 10 min the supernatant obtained was designated as free form of nuclear RNA polymerase. Pellet obtained was suspended in 0.34 M sucrose - 0.5 mM PMSF and was designated as engaged form of the enzyme.

2.2.2.4. Isolation of nuclear RNA:

Nuclear RNA was purified from crude nuclear pellet (occasionally also from purified nuclei) by a procedure

evolved in this laboratory from several methods available in literature (Schutz et al. 1968; Wilkinson et al. 1971; Markov and Arion 1973). Crude nuclear pellet (2.5 g liver equivalent) was suspended in 4 vol of SDS medium [0.3% SDS, 0.1 M NaCl, 0.05 M sodium acetate buffer (pH 5.0)]. The suspension was extracted by vigorous shaking at 60° for 10 min, with 0.5 vol of phenol mixture (phenol : water : m-cresol = 64 : 16 : 20 containing 0.1% 8-hydroxyquinoline). The mixture was cooled rapidly in ice and was further extracted at room temperature for 10 min. Aqueous phase obtained by centrifugation at 12,000 x g for 10 min was reextracted with the phenol mixture at room temperature till all proteins were removed. Final aqueous phase was made to 3% with potassium acetate and RNA was precipitated by addition of 2.5 vol of cold ethanol and the mixture was left at -40° for at least 3 h. RNA was collected by centrifugation at 12,000 x g for 10 min and was dissolved in 0.01 M NaCl (2.5 ml). It was reprecipitated with ethanol as above and final RNA precipitate was dissolved in 1.0 ml of 0.01 M NaCl.

2.2.2.5. In vivo incorporation of [6-¹⁴C]orotic acid into hepatic nuclear RNA:

Animals treated with BP or DMSO as specified earlier were administered i.p. [6-¹⁴C]orotic acid (dissolved in warm 0.9% NaCl) at the dose of 25 µCi Kg⁻¹ body weight, half an hour prior to sacrifice. Livers obtained from two identically

treated animals were pooled together for isolation of nuclear RNA. RNA was assayed spectrophotometrically or chemically by orcinol method. Radioactivity was analyzed by direct counting. Results were expressed as cpm mg^{-1} RNA.

2.2.2.6. In vitro RNA synthesis:

(a) Nuclear RNA synthesis:

(i) Mg^{2+} - and Mn^{2+} plus $(\text{NH}_4)_2\text{SO}_4$ -assays: Purified nuclei isolated by osmotic method were assayed for RNA synthesis under different ionic conditions to determine Mg^{2+} - and Mn^{2+} plus $(\text{NH}_4)_2\text{SO}_4$ -dependent activities according to the procedure of Zieve (1972). Reaction mixture (100 μL) for Mg^{2+} -dependent assay contained 50 mM Tris-HCl (pH 8.5), 5 mM MgCl_2 , 20mM 2-mercaptoethanol, 4 mM NaF, 0.6 mM each of ATP, GTP and CTP, 0.04 mM $[5,6\text{-}^3\text{H}]\text{UTP}$ (sp.act. 0.2 mCi mmol^{-1}) and 20 μL nuclear suspension (~ 80 μg DNA). For Mn^{2+} plus $(\text{NH}_4)_2\text{SO}_4$ -dependent activity, the above reaction mixture was modified to contain 50 mM Tris-HCl (pH 7.5), 4mM MnCl_2 , 400 mM $(\text{NH}_4)_2\text{SO}_4$, nucleoside triphosphates and nuclear suspension as stated above. After incubation at 37° for 5 min (45 min for Mn^{2+} -dependent activity) an 80 μL aliquot was removed on Whatman 3 MM filter disk to determine acid-insoluble radioactivity by the method of Bollum (1968). Initial wash with 10% TCA-1% pyrophosphate (4° for 30 min) was followed by 2 washes with 5% TCA-1% pyrophosphate (4° for 10 min) and a

wash each (4° for 5 min) with ethanol, ethanol : ether (1:1) and ether before counting for radioactivity. DNA was estimated by indole method. Results were expressed as pmol $[^3\text{H}]\text{UMP}$ incorporated mg^{-1} DNA.

Both assays were also conducted with limiting concentration of DNA (30 μg instead of 80 μg). In these cases reactions were allowed to proceed at 37° for 5 min. Since Mn^{2+} -dependent assay showed linearity only upto 15 min, the reduced time was to ensure that differences in activity were not masked in plateau region.

(ii) Combined RNA polymerases assay: Nuclei purified by hypertonic sucrose method were assayed for total nuclear RNA synthesis according to the method of Yu (1975). Reaction mixture (100 μL) contained 75 mM Tris-HCl (pH 7.9), 1.5 mM MnCl_2 , 60 mM $(\text{NH}_4)_2\text{SO}_4$, 21 mM 2-mercaptoethanol, 0.6 mM each of ATP, CTP and UTP, 0.04 mM $[8\text{-}^3\text{H}]\text{GTP}$ (sp.act. 0.2 mCi mmol^{-1}), and 20 μL nuclear suspension (~ 80 μg DNA). Following incubation at 37° for 10 min, acid-insoluble radioactivity in an aliquot was counted by the filter-disk method. DNA was estimated by diphenylamine method after perchloric acid (PCA) hydrolysis. For some experiments radioactive nucleotide used was $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ (sp.act. 0.125 mCi mmol^{-1}).

The assays were performed in absence and presence of two concentrations (0.01 μM and 0.01 mM) of α -amanitin

(2 mM stock in water) to determine contribution by each of the three forms of RNA polymerases according to the method of Liberator and Bresnick (1981a). Total activity was that which was obtained in the absence of α -amanitin. Polymerase I represented activity resistant to 0.01 mM α -amanitin, polymerases II and III represented activities which were sensitive to 0.01 μ M and 0.01 mM α -amanitin respectively. Results were expressed as pmol [3 H]GMP (or [α - 32 P]UMP) incorporated mg^{-1} DNA.

(b) Nucleolar RNA synthesis:

Transcriptive activity of purified nucleoli was assayed by the method of Yu (1975) for combined RNA polymerase except that nuclei were replaced in each assay by nucleoli containing ~ 30 μ g nucleolar DNA. Results were expressed as pmol [3 H]GMP incorporated mg^{-1} nucleolar DNA.

(c) Engaged enzyme-directed RNA synthesis:

Engaged enzyme fraction of nuclei consisting of both chromatin and the enzyme was assayed for its transcriptive activity by the method of Yu (1975) using [α - 32 P]UTP except that nuclei were replaced by engaged enzyme preparation (~ 100 μ g DNA per assay). Results were expressed as pmol [α - 32 P]UMP incorporated mg^{-1} DNA or g^{-1} liver.

(d) Free enzyme-directed RNA synthesis:

Free enzyme fraction of nuclei containing only the RNA polymerases was assayed for enzyme activity according to Yu (1975), except that nuclei were omitted and free enzyme preparation (in general 150 μg protein per assay) was added to transcribe external template, calf thymus DNA (25 μg in SSC). Protein content of the free enzyme fraction was assayed by Folin-phenol method. Results were expressed as pmol [α - ^{32}P]-UMP incorporated mg^{-1} protein or g^{-1} liver.

2.2.2.7. Isolation and partial purification of RNA polymerases:

Total RNA polymerases were solubilized and partially purified by a method derived from Yu's (1975) modification of the method of Roeder and Rutter (1970). Entire procedure was carried out at 4° . Nuclei purified by hypertonic sucrose method were suspended in 20 ml TSM-PMSE [10 mM Tris HCl (pH 7.9) - 1 M sucrose - 5 mM MgCl_2 - 20 mM 2-mercaptoethanol - 0.5 mM PMSE]. Suspension was raised to 0.3 M with respect to salt concentration by addition of 1.62 ml 4 M $(\text{NH}_4)_2\text{SO}_4$ (pH 7.9). After stirring for 15 min, the viscous solution was sonicated by a cell disruptor from Heat systems - Ultrasonics Inc., Plainview, N.Y., USA (output 5, scale 22, 6 bursts of 10 sec each, with 30 sec cooling in between). The sonicated solution was rapidly diluted with 2 vol of TGMEM [50 mM Tris-HCl (pH 7.9) - 25% glycerol - 5 mM MgCl_2 - 0.1 mM EDTA (pH 7.2)]

- 20 mM 2-mercaptoethanol]. After stirring for 0.5 h, the solubilized enzyme was separated from chromatin by centrifugation at $100,000 \times g_{av}$ for 1 h. The enzymes along with other proteins present in supernatant were salted out by addition of $(NH_4)_2SO_4$ (0.42 g ml^{-1}) with constant stirring and maintaining pH at 7.9 with liq. NH_3 . The protein precipitate recovered by centrifugation at $100,000 \times g$ for 1 h was suspended in small vol of TGMEM and excess salt was reduced by dialysis against two changes of TGMEM (0.03) (i.e. TGMEM containing 0.03 M $(NH_4)_2SO_4$). The dialysate was spun at $160,000 \times g_{av}$ for 1 h to obtain clear solubilized enzyme preparation referred to as fraction IV by Roeder and Rutter (1970). This was stored at -70° until further use (upto 2 weeks).

The preparation of fraction IV from the engaged and free enzyme fractions of the nuclei was carried out essentially as per the method for nuclei except that initial preparation was different as follows. For engaged enzyme, the engaged enzyme pellet suspended in TSM-MPMSF, and for free enzyme, supernatant obtained in 0.32 M sucrose and diluted with equal volume of 2 x TSM-MPMSF were treated as starting materials for solubilization of the enzymes.

The three different forms of RNA polymerases were partially purified by chromatography on DEAE-Sephadex A-25 ion-exchange column (0.9 cm x 21 cm). Sample (fraction IV)

containing proteins from ~10 g liver was loaded on the column which was pre-equilibrated with TGMEM [0.03] buffer at a flow rate of 17.5 ml h^{-1} . Column was washed with 2 vol (1 vol \approx 14 ml) of same buffer to remove unadsorbed proteins. Enzymes were eluted from the column with a 50 ml linear gradient of 0.03 to 0.5 M $(\text{NH}_4)_2\text{SO}_4$ in TGMEM buffer and collected as fractions of 1.35 ml each (55 drops). Each fraction was assayed for RNA polymerase activity as described in the following section (2.2.2.8). The peak fractions were pooled together and incorporation values were integrated from the individual values of fractions. The three forms of enzymes in order of their elution from the column were referred to as polymerases I, II and III. These were characterized by their respective sensitivity to α -amanitin. Protein was estimated by a modification of Bradford's method and concentration of $(\text{NH}_4)_2\text{SO}_4$ was determined by conductivity measurements. Results were expressed as pmol $[^3\text{H}]\text{GMP}$ incorporated per fraction or mg^{-1} protein.

2.2.2.8. Assay of partially purified RNA polymerases:

The enzymes eluted from DEAE-Sephadex A-25 column were assayed according to Yu's method (1977). An aliquot (0.5 ml) of each column fraction was mixed with 0.05 ml BSA (20 mg ml^{-1} in H_2O) and a 50 μL of this mixture was added to 50 μL cocktail containing remaining assay components with the omission of $(\text{NH}_4)_2\text{SO}_4^*$. After incubation at 37°C and addition of 0.1 unit poly[AdIC- α]

for 20 min, the acid-insoluble radioactivity was estimated in an aliquot by the filter disk method.

2.2.2.9. In vitro model to characterize inhibition of transcription in nuclei:

Purified nuclei obtained by hypertonic sucrose method were reacted with specified concentrations of BPDE I (dissolved in distilled DMSO) in 50 μ L reaction mixture at room temperature for 10 min. After this preincubation, it was mixed with 50 μ L cocktail containing remaining components of Yu's assay (1975) for total RNA synthesis. Transcription was allowed to proceed at 37°C for 10min and an aliquot was withdrawn for measurement of acid-insoluble radioactivity by filter disk method.

When required, actinomycin-D dissolved in acetone : water : glycerol (2:1:1) and poly[d(I-C)] dissolved in 0.1 M NaCl - 0.02 M sodium phosphate buffer (pH 7.0), were added to the reaction mixture. Results were expressed as pmol ^3H /GMP incorporated per assay.

2.2.3. ANALYTICAL PROCEDURES

2.2.3.1. Estimation of RNA:

RNA isolated from nuclei was analyzed either spectrophotometrically or by chemical method as follows: An aliquot of RNA dissolved in 0.01 M NaCl was diluted

suitably with water to measure absorbance at 260 nm and 280 nm in a Beckman DU Spectrophotometer. A ratio of above 1.8 for A_{260}/A_{280} ensured preparation to be substantially free of proteins. RNA was estimated from A_{260} using extinction coefficient $E_{260}^{1\%} = 230 \text{ cm}^{-1}$ (Jennette et al. 1977).

Chemical estimation of RNA by orcinol was carried out according to Ceriotti (1955). An aliquot of RNA (10 - 100 μg) made to 5.0 ml with water was reacted with 5 ml orcinol reagent (200 mg orcinol and 6.1 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ dissolved fresh in 100 ml conc HCl). The reaction was carried out in boiling water bath for 45 min. The contents were cooled and vigorously extracted with 5 ml isoamyl alcohol. The organic phase was separated after standing for 4-5 h at room temperature or by centrifugation and its absorbance was read at 675 nm in Beckman DU or Hitachi-101 Spectrophotometer. RNA was estimated by comparison with the absorbance of yeast soluble RNA which was used as standard after similar treatment. In samples containing sucrose (eg. nuclear preparation) RNA was first hydrolyzed after acid precipitation, by the procedure outlined in the next section, before performing orcinol assay.

2.2.3.2. Estimation of nuclear DNA:

DNA was hydrolyzed adopting a method derived from Munro and Fleck (1966). An aliquot of nuclear suspension (20 - 100 μL containing 50-500 μg DNA) was chilled for 10 min

before mixing with cold 0.6 N PCA (5 ml for macro-method or 0.6 ml for micro-method). The mixture was allowed to stand in ice for 10 min, before the precipitate was obtained by centrifugation (5 min at 3000xx g for macro- or 2 min in an Eppendorf centrifuge for micro-method). The precipitate was washed twice with cold 0.2 N PCA (5 ml or 0.6 ml) each time resuspending the pellet to remove sucrose and other contaminants. RNA from the washed precipitate was hydrolyzed in 0.3 N KOH (2 ml or 0.4 ml) at 37° for 1 h. After cooling for 5 min, the alkali was neutralized and DNA was precipitated along with sodium perchlorate salt by addition of cold 1.2 N PCA (1.25 ml or 0.25 ml). Supernatant containing alkali hydrolyzed RNA was subjected to estimation by the orcinol method as described in the previous section. Precipitate containing DNA was hydrolyzed with 1 N PCA (2 ml or 0.6 ml) at 70° for 15 min. The hydrolyzed DNA collected as supernatant after centrifugation was estimated chemically by diphenylamine method or by indole method as follows. In case of need for exclusive DNA estimation, the alkali hydrolysis step was excluded in the procedure.

In indole method of estimation (Ceriotti, 1952), acid hydrolysate of DNA (containing 10-60 µg DNA) in 2 ml 1 N PCA was mixed with 1 ml indole reagent (40 mg indole and 15 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ dissolved in little warm water and made to 100 ml with water) and 1 ml conc HCl. The mixture was

treated for 10 min in boiling water bath and cooled to room temperature. It was then extracted repeatedly with equal vol of chloroform till all interfering coloring matters (generally pink when direct unhydrolyzed nuclear matter was used) were removed in organic phase. Final aqueous phase was read at 490 nm in a Hitachi 101 spectrophotometer to estimate DNA content using PCA-hydrolyzed calf thymus DNA as standard.

The more routinely followed diphenylamine method (Burton 1956) was as follows. An acid hydrolyzed DNA preparation in 1 ml of 1 N PCA (containing 10-80 μ g DNA) was mixed with 2 ml freshly prepared diphenylamine reagent [1.5 g recrystallized diphenylamine dissolved in 100 ml glacial acetic acid and mixed with 1.5 ml conc H_2SO_4 . Aqueous acetaldehyde (distilled, 16 mg ml^{-1}) was added at 0.1 ml for 20 ml above mixture]. The mixture was allowed to stand overnight at room temperature in the dark. The blue color was read at 600 nm in Beckman DU or Hitachi-101 spectrophotometer to estimate DNA content. PCA-hydrolyzed calf thymus DNA was used as standard.

2.2.3.3. Estimation of protein:

Protein content of nuclear samples was generally estimated by Folin-phenol method of Lowry et al. (1951) using BSA as standard.

For samples of enzyme in TGMEM buffer, the interference from components of the buffer prevented effective estimation of protein by Lowry's method or by spectrophotometric method. The protein in these samples was estimated by a modification of Bradford's method (1976). An aliquot of protein preparation (0.1 ml containing 1 - 10 μg protein) was mixed with 0.9 ml of modified reagent (1 g Brilliant Blue-G dissolved in 100 ml of 88% phosphoric acid and diluted to 1 L with water). Five min after mixing (and before 1 h) the absorbance was measured at 595 nm in plastic cuvettes using a Shimadzu UV/VIS spectrophotometer. Cuvettes were rinsed in ethanol and water after every measurement. Protein was estimated from the absorbance value after subtracting appropriate blank values and by comparison with BSA as standard. Generally observed value for standard was $0.021 A_{595} \mu\text{g}^{-1}$ BSA.

2.2.3.4. Measurement of specific conductance:

In order to estimate concentrations of $(\text{NH}_4)_2\text{SO}_4$ in enzyme fractions eluted from DEAE-Sephadex column, the specific conductivity of 30 μL aliquot mixed in 50 ml water was measured in μMHO units in a Systronics conductivity meter, Type 303 at cell constant of 0.95. This was compared against a range of $(\text{NH}_4)_2\text{SO}_4$ concentrations in TGMEM buffer to get estimate of $(\text{NH}_4)_2\text{SO}_4$ concentrations in each enzyme fraction. Generally observed value for standard was 23 μMHO for every 0.1 M $(\text{NH}_4)_2\text{SO}_4$ in TGMEM buffer.

2.2.3.5. Radioactivity measurement:

Soft β emissions from [^3H] and [^{14}C] sources were counted in liquid scintillation counters using dioxane based cocktail (5 g PPO, 200 mg dimethyl-POPOP and 80 g naphthalene made to 1 L in dioxane). Hard β emissions from [^{32}P] were counted as Cerenkov radiations in distilled water. The counting efficiency for [^3H] was 45% and for [^{14}C] around 98% in Beckman LS-100 liquid scintillation spectrometer and it was 65% for [^3H] in LKB Rackbeta 1217 liquid scintillation spectrometer. For [^{32}P], counting efficiency of Cerenkov radiation was 40% in the Beckman instrument.

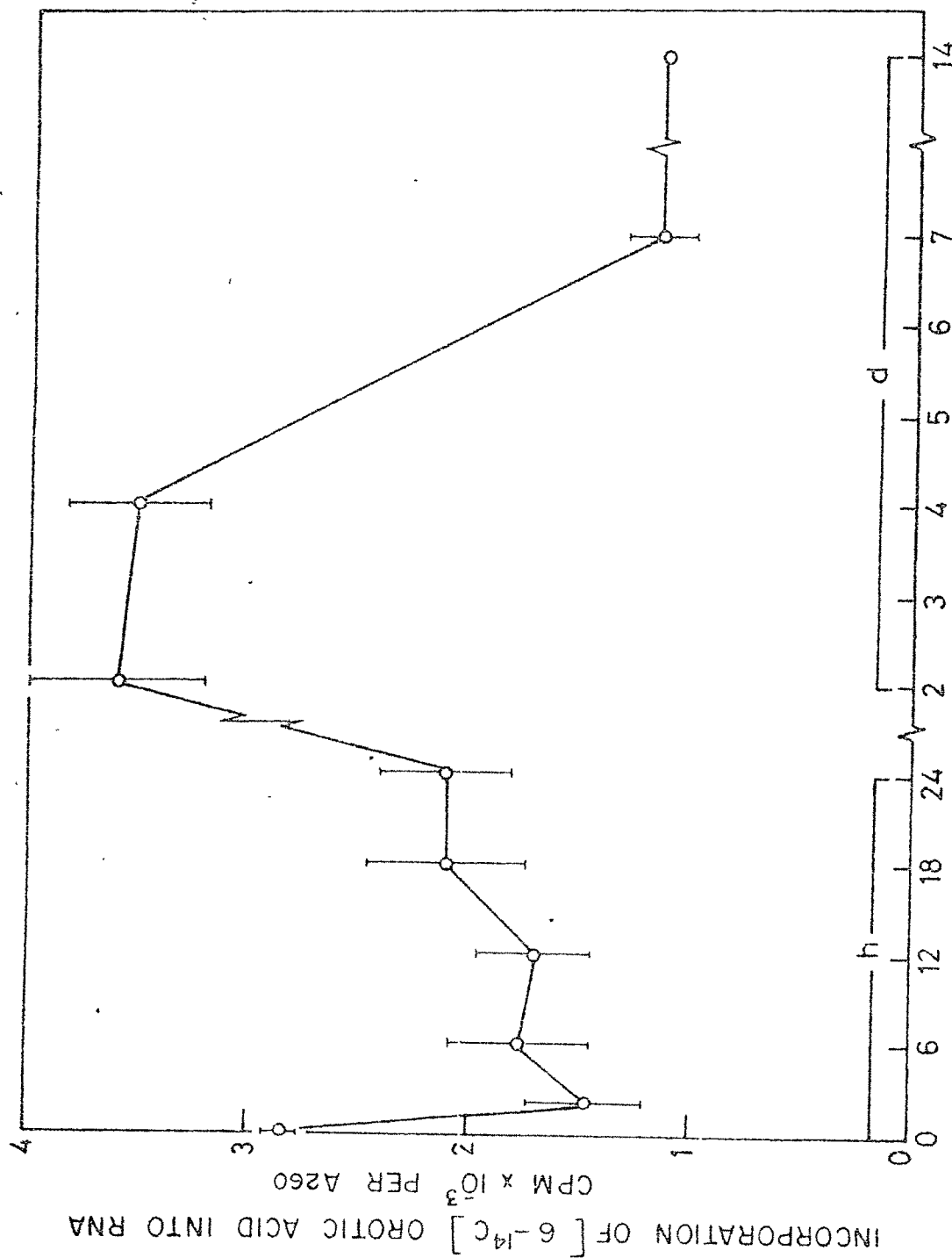
Some samples, viz. filter disks from all the transcription assays and aliquot of [^{14}C]RNA, were counted directly in cocktail (5 ml or 10 ml). Alkali digest of RNA, mixed with cocktail was stabilized overnight in the dark before counting. The sample values were corrected for quenching using external standard (18% quench correction was applied).

2.3. RESULTS

2.3.1. Incorporation of [6-¹⁴C]orotic acid into hepatic nuclear RNA in response to BP administration:

Gross transcription in liver measured as incorporation of [¹⁴C]orotic acid into nuclear RNA showed marked changes at various time points after i.p. administration of BP as shown in Fig. 2.1. In these studies comparison was made with untreated controls. Transcription was inhibited by nearly 50% of control at 2 h, which was gradually reversed to a stimulation (20% over control) at 2 days and continued at this level upto 4 days. Transcriptional activity in liver once again declined to 50% of control activity by 7 days and remained declined even at 14 day subsequent to BP-administration.

The solvent (DMSO) effect on inhibition observed at 2 h was ruled out by comparing data on [¹⁴C]orotic acid incorporation in RNA from BP-treated rats with those from DMSO-treated ones and untreated controls. Similarly, the possibility of isotopic contamination of radioactivity from non-RNA sources was eliminated by analyzing RNA directly as well as after alkali hydrolysis. All these results are presented in Table 2.1. It was observed that direct estimation of RNA as well as of alkali digest gave similar results indicating presence of [¹⁴C] from RNA alone. DMSO per se



TIME AFTER BENZO(a)PYRENE ADMINISTRATION

FIG. 2.1. Incorporation of (6-¹⁴C)orotic acid into hepatic nuclear RNA at various time after BP administration; Each time point represents mean value + S.E. obtained from six independent observations. Zero time group did not receive any injection other than (6-¹⁴C)orotic acid (2.5 μ Ci/100 g body weight).

TABLE 2.1

Comparison of in vivo incorporation of (6-¹⁴C)orotic acid in nuclear RNA 2 h post administration of BP or DMSO

Treatment	Incorporation (cpm mg ⁻¹ RNA) measured in	
	RNA (Spectral)	Alkali digest
Control	58,765 (100)	66,393 (100)
DMSO	54,694 (92.5)	61,422 (93.1)
BP	34,960 (58.3)	38,672 (59.5)

Each value is average of 3 experiments. Figures in parenthesis represent per cent of respective control value.

(¹⁴C)Orotic acid was administered at 0.017 µCi per g body weight. Nuclear RNA isolated from livers was analyzed either spectrophotometrically or by orcinol method after alkali hydrolysis. Radioactivity was measured either in the dissolved sample of RNA or in the alkali digest.

did bring about inhibition to the extent of 7% of control but BP effect was observed to be much more than that (41% inhibition).

2.3.2. Effect of BP on nuclear transcription in vitro
assayed under different ionic conditions:

Osmotonic nuclei from livers of animals administered BP or DMSO obtained at various time periods ranging from 2 h to 3 days were assayed for RNA polymerase activity in vitro under different ionic conditions as described in Methods (2.2.2.6.a.i). Results are shown in Fig. 2.2. The same results have also been expressed as % of DMSO-control and are shown in Fig. 2.3. At 2 h after administration of BP the expression of polymerase I did not show much difference over DMSO-control, while expression of polymerase II did show inhibition to the extent of 25% of DMSO-control. Both the enzyme activities were stimulated above control at 6 h post administration of BP (58% and 32% stimulation over respective DMSO-control for I and II). The two activities declined to near control values at 16 h and remained within 10% of control upto 3 days of BP-treatment. It is worthwhile to note that DMSO itself affects the transcription at all time points.

The incorporation assays were also carried out with limiting concentration of DNA (30 µg) as described in Methods (2.2.2.6.a.i). Results are shown in Table 2.2.

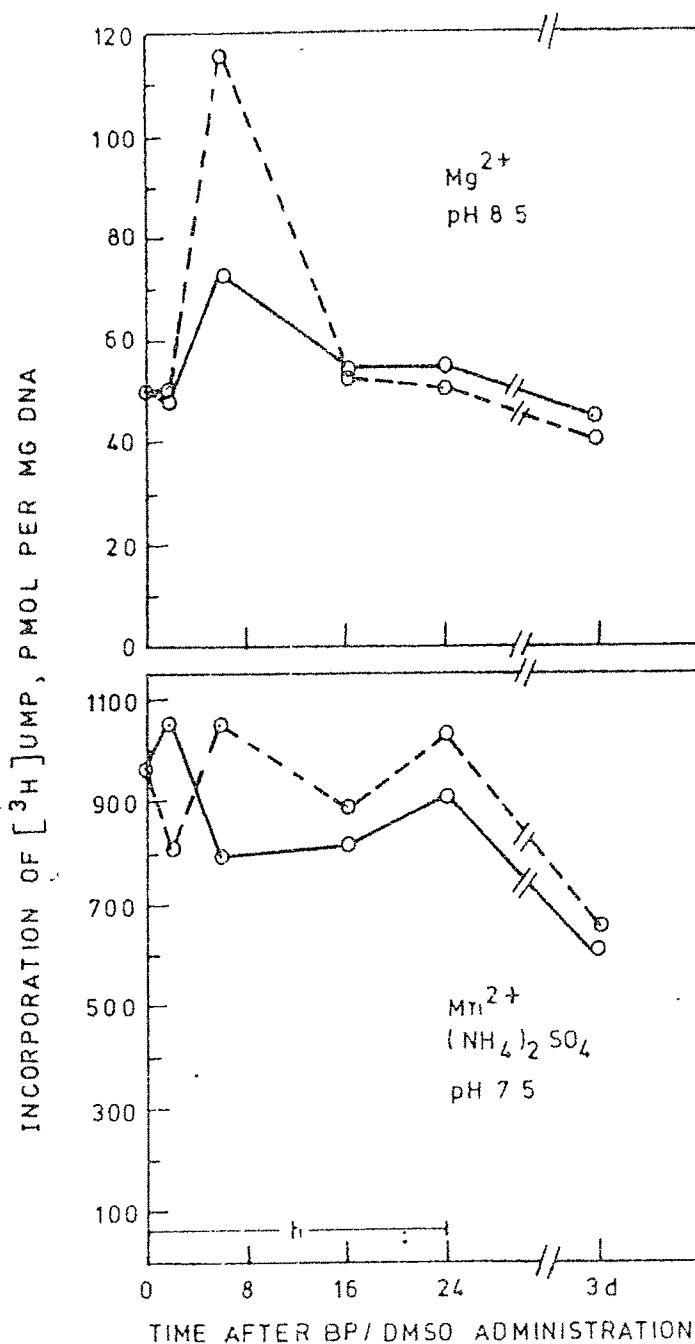


FIG. 2.2. Effect of BP on nuclear transcription under different ionic conditions:

Incorporation of $[5,6-^3H]UMP$ into TCA-insoluble fraction was measured in response to exposure to BP or DMSO for various time periods. For each assay, $\sim 80 \mu g$ DNA was used and incubation time was 5 min for Mg^{2+} -dependent activity and 45 min for $Mn^{2+} + (NH_4)_2SO_4$ -dependent activity. Each value is average of three experiments. Solid line (—) represents DMSO values and broken line (---) denotes BP values.

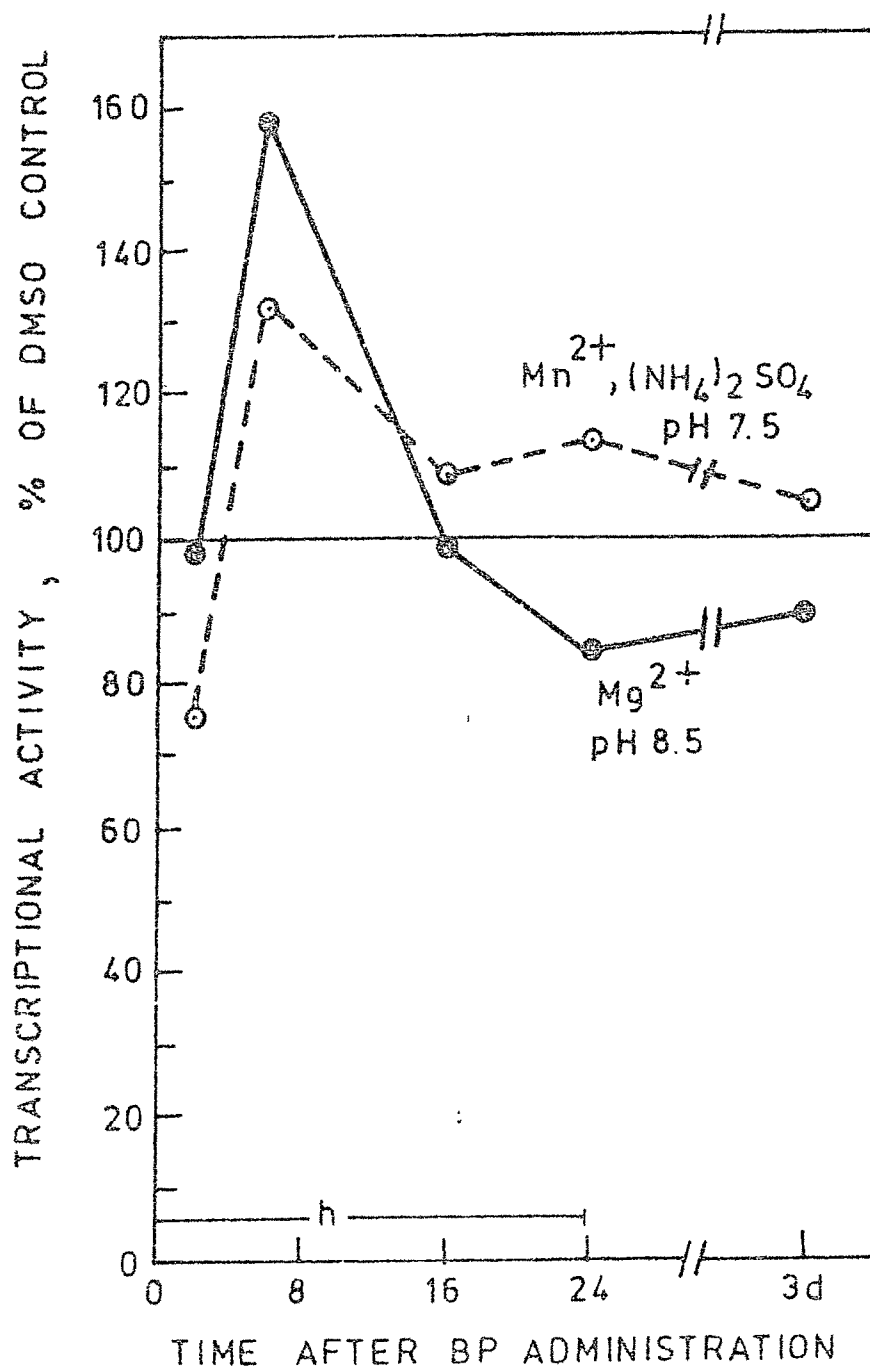


FIG. 2.3. Effect of BP on relative transcriptional activity of nuclei under different ionic conditions:

The values observed in Fig. 2.2 for BP-treatment are expressed as % of respective DMSO-control. Filled circles (●) represent Mg²⁺-dependent activity and open circles (○) represent Mn²⁺ + (NH₄)₂SO₄-dependent activity.

Expression of polymerase I (Mg^{2+} -dependent) activity did not show any difference over control, while polymerase II [Mn^{2+} + $(\text{NH}_4)_2\text{SO}_4$ -dependent] did show about 11% inhibition over DMSO-control. However, the expression of activity was different from earlier experiments (Fig. 2.2) due to use of lesser amount of DNA per assay. At 2 h, Mg^{2+} -dependent activity was $\sim 160 \text{ pmol mg}^{-1} \text{ DNA}$ at $30 \text{ }\mu\text{g}$ DNA per assay, while it was $\sim 50 \text{ pmol mg}^{-1} \text{ DNA}$ at $80 \text{ }\mu\text{g}$ DNA per assay. Since both assays for Mg^{2+} -dependent activity were carried out for the same time (5 min), it can be surmised that increasing amount of DNA from 30 to $80 \text{ }\mu\text{g}$ did not increase the incorporation of $[^3\text{H}]/\text{UMP}$ under given circumstances and hence ratio of $\text{pmol mg}^{-1} \text{ DNA}$ registered a decline to the extent of one-third by increase in DNA. For Mn^{2+} -dependent activity at 2 h, when DNA was decreased from $80 \text{ }\mu\text{g}$ to $30 \text{ }\mu\text{g}$ and assay was conducted for 5 min instead of 45 min, it was observed that incorporation of $[^3\text{H}]/\text{UMP}$ into TCA-insoluble fraction decreased from $\sim 1000 \text{ pmol mg}^{-1} \text{ DNA}$ (Fig. 2.2) to $\sim 650 \text{ pmol mg}^{-1} \text{ DNA}$ (Table 2.2). This may be explained by the fact that with higher amount of DNA and 45 min for the assay, the incorporation of $[^3\text{H}]/\text{UMP}$ could be better to the extent of 35%. However, it certainly did not register 2-3-fold increase in activity after increasing DNA from 30 to $80 \text{ }\mu\text{g}$ per assay, indicating inherent limitations of the assay system to support transcription. At the same time, while registering expression of one activity, the overlap by expression of other activity could

TABLE 2.2

Effect of BP on Mg^{2+} - and Mn^{2+} plus $(NH_4)_2SO_4$ -dependent RNA polymerase activities in vitro in liver nuclei

RNA polymerase dependent on	pmol (5,6- ³ H)UMP incorporated mg ⁻¹ DNA	
	DMSO	BP
Mg^{2+} , pH 8.5	161.6 (100)	163.1 (100.9)
Mn^{2+} + $(NH_4)_2SO_4$, pH 7.5	667.0 (100)	591.8 (88.7)

The purified nuclei from livers of BP or DMSO-treated (2 h) rats were assayed under different ionic conditions. Each value is average of 5 experiments. Figures in parenthesis denote per cent of DMSO-control value. For each assay ~ 30 µg DNA was used and both assays were carried out for 5 min.

not be ruled out and this might result in less clear expression of the true situation.

2.3.3. Effect of BP on total nuclear transcription in vitro and on the activities of RNA polymerase I, II and III:

Hypertonic nuclei from livers of animals administered BP or DMSO obtained at various periods of time ranging from 2 to 7 days were assayed for total and individual RNA polymerase activity using [α -³²P]UTP, as described in Methods (2.2.2.6.a.ii). Results as shown in Fig. 2.4 represent absolute activity of total as well as of three forms of the enzyme with respect to administration of BP and DMSO. Comparison of transcription in nuclei from BP-administered animals have also been made with respect to DMSO-control. These are depicted in Fig. 2.5. From these data, it becomes apparent that total activity as well as individual polymerase activities vary considerably with time of administration of either BP or DMSO. But when effect of BP was compared with that of DMSO (Fig. 2.5), the results show that at 2 h, total activity was inhibited by 15%, while polymerase I was stimulated by 5%. However, it was polymerases II and III which registered inhibition to the extent of 43% and 8% respectively. At 1 day after BP-treatment total activity still showed inhibition, although at a lower magnitude (10%), while the activities of polymerases I and II were stimulated to the extent of 4%

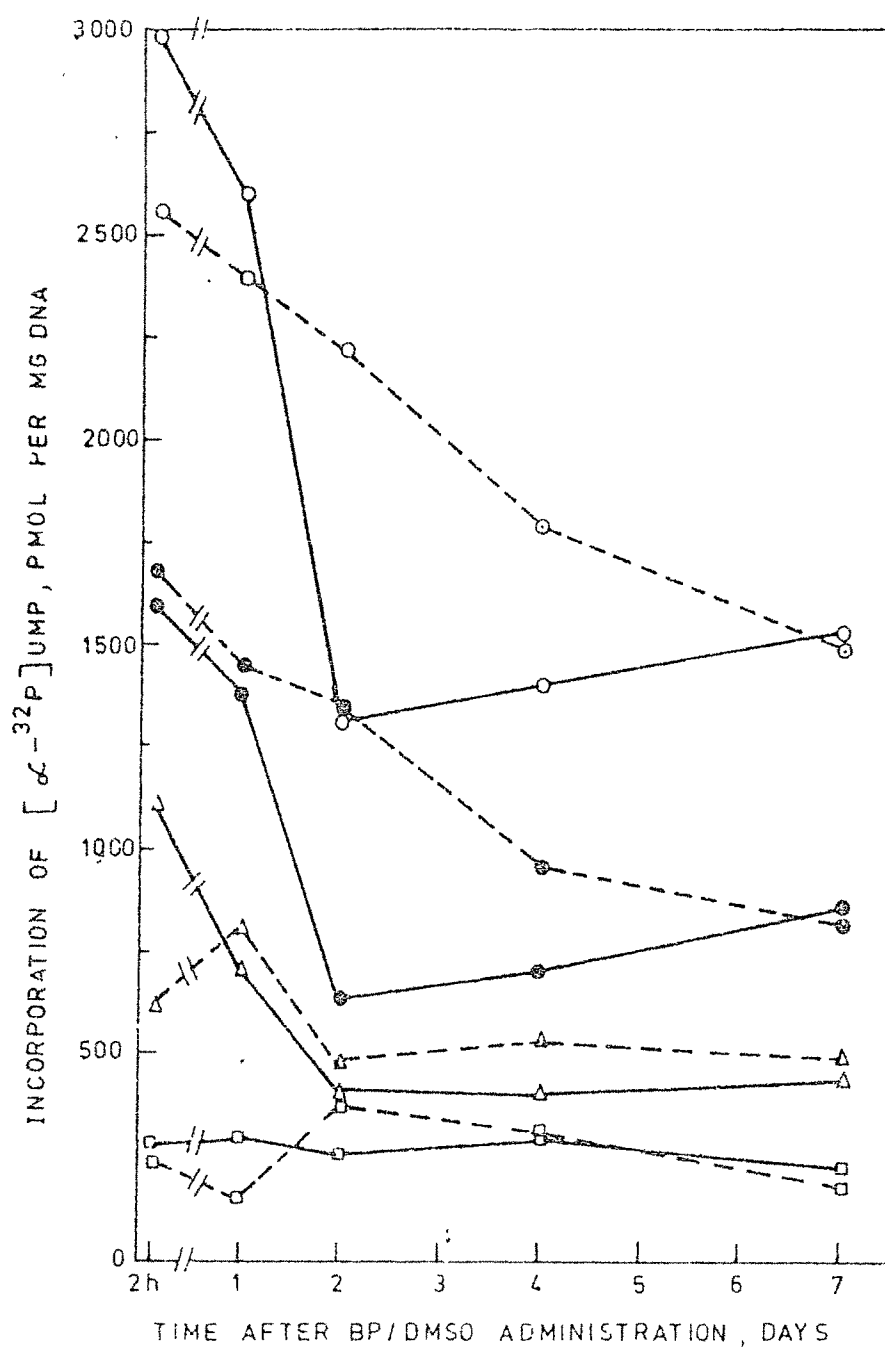


FIG. 2.4. Activity of different RNA polymerases in vitro in liver nuclei from rats administered BP or DMSO: Each result is average of two independent experiments. Results are expressed as pmol $[\alpha\text{-}^{32}\text{P}]\text{UMP}$ incorporated per mg DNA. Solid line (—) represents DMSO value while broken line (---) refers to BP values. Individual activities are referred to as follows. Open circles (o): total activity; filled circles (●): polymerase I; open triangles (△): polymerase II and open squares (□): polymerase III.

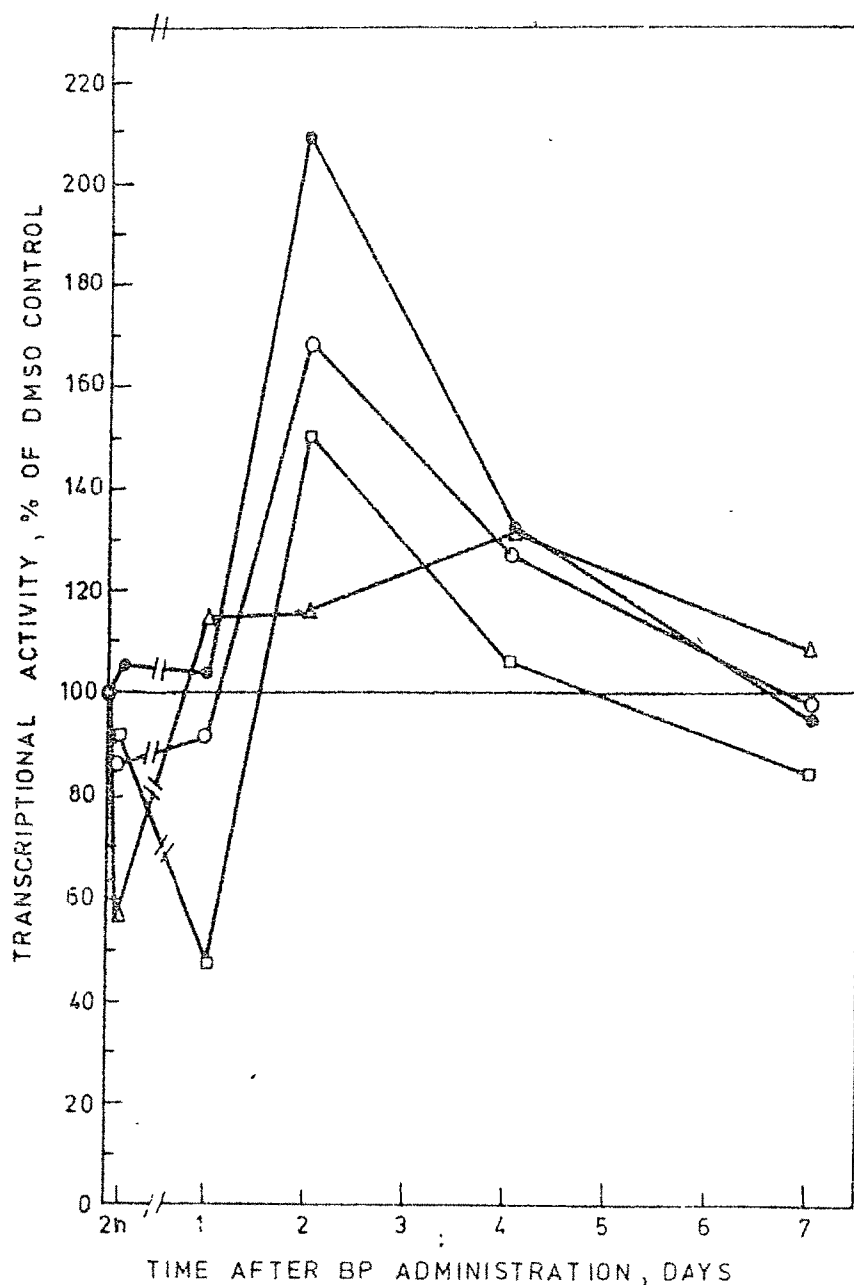


FIG. 2.5. Relative activity of different RNA polymerases in vitro in liver nuclei in response to BP administration: Each point is BP value represented as % of corresponding DMSO value observed in Fig. 2.4. Individual activities are referred to as follows. Open circles (o): total activity; filled circles (●): polymerase I; open triangles (△): polymerase II and open squares (□): polymerase III.

and 15% respectively. In contrast to all these, the activity of polymerase III showed ~52% inhibition. At 2 days, total activity was stimulated by 69%, which reflected in the rise in activities of all three polymerases. The rise was to the extent of 110%, 15% and 50% over the respective controls of the three forms. On 4th day, total activity scaled down but was still 28% above control. This was once again due to increased activities of the three enzymes above control to the extent of 33%, 32% and 5% respectively. On the 7th day, total activity returned almost to DMSO-value (only 3% inhibition) while the individual activity showed 5% and 15% inhibition for polymerases II and III respectively and 8% stimulation for polymerase I.

Since the above results were average of only two independent experiments and since 2 h-inhibition in polymerase II and III was obvious while polymerase I did not show any inhibition, it was decided to reconfirm the 2 h results by measuring incorporation of a different nucleotide. In these assays [γ - ^3H]/GTP was used instead of [α - ^{32}P]/UTP. Results obtained from six independent experiments are shown in Table 2.3. The incorporation of [^3H]/GMP into TCA-insoluble fraction appeared in the range of 900 pmol mg^{-1} DNA for total activity while comparable [^{32}P]/UMP incorporation was ~3000 pmol mg^{-1} DNA, indicating apparent changes in the levels of expression of transcription because of a change

in the radioactive nucleotide. This could perhaps be explained by base composition of RNA synthesized under given circumstances. However, relative results of incorporation in the two systems are comparable. It was observed from the results of [^3H]/GMP incorporation (Table 2.3) that the total enzyme activity was inhibited to the tune of 10% of DMSO-control. This was mainly due to inhibition in the activities of polymerases II and III to the extent of 37% and 25% respectively, while polymerase I activity showed marginal stimulation to the extent of 17%. Since, under the assay conditions employed polymerase I contributes 50% of total activity, the inhibition showed by other two enzymes could be masked due to stimulation of polymerase I resulting in only 10% inhibition of total activity.

2.3.4. Modulation of nucleolar transcription in vitro by BP:

Nucleoli isolated from liver nuclei of 2 h-BP or DMSO-treated animals were assayed for their ability to incorporate [^3H]/GMP into TCA-insoluble fraction. Results are expressed in Table 2.4. It was observed that total nucleolar activity was inhibited by 18%. However, since nucleoli are rich in polymerase I activity, the enzyme activity when monitored in presence of 0.01 mM α -amanitin, was inhibited by $\sim 10\%$ of DMSO-control.

TABLE 2.3

Activity of different RNA polymerases assayed in vitro in liver nuclei from rats 2 h post administration of BP

RNA polymerases	pmol (8- ³ H)GMP incorporated mg ⁻¹ DNA	
	DMSO	BP
Total activity	795.4 ± 43.9 (100)	715.4 ± 38.9 (90.0)
Polymerase I	352.3 ± 27.4 (100)	412.3 ± 32.6 (116.8)
Polymerase II	304.6 ± 50.8 (100)	192.3 ± 29.3 (63.4)
Polymerase III	147.7 ± 10.0 (100)	115.5 ± 33.7 (75.5)

Each value represents mean ± S.E. from 6 independent experiments. Figures in parenthesis represent per cent of DMSO-control value. Assays were carried out with purified nuclei in presence of 0, 0.01 µM and 0.01 mM α-amanitin. Total activity is that which is obtained in the absence of α-amanitin. Polymerase I represents activity resistant to 0.01 mM α-amanitin. Polymerase II and III represent activities which are sensitive to 0.01 µM and 0.01 mM α-amanitin respectively.

TABLE 2.4

Effect of BP (2 h) on nucleolar transcription in vitro

Nucleolar activity	pmol (8- ³ H)GMP incorporated mg ⁻¹ DNA	
	DMSO	BP
Total activity	2330.0 (100)	1909.9 (82.0)
+ 0.01 mM α -amanitin	2322.0 (100)	2085.7 (89.8)

Each result is average of three independent experiments. Figures in parenthesis represent per cent of DMSO-control values. Nucleolar transcription was measured in absence and presence of 0.01 mM α -amanitin to represent total and polymerase I activity respectively.

2.3.5. Effect of BP on free and engaged RNA polymerase fractions of nuclei:

Free and engaged RNA polymerase fractions of intact nuclei were prepared from 2 h-BP or DMSO-treated animals as described in Methods (2.2.2.3). Free enzyme fraction, supposedly constituting free form of RNA polymerases, was assayed in presence of calf thymus DNA as described in Methods (2.2.2.6.d). It was observed that free enzyme from BP-nuclei was inhibited to the extent of 8.5% as compared to the same fraction from DMSO-nuclei, when the activity was expressed on per mg protein basis (Table 2.5). Engaged enzyme fraction of nuclei, consisting of endogenous template as well as enzyme, was assayed as described in Methods (2.2.2.6.c), but expressed as incorporation per mg DNA. On this basis, BP-derived engaged enzyme fraction expressed about 18% less than DMSO-derived fraction (Table 2.5). Thus, both free as well as engaged enzyme fractions of nuclei show 8% and 18% inhibition respectively due to BP-treatment. When compared on the basis of weight of liver, free enzyme showed BP-induced increase in activity (30%), while engaged enzyme was inhibited to the extent of 30% of DMSO-control.

2.3.6. Effect of BP on partially purified nuclear RNA polymerases I, II and III:

(a) Total nuclear enzymes:

Fraction IV containing the different forms of RNA

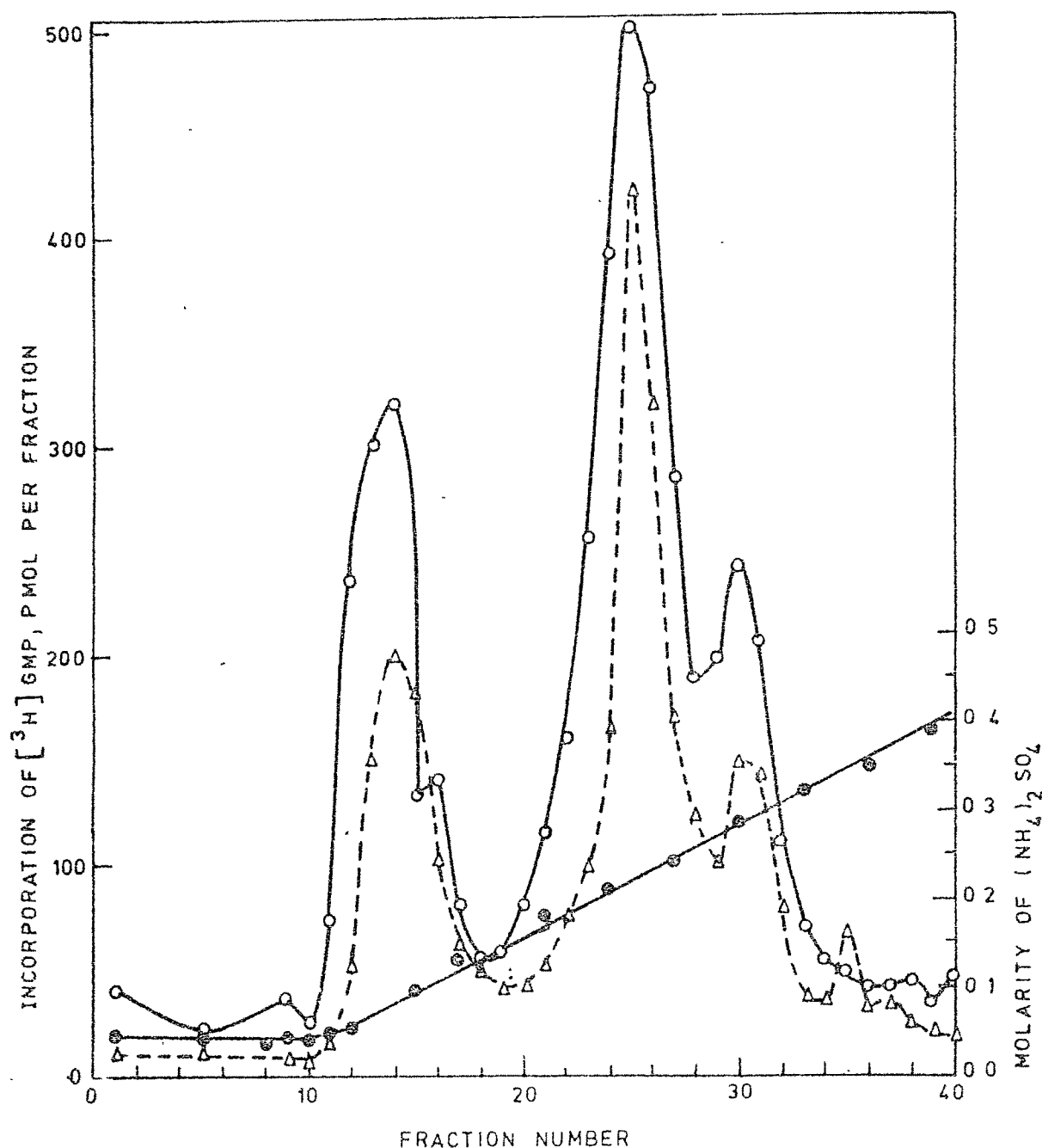


FIG. 2.6. DEAE-Sephadex A-25 column chromatography of total nuclear RNA polymerase activities:

Total nuclear RNA polymerase enzymes solubilized from 13.6 g liver (~ 13 mg protein) from DMSO (2 h) or BP (2 h) treated rats were resolved by chromatography on DEAE-Sephadex A-25 column (0.9 cm x 21 cm) with 50 ml gradient of $(\text{NH}_4)_2\text{SO}_4$ in TGMEM buffer. Each fraction was assayed for transcriptive ability using poly[d(I-C)] as described (2.2.2.8). Solid line (—) joining open circles (o) represents DMSO values, while broken line (---) joining open triangles (Δ) represents BP values. Filled circles (\bullet) represent molarity of $(\text{NH}_4)_2\text{SO}_4$.

TABLE 2.6

Effect of BP (2 h) on total nuclear RNA polymerases I, II and III

Polymerase	Parameter	DMSO		BP	
I	mg protein	1.530 (100)		1.529 (99.9)	
	pmol ^3H GMP	1285	(100)	841	(65.4)
	pmol mg^{-1} prot.	839.6	(100)	549.9	(65.5)
II	mg protein	0.525 (100)		0.472 (90.0)	
	pmol ^3H GMP	2069	(100)	1366	(66.0)
	pmol mg^{-1} prot.	3867.1	(100)	2891.6	(74.8)
III	mg protein	0.132 (100)		0.105 (79.6)	
	pmol ^3H GMP	644.7	(100)	413.8	(64.2)
	pmol mg^{-1} prot.	4884.0	(100)	3940.4	(80.7)

Different forms of total nuclear RNA polymerase from 13.6 g liver (~13 mg protein) were separated on DEAE-Sephadex A-25 as shown in Fig. 2.6. The peak fractions were pooled together and designated as polymerase I, II and III respectively. Figures in parenthesis represent per cent of respective DMSO-value.

extent of 35%. Since protein content of the peaks were same, the specific activity showed 35% inhibition. Pooled peak II from BP-preparation was 10% less in protein content as compared to DMSO, and the enzyme activity was 34% less. Specific activity of BP-enzyme was, therefore, inhibited by 25% with respect to DMSO-enzyme. Pooled peak III had 20% less protein content and 35% less enzyme activity in BP-preparation than in DMSO-preparation. Therefore specific activity of this fraction was inhibited by 20% due to BP-treatment. Thus, all the three forms of RNA polymerases from BP-preparation showed reduced enzymatic activity when compared to DMSO-control.

(b) Free enzymes:

The fraction IV enzyme from free fraction of 10.9 g liver from each of DMSO or BP-treated animals was chromatographed on DEAE-Sephadex A-25 column. Out of 2 mg protein loaded on the column only 41 µg appeared in the peak fractions for each of BP or DMSO-preparations. As revealed in Table 2.7 polymerase I obtained from BP-treated preparation showed 15% inhibition in enzyme activity while protein content was only 7% less with respect to DMSO-control. Hence, specific activity was inhibited by about 10% due to BP. The activity of polymerase II from BP-treated preparation was drastically reduced (42%) and since protein content of the peak was actually 35% above DMSO preparation, the resultant specific activity of BP-derived polymerase II was 57% less as compared to

TABLE 2.7.

Effect of BP (2 h) on free RNA polymerases I, II and III

Polymerase	Parameter	DMSO	BP
I	µg protein	30.86 (100)	28.75 (93.2)
	pmol $\text{[}^3\text{H]GMP}$	28.64 (100)	24.16 (84.3)
	pmol mg^{-1} prot.	928.2 (100)	840.0 (90.5)
II	µg protein	7.07 (100)	9.59 (135.6)
	pmol $\text{[}^3\text{H]GMP}$	38.01 (100)	22.24 (58.0)
	pmol mg^{-1} prot.	5422.5 (100)	2319.6 (42.8)
III	µg protein	3.86 (100)	2.85 (73.9)
	pmol $\text{[}^3\text{H]GMP}$	88.04 (100)	81.85 (93.0)
	pmol mg^{-1} prot.	22807.4 (100)	28693.9 (125.8)

Different forms of RNA polymerase from free enzyme fraction of nuclei from 10.9 g liver (~ 2 mg protein) of BP or DMSO-treated animals were separated by chromatography on DEAE-Sephadex A-25 column as described in Fig. 2.6. After the enzyme assay of each fraction, the peak fractions were pooled together and analyzed for protein content. Figures in parenthesis represent per cent of respective DMSO-value.

DMSO-control. For polymerase III, enzyme activity was inhibited in BP-preparation to the extent of only 7% but since protein content was reduced by 26%, the net specific activity was in fact increased by 20% above DMSO-control. Thus, among the free forms of enzymes, it was polymerase II which was maximally inhibited, followed by polymerase I and III.

(c) Engaged enzymes:

Different forms of RNA polymerases from engaged fraction of 11.8 g liver from each of DMSO- and BP-treated animals were separated on DEAE-Sephadex A-25 column. Out of ~8.8 mg of fraction IV protein from DMSO-preparation loaded on the column, only about 1 mg was recovered in peak fractions. Fraction IV from BP-preparation carrying 12.2 mg protein also yielded about 1 mg protein in peak fractions, indicating a higher level of non-polymerase protein content in engaged preparation from BP-treated animals. As shown in Table 2.8, the enzyme activity of polymerase I from BP-preparation was 14% above control level, while the protein content was nearly same as control. The net result was an increase of 14% in specific activity of BP-treated enzyme. Engaged polymerase II from BP-treated preparation was enzymatically 56% more active while only 7% less in protein content, yielding 68% better specific activity with respect to engaged polymerase II from DMSO-controls. For engaged polymerase III both enzyme

TABLE 2.8

Effect of BP (2 h) on engaged RNA polymerases I,II and III

Polymerase	Parameter	DMSO	BP
I	µg protein	599.2 (100)	597.6 (99.7)
	pmol (³ H)GMP	282.3 (100)	320.4 (113.5)
	pmol mg ⁻¹ prot.	471.1 (100)	536.1 (113.8)
II	µg protein	311.8 (100)	290.4 (93.1)
	pmol (³ H)GMP	435.4 (100)	680.7 (156.4)
	pmol mg ⁻¹ prot.	1396.3 (100)	2344.1 (167.9)
III	µg protein	91.3 (100)	89.04 (97.5)
	pmol (³ H)GMP	560.7 (100)	551.6 (98.4)
	pmol mg ⁻¹ prot.	6140.8 (100)	6194.7 (100.9)

Different forms of RNA polymerases from engaged enzyme fraction of nuclei from 11.8 g liver (~8.8 mg protein) of BP or DMSO-treated animals were separated by chromatography on DEAE-Sephadex A-25 column as described for total nuclear enzymes (Fig. 2.6). After the enzyme assay of each fraction, the peak fractions were pooled together and analyzed for protein content. Figures in parenthesis represent per cent of respective DMSO-values.

activity and protein content showed decrease by about 2%, as compared to control. Therefore, net specific activity was same as DMSO-control. Thus, among the engaged polymerases, polymerase II was markedly stimulated by BP, while polymerase I showed slight increase in activity, and polymerase III did not show any change with respect to DMSO-control.

2.3.7. Direct inhibitory effects of BPDE I on nuclear transcription in vitro:

Nuclei from livers of untreated control animals prepared by hypertonic sucrose method and suspended in 0.32 M sucrose-1 mM MgCl_2 were used for in vitro transcription as described in Methods (2.2.2.6.a). In order to check integrity of nuclei, response of the assay system to increasing amounts of nuclear suspension ($14.22 \text{ mg DNA ml}^{-1}$) was examined as shown in Fig. 2.7. There was a linear increase in the incorporation of $[^3\text{H}]\text{GMP}$ into TCA-insoluble fraction upto the concentration of $\sim 200 \text{ }\mu\text{g}$ nuclear DNA. From 200 to $430 \text{ }\mu\text{g}$, the rate of increase was low and from 430 to $600 \text{ }\mu\text{g}$ nuclear DNA, there was once again better response and linearity of transcription with increasing DNA content. Thus, the entire dose-range in which all subsequent experiments had been performed, showed reasonable transcriptional sensitivity to the amount of nuclear DNA in the assay.

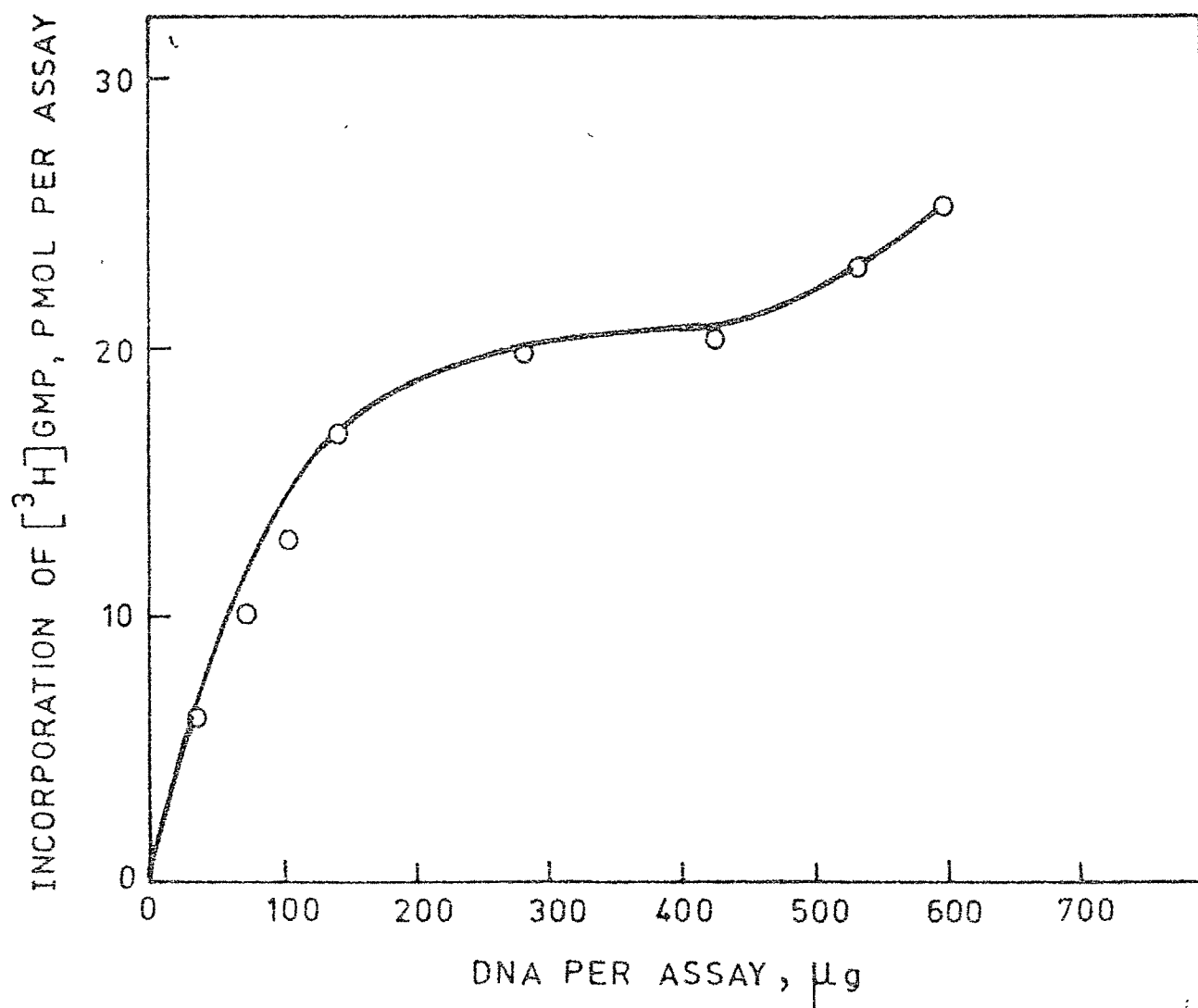


FIG. 2.7. Response of nuclear transcription in vitro to amount of nuclear DNA:

Control nuclei were assayed for transcription by method of Yu (1975) using varying concentrations of nuclear DNA. Incorporation of [³H]GMP into TCA-insoluble fractions was monitored as described (2.2.2.9). Results expressed here are representative set from a series of four such experiments.

(a) Effect of preincubation of nuclei with BPDE I on transcriptional activity:

Preincubation of nuclear suspension with BPDE I or DMSO was carried out at room temperature for varying time periods. It was observed that preincubation with DMSO (5% v/v) showed only 5% inhibition of transcription at 5 min which remained at the same level for upto 30 min (data not shown). On the other hand, preincubation with BPDE I (dissolved in DMSO) showed considerable inhibitory response at 5 min and it remained at the same level upto 15 min (data not shown). Hence, for all subsequent preincubations of nuclei with BPDE I or DMSO, room temperature for 10 min was chosen.

The preincubation of nuclear preparation was carried out in 50 μ L volume with 142.2 μ g nuclear DNA (10 μ L suspension) and varying concentrations of BPDE I from 0.05 - 75 μ M, keeping volume of DMSO at 5%. After preincubation at room temperature for 10 min, this was assayed for transcription as described in Methods (2.2.2.9). Results as shown in Fig. 2.8, showed a clear case of concentration-dependent inhibition by BPDE I in the transcriptive ability of nuclei. At 0.5 μ M, inhibition was just 1.5% with respect to DMSO-control, while at 5, 25, 50 and 75 μ M concentrations, BPDE I inhibited the transcription to the extent of nearly 25, 48, 55 and 63% with respect to DMSO-control. The control assay involved preincubation with 2.5 μ L DMSO (5% v/v) and was observed to

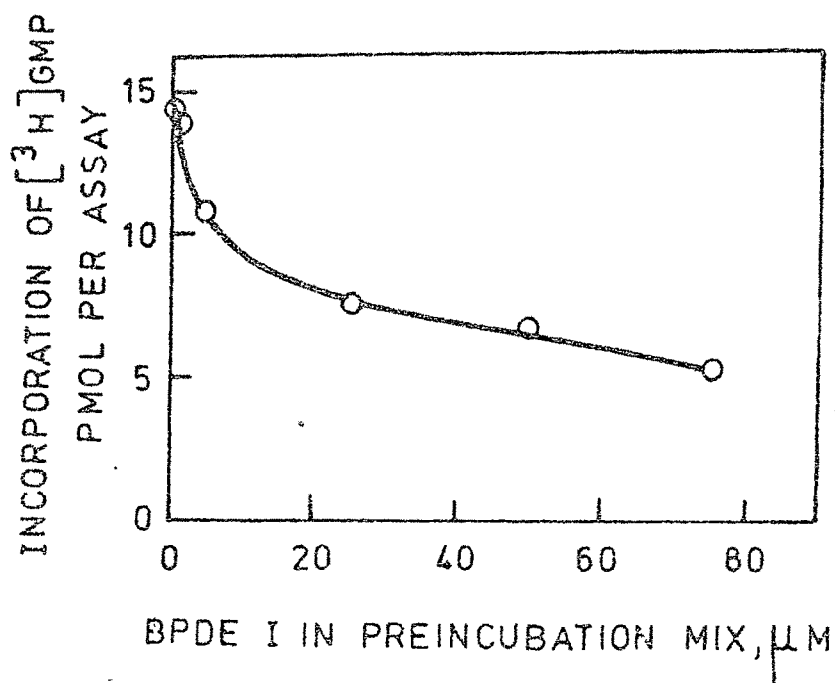


FIG. 2.8. Inhibitory effect of BPDE I on nuclear transcription in vitro:

Nuclei containing 142.2 μg DNA were preincubated at room temperature for 10 min with varying concentrations of BPDE I. The transcriptive ability of such nuclei was monitored as described earlier (2.2.2.9). Results represented here are a set from series of four such experiments.

give 14.44 pmol [^3H]/GMP incorporation into TCA-insoluble fraction per assay.

(b) Reversal of BPDE I effect with increasing concentrations of nuclei:

In order to rule out any effect contributed by DMSO and also to see if increasing nuclear concentration could reverse the inhibition induced by BPDE I, the preincubation of different amounts of nuclear suspension ($\sim 142.2 - 530 \mu\text{g}$ nuclear DNA) was carried out with DMSO or BPDE I ($75 \mu\text{M}$). DMSO concentration in each case was 5% v/v. Transcription assay was performed as usual after preincubation. It was observed as shown in Fig. 2.9, that incorporation observed in presence of DMSO remained near control value (in absence of DMSO) at all concentrations of nuclear DNA tested. On the other hand, inhibition observed due to $75 \mu\text{M}$ BPDE I at $142.2 \mu\text{g}$ DNA (66% of DMSO-control) was decreased gradually with increase in nuclear DNA concentration so that at $530 \mu\text{g}$ DNA, the inhibition was only 18%. This is more evident from the data represented in Fig. 2.10. By increasing the amount of nuclear DNA per assay from $142.2 \mu\text{g}$ to 280, 425 and $530 \mu\text{g}$, the inhibition due to BPDE I was decreased from 66% to 40, 38 and 18% of DMSO-control. In other words, in the presence of nearly 4-fold excess DNA during treatment with $75 \mu\text{M}$ BPDE I almost 80% of the transcriptional activity of nuclei was retained.

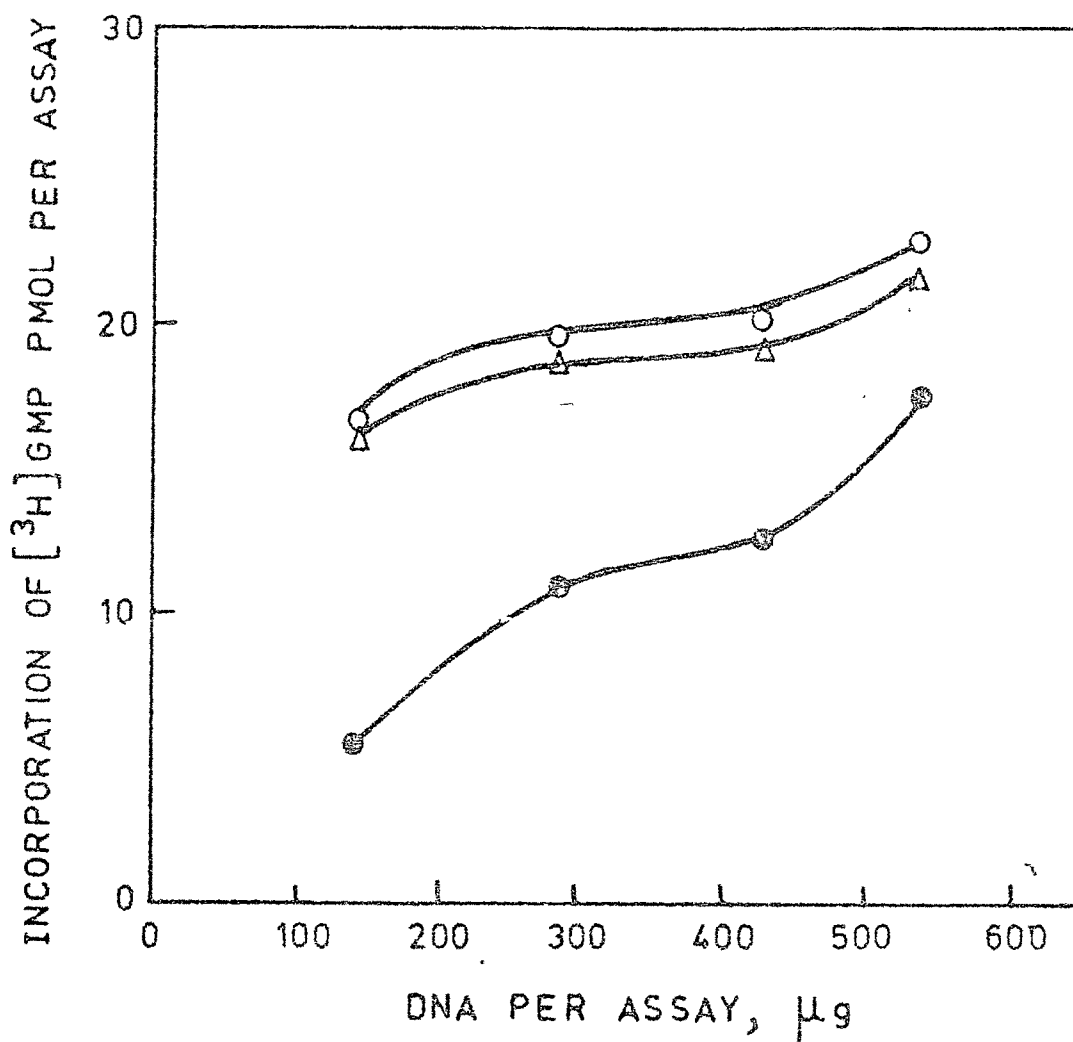


FIG. 2.9. Effect of preincubation with DMSO or BPDE I on nuclear transcription in response to amount of nuclear DNA: Varying amount of nuclei were preincubated with either DMSO (5% v/v) or BPDE I (75 μ M) and their subsequent transcriptive ability was monitored as usual. Open circles (o) represent values without preincubation, open triangles (Δ) represent DMSO treated values and filled circles (\bullet) represent BPDE I preincubated values.

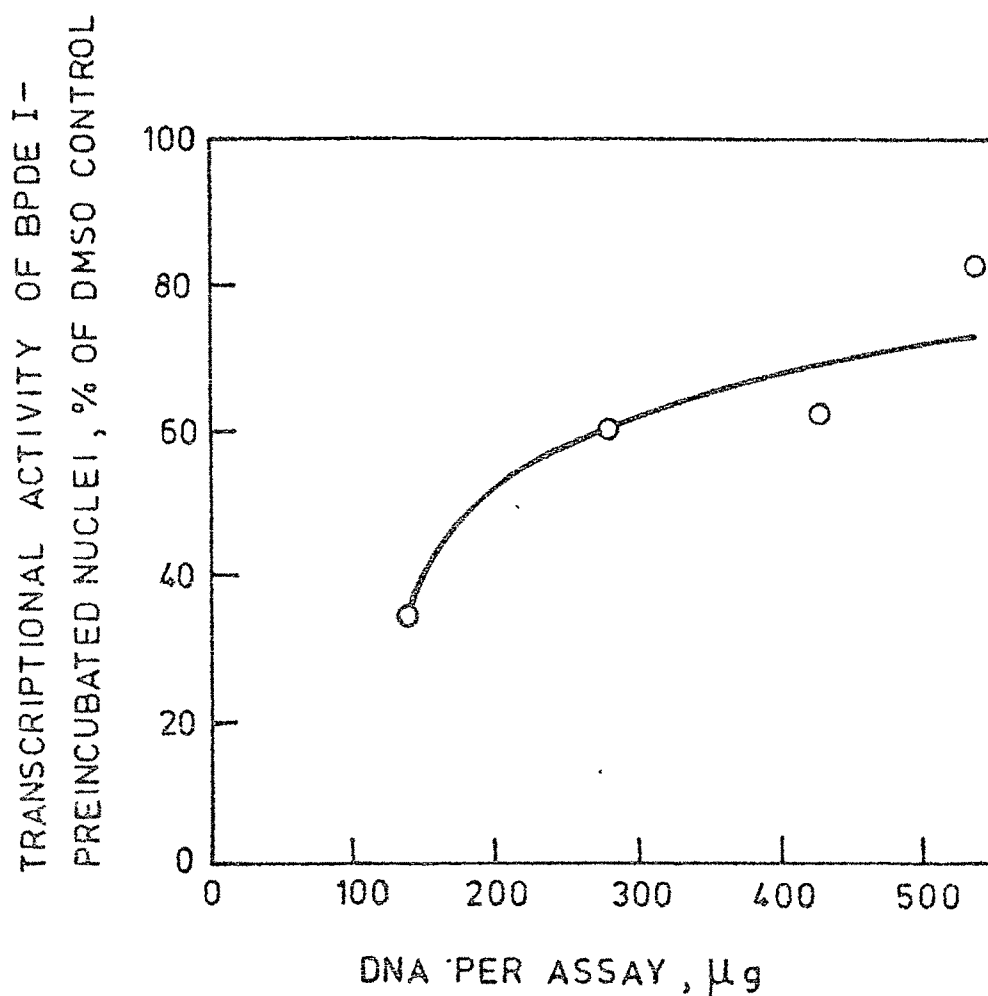


FIG. 2.10. Reversal of BPDE I-induced inhibition of nuclear transcription with increasing concentrations of nuclear DNA: The values of BPDE I preincubated nuclei is represented as % of DMSO values from Fig. 2.9.

(c)Distinction between chromatin template and RNA polymerase activities of nuclei during treatment with BPDE I:

In order to differentiate the effects of BPDE I on the endogenous enzymes from its effects on total transcription, the endogenous template was made inactive by addition of actinomycin-D during the transcription assay. In order to select a desired concentration of actinomycin-D, some preliminary experiments were conducted. A concentration-dependent inhibition of transcription was evident by addition of actinomycin D from 10 ng to 1250 ng per assay (Fig. 2.11). The extent of inhibition ranged from 27% at 10 ng to 48, 66, 78 and 82.5% at 100, 250, 625 and 1250 ng actinomycin-D per assay respectively.

Actinomycin-D-inhibited nuclei containing inactive endogenous template but active endogenous enzymes were then assayed for transcription activity of these enzymes in presence of externally added synthetic DNA template poly[d(I-C)], since the latter is insensitive to actinomycin-D effects. This system was standardized by measuring response of endogenous enzymes to various concentrations of exogenous template. The inhibition (66%) of transcription activity of nuclei (~142 µg DNA) in the presence of 250 ng actinomycin-D was found to be increasingly revived with increasing concentrations of poly[d(I-C)]. The concentration-dependent revival by this exogenous template of actinomycin-D-induced inhibition

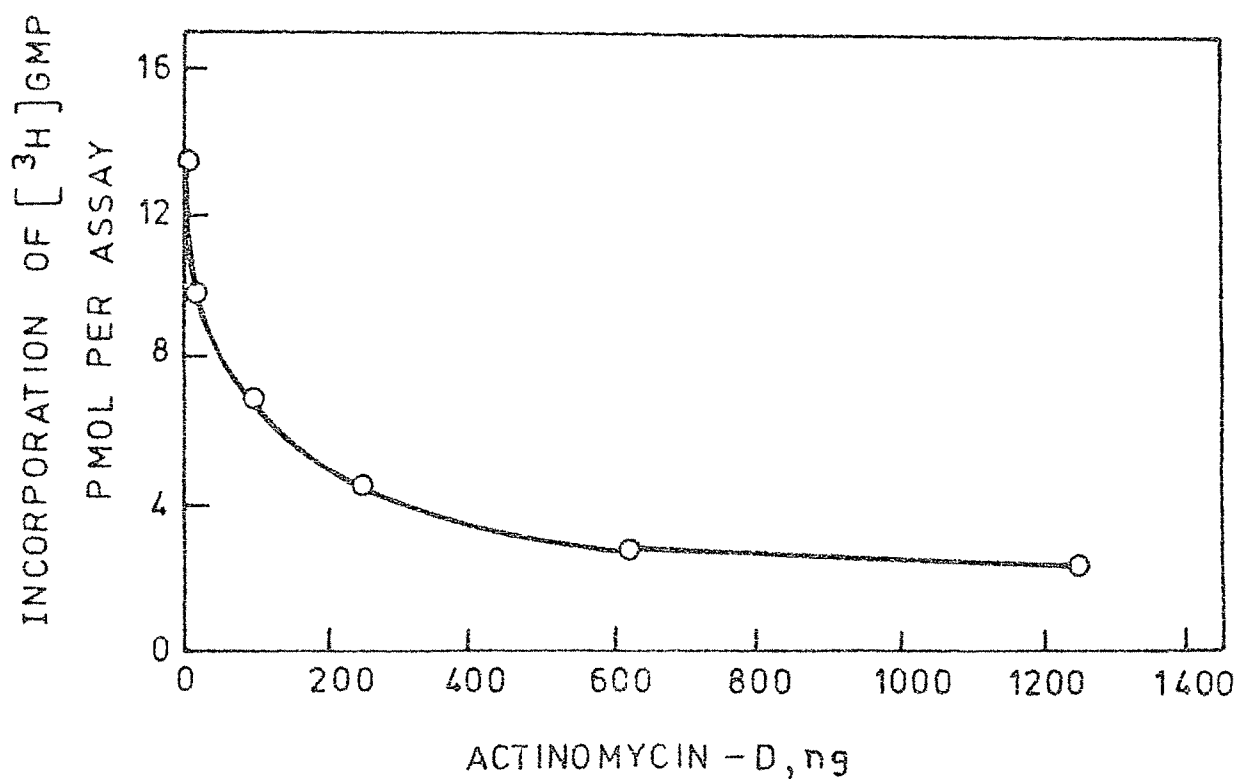


FIG. 2.11. Inhibitory response of nuclear transcription in vitro to actinomycin-D:

Nuclei containing 142.2 μ g DNA were assayed for transcription in presence of varying amounts of actinomycin-D.

of activity is shown in Fig. 2.12. Thus, when assayed in presence of 0.05, 0.1, 0.15 and 0.2 units of poly[d(I-C)], the inhibition was reduced to 61, 49, 26 and 16% respectively, showing 84% revival of transcription activity at the highest concentration tested. Characterization of this model system was necessary in order to fully understand the effect of BPDE I on nuclear transcription.

Nuclear preparation (~ 142 μ g DNA) was preincubated with BPDE I (75 μ M) as usual and in subsequent incubations, incorporation of [3 H]GMP into TCA-insoluble fraction was monitored by filter-disk method as described earlier. The three polymerases were differentiated by addition of 0.01 μ M and 0.01 mM concentrations of α -amanitin during the assay. Results are tabulated in Table 2.9. Total polymerase activity was inhibited by BPDE I-treatment to the tune of 71% of DMSO-control. When differentiated by α -amanitin, it was seen (2 v/s 1) that polymerase I and II bore the brunt of the BPDE I effect by showing inhibition to the extent of 76 and 69% of control respectively. Whereas, polymerase III was inhibited by the same treatment to the extent of 28% of DMSO-control. Addition of 650 ng actinomycin-D during the assay showed 83% inhibition for DMSO-preparation (2.84 pmol v/s 16.54 pmol, 3 v/s 1) and 39% for BPDE I-treated nuclear preparation (2.95 pmol v/s 4.84 pmol, 4 v/s 2). This latter effect was a further inhibition in a preparation already inhibited by

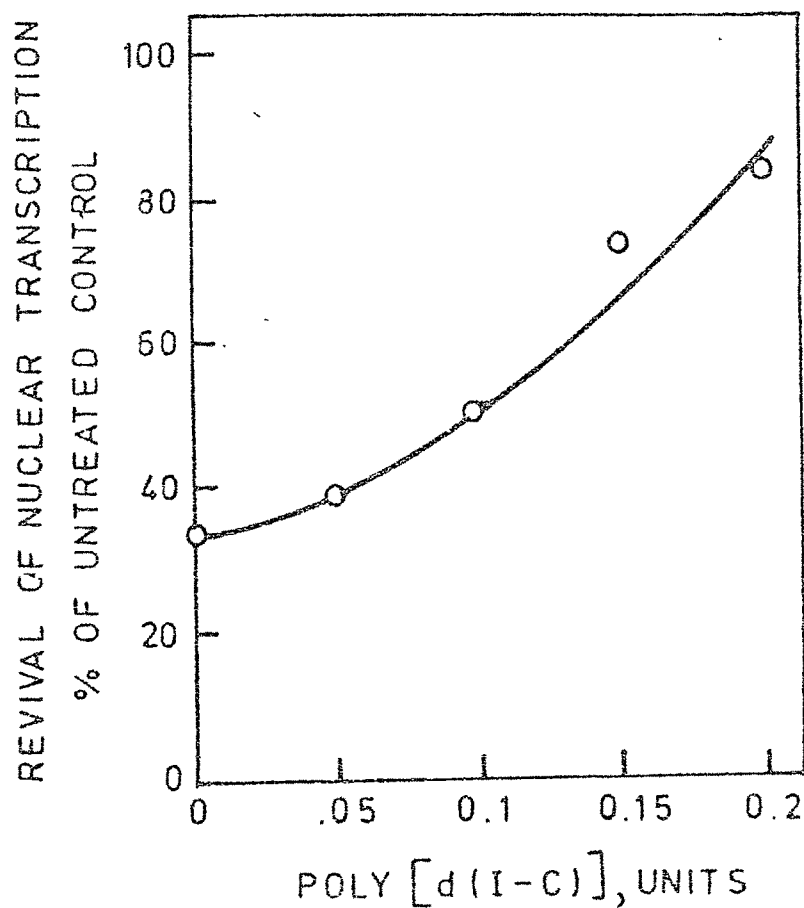


FIG. 2.12. Revival of actinomycin-D induced inhibition of nuclear transcription in vitro by poly[d(I-C)]:

Nuclei containing 142.2 μg DNA were assayed in presence of 250 ng actinomycin-D and varying amounts of poly[d(I-C)] to determine revival of transcriptive activity.

TABLE 2.9

Effect of BPDE I on nuclear transcription in vitro

Expt.	Treatment		pmol [3 H]GMP incorporated per assay			
	Preincubation	Assay	Total polymerases	Polymerase I	Polymerase II	Polymerase III
1	DMSO	-	16.54 (100)	12.65 (100)	2.20 (100)	1.69 (100)
2	BPDE I	-	4.84 (29.3)	2.95 (23.3)	0.68 (30.9)	1.21 (71.6)
3	DMSO	Actinomycin-D	2.84 (100)			
4	BPDE I	Actinomycin-D	2.95 (103.9)			
5	DMSO	Actinomycin-D Poly[G(I-C)]	13.28 (100)	8.45 (100)	0.91 (100)	3.93 (100)
6	BPDE I	Actinomycin-D poly [d(I-C)]	10.01(75.3)	6.60 (78.2)	0.93 (102.7)	2.48 (63.01)
7	5 minus 3		10.45 (100)			
8	6 minus 4		7.06 (67.6)			

Nuclei, containing 142.2 μ g DNA preincubated at room temperature for 10 min with either DMSO (5% v/v) or BPDE I (75 μ M) were assayed for transcription. During the assay (final vol 100 μ L), following components were added (when required): α -amanitin: 0.01 μ M and 0.01 mM; actinomycin-D: 650 ng per assay and poly[G(I-C)]: 0.25 units per assay. Results are expressed as pmol [3 H]GMP incorporated into TCA-insoluble fraction per assay. Figures in parenthesis represent per cent of respective control values.

BPDE I-treatment (4 v/s 1). For both DMSO-and BPDE I-preparations actinomycin-D-inhibited nuclei exhibited same level of final transcription viz. ~ 2.9 pmol per assay (3 and 4).

The expression of nuclear enzymes in actinomycin-D-inhibited nuclei was observed by addition of 0.25 units of synthetic template poly[d(I-C)] during the assay. Addition of this actinomycin-D-resistant template evoked transcriptive response in both DMSO-or BPDE I-pretreated preparations. However, the DMSO-preparation was revived from 17% of control activity (83% inhibition due to actinomycin-D, 3 v/s 1) to 80% of control (13.28 pmol v/s 16.54 pmol, 5 v/s 1). The revival in BPDE I-inhibited nuclei, however, was not to the same extent as for DMSO-control. It was observed to be 75% of control (10.01 pmol v/s 13.28 pmol, 6 v/s 5). When poly[d(I-C)]-revived activity was differentiated by addition of α -amanitin, it was observed that polymerase I and III showed inhibition (due to BPDE I) to the extent of 22 and 37% of DMSO-control, while polymerase II was nearly same as DMSO-control (6 v/s 5). When activity exclusively in response to added poly[d(I-C)] (7 and 8) was deduced for both DMSO-preparation (5 - 3) and BPDE I-preparation (6 - 4), it was observed that BPDE I-treated preparation was inhibited by 32% as compared to DMSO-control (8 v/s 7).

2.4. DISCUSSION

The objective of this work was to probe at various levels of organization, the effect of BP on transcription machinery in rat liver. Single intraperitoneal administration of BP at 20 mg kg⁻¹ dose level, brought about modulation in gross transcription over a period of 14 days after administration of the carcinogen. Incorporation of radioactive precursor [⁶⁻¹⁴C]orotic acid into nuclear RNA of liver - the work pioneered by Hurlbert and Potter (1954) - was monitored to obtain an idea about the status of nascent RNA synthesis at a given time of BP treatment. BP caused drastic (50%) inhibition in RNA synthesis at 2 h (Fig. 2.1) which was then gradually reversed to a stimulation (20%) between 2-3 days. Such early reversible inhibition of transcription in mouse skin in response to topical application of BP was observed by Alexandrov et al. (1970). Similar early reversible inhibition of transcription was observed with other carcinogens like DMBA (Flamm et al. 1966; Alexandrov et al. 1970), AFB₁ (Gelboin et al. 1966) and N-OH-AAF (Grunberger et al. 1973). Most significant observation by Alexandrov et al. (1970) was that this early inhibition was not caused by noncarcinogenic BeP, though it did cause stimulation in transcription as observed with carcinogens BP and DMBA. Alexandrov and Vendrely (1972) also observed that only the carcinogenic subfractions of cigarette smoke (including the BP-bearing

subfraction) caused early inhibition and the noncarcinogenic subfraction did not do so. Another parallel observation (deAngelo et al. 1978) was that DMBA caused inhibition in RNA synthesis in mammary gland cells of only susceptible strain of rat (Sprague-Dawley) and not of resistant-strain (Long-Evans). All these observations necessitated investigation of this early reversible inhibition in greater details.

BP is also an inducer of xenobiotic metabolizing enzymes and hence the 2-3 day stimulation in RNA synthesis could be due to synthesis of these enzymes. MC, another stimulator of metabolism was observed to induce such increase in RNA synthesis (Bresnick 1966; Gelboin 1967). The subsequent decline in RNA synthesis from 7-14 days as observed with BP in the present studies could be due to turnover and reduction in synthesis of RNA required for metabolizing enzymes. The inhibitory effect observed at 2 h was not due to vehicle DMSO and it truly reflected incorporation of precursor into RNA. This was confirmed by comparing RNA (before and after alkaline hydrolysis) obtained from DMSO- and BP-treated rats against untreated controls (Table 2.1).

The inhibition at 2 h and subsequent stimulation (2-4 d) may be due to corresponding depression or stimulation in synthesis of different types of RNA. Markov and Arion (1973) followed similar short pulse of [^{14}C]orotic acid followed by high temperature extraction of RNA and observed

that in rat liver or even in ascites tumor cells, the radioactivity was incorporated mainly in precursor rRNA and in "DNA-like" RNA i.e. mRNA molecules. Parsons and McCarty (1968) observed rapid labeling of mRNA-protein complex of rat liver nuclei, shortly after administration of [^{14}C]orotic acid. Similarly, short pulse of [^3H]uridine to HeLa cells, was incorporated into rapidly labeled precursor rRNA and HnRNA i.e. precursor mRNA (Soiero et al. 1968). Thus, the inhibition in RNA synthesis by BP at 2 h, could be due to inhibition in synthesis of rRNA or due to selective or generalized inhibition in mRNA population. Attempts were made to differentiate the inhibited population by two different means of separation. Labeled RNA was subjected to acrylamide-agarose (2.4 : 0.6%) slab-gel electrophoresis (Peacock and Dingman 1967). [^{14}C]Orotic acid was observed to be incorporated into many high MW RNA molecules including some rRNA bands, but no clear difference emerged between RNA from BP and DMSO preparations, at least under experimental conditions followed in these studies (data not shown). The (poly A⁺)messenger RNA populations (labeled with [^{32}P]orthophosphate) from control and BP-treated animals were isolated by Oligo(dT)-cellulose affinity column chromatography (Aviv and Leder 1972). However specific activity of different populations was not clearly distinguishable from each other (data not shown). The possibility of BP preventing entry of hot orotic acid into nucleotide pool also cannot be ruled out.

In order to answer these doubts and understand the mechanism of this early (2 h) reversible inhibition, nuclei purified from treated animals were assessed in vitro for transcriptive ability by a variety of procedures. Widnell and Tata (1964) were the first to use different ionic conditions to elicit differential transcriptive response from purified nuclei. When nuclei from BP (2 h)-treated animals were assayed, the Mg^{2+} -dependent activity was unaffected while the $Mn^{2+} + (NH_4)_2SO_4$ -dependent activity was inhibited (Fig. 2.3). The latter activity showed inhibition even under conditions of limiting DNA and reduced time of incubation (Table 2.1). Since $Mn^{2+} + (NH_4)_2SO_4$ assay very roughly expresses RNA polymerase II activity and Mg^{2+} assay generally expresses RNA polymerase I activity, it may be surmised that BP inhibited transcription by RNA polymerase II and not by I. However, overlap of the expression of one enzyme during assay of other cannot be ruled out. Cedar and Felsenfeld (1973) observed that high $(NH_4)_2SO_4$ prevents reinitiation of transcription but it facilitates propagation of RNA synthesis of preinitiated transcripts. Thus, such conditions as $Mn^{2+} + (NH_4)_2SO_4$, pH 7.5 need not exclusively represent RNA polymerase II activity. Zieve (1972) while probing into the transcription inhibitory effect of N-OH-AAF under such assay conditions pointed out the possibility of carcinogen affecting factors other than template or the enzyme viz. increased RNase activity of nuclei, decreased permeability of nuclear

membranes to NTP, alteration in the level of activators or inhibitors of transcription or change in NTP pools in nuclei. Under these assay conditions, both the activities showed peak activity at 6 h after BP-treatment (Fig. 2.3). The activities surprisingly declined at 16 h to near control values and remained so at 1 d and 3 d. However, since 2 d activity was not monitored, it is difficult to correlate with the 2-3 d peak of [^{14}C]orotic acid incorporation.

Nuclei used in above studies were purified by osmotic method of Blobel and Potter (1966). However, Yu (1975) improved the method of isolation of nuclei by using hypertonic sucrose and claimed maximum retention of RNA polymerases by this method. When nuclei isolated by this method were assayed for transcription under moderate conditions of pH, $(\text{NH}_4)_2\text{SO}_4$ and other ions, expression of nuclei from BP-administered animals was found to be 10-15% less than control (Fig. 2.5 or Table 2.3). Since DNA of nucleus is a heterogeneous template and multiple forms of RNA polymerases exist in rat liver nuclei (Roeder and Rutter 1969), the total assay provides a crude overall picture. Hence, an inhibitor of transcription, α -amanitin (Weiland 1968), the cyclic octapeptide from mushroom *Amanita phalloides*, was used to differentiate the expressions due to different forms of RNA polymerase. The differential sensitivity of polymerases II and III and resistance of I, as exploited by Liberator and Bresnick (1981a) was employed to understand

the mechanism of modulation of transcription by BP. At 2 h after BP treatment nuclei exhibited inhibition in expression of polymerases II and III but not in that of I (Fig. 2.5). In fact, polymerase I activity was marginally above control value. Use of $[8-^3\text{H}]\text{GTP}$ instead of $[\alpha-^{32}\text{P}]\text{UTP}$ in this assay showed similar pattern of effect by BP (Table 2.3). However, the polymerase III inhibition was more pronounced with GTP than with UTP. The fact that base composition of the specific DNA read by each of the three forms of RNA polymerase is different may be the cause of differential expression of activity observed while using different hot nucleotides. These apparent differences may also have arisen due to difference in activities at 0.01 μM and 0.01 mM α -amanitin. Thus, calculating contribution of each enzyme by difference may be the reason for high standard error values in Table 2.3. Therefore, many workers prefer to express the polymerase activities as activity resistant to 1-3 μg α -amanitin ml^{-1} (i.e. $\sim 0.01 - 0.03 \text{ mM}$) which may be correlated as polymerase I and activities which are susceptible to α -amanitin (polymerase II & III).

Individual polymerase activity showed increased value 2 d after BP treatment (Fig. 2.5); polymerase III, however showed maximum activity at 4 d. These enhanced activities returned to near control levels at 7 d. This correlated very well with $[^{14}\text{C}]\text{orotic acid}$ incorporation.

From results of these assays, it was apparent that the initial inhibition by BP was due to inhibition of polymerase II and III. However, polymerase I - which contributes nearly 50% of activity expressed under such assay conditions (Fig. 2.4), was marginally stimulated and hence total polymerase activity registered a modest inhibition due to BP.

Nucleolus is a major site of rRNA synthesis in eukaryotes and it is also the seat of RNA polymerase I. Nucleoli isolated from livers of BP treated (2 h) animals exhibited 18% inhibition in transcription (Table 2.4). Use of 0.01 mM α -amanitin in the assay could bring down the inhibition to 10% of control. Thus expression of polymerase I enzyme of nucleoli was marginally inhibited in response to BP. This was in contrast to marginal stimulation in polymerase I activity in nuclei in combined assay (Table 2.3). This could be due to specific inhibition in nucleolar template or enzyme localized in nucleolus, which would get maximally expressed in isolated nucleoli, but may be masked in total nuclear assay. Thus caution must be exercised in interpreting results obtained from only one type of assay condition e.g. using specific ionic strength, pH or inhibitor. It is worthwhile to note that specific activity of nucleolar transcription (~ 2000 pmol mg^{-1} DNA) was at least 6 x better than that of nuclear polymerase I (~ 350 pmol mg^{-1} DNA) (compare Tables 2.4 v/s 2.3). This can be explained from the facts

that nucleolar DNA constitutes just 4-5% of total nuclear DNA (Muramatsu et al. 1963) and ribosomal DNA is less than 1% of total DNA (Steel 1968), while transcription activity expressed under the assay conditions maximally emphasizes polymerase I activity (~50% total activity), thereby increasing the specific activity of transcription per mg DNA by a factor of five to ten.

Further studies by studying isolated enzymes to understand the mechanism of BP induced inhibition in transcription were attempted. Yu (1974) suggested existence of nuclear RNA polymerases in two forms: free and engaged to the template. These two forms were separated by a simple technique (Yu 1975) and were assayed for transcription in absence (for engaged enzyme) or presence of exogenous templates like calf thymus DNA or synthetic polynucleotide. Free enzyme displayed 9% inhibition (per mg protein basis) in response to BP, while engaged enzyme was inhibited to the extent of 18% (per mg DNA basis). Since the total nuclei displayed about 10-15% inhibition (Table 2.5), this could be contributed by both the free and the engaged forms of the enzyme. However, when each fraction is compared on unit-weight of liver basis, engaged activity was reduced by 30% while free activity was 30% above control. BP may have altered the template bringing about release of engaged form of enzyme into the free pool as was observed for AFB₁ (Yu 1977) and thus causing an apparent

increase in free activity, even though calculated per mg protein basis free activity showed marginal inhibition. Leffler et al. (1977) used DNA extensively modified by BPDE as template for transcription in vitro and observed decreased chain length of RNA. This was due to premature chain termination during RNA synthesis from modified template. Such situation would surely release the engaged enzyme into free pool. In these studies DNA was modified by BPDE at the level of 1 in 100 nucleotides, while in vivo level of modification is 1 in 10^5 nucleotides. Nevertheless, such modification could bring about decreased activity of engaged enzymes.

The effect of BP was then sought to be tracked down at the level of partially purified enzymes. The enzymes from whole nuclei, free and engaged fraction were solubilized and separated into three forms. Total enzyme from BP-treated animals was uniformly 35% less active compared to the activity of equal weight of liver from control animals (Fig. 2.6). This could be either due to reduced activity of the enzyme or reduced extraction of proteins from the BP nuclei - a plain procedural difference. However, the total amount of protein from equivalent weight of liver in each case was nearly same (~ 13 mg protein). Moreover, the amount of protein recovered in each peak was also measured and when specific activity of each enzyme was expressed per mg protein basis, all the three enzymes were still inhibited (Table 2.6) although the extent

of inhibition observed with individual enzymes was variable. Thus, polymerases I, II and III exhibited inhibition to the extent of 35%, 25% and 20% respectively. It is appropriate to discuss yield of protein and enzyme from the column as there appears to be controversy among workers in this field. Yu (1977) observed 100% recovery of control enzymes from the column and assumed the same for treated (AFB₁) preparation, while Shields and Tata (1976) found variable recovery of the different forms of enzyme from control and treated (cortisone) preparations. The latter authors also cautioned against overloading DEAE-Sephadex A25 column resulting in reduced retention of the enzyme in the column and suggested maximum load of 1 - 1.5 mg protein ml⁻¹ bed volume. We have loaded maximum of 13 mg protein for 14 ml column. In general, for both the preparations, out of 13 mg loaded nearly 8 mg (61.5%) was unadsorbed. Only 2.1 mg (16%) of protein was eluted in peak fractions, while the remaining 22% protein could have come out in non-peak fractions or was washed out at the end of each column run with 0.5 M (NH₄)₂SO₄. Protein yield in peak fractions was nearly the same for both the preparations. When recovery of enzyme activity was calculated, it was always observed that recovery of enzyme was more than what was expressed in Fraction IV. This was due to an unavoidable situation where assay of each fraction was conducted in different amount of (NH₄)₂SO₄-depending upon the stage of the (NH₄)₂SO₄ gradient. Under these circumstances fraction IV

in 0.03 M $(\text{NH}_4)_2\text{SO}_4$ expressed itself much below its maximal expression while polymerase II and III peak fractions eluted at higher $(\text{NH}_4)_2\text{SO}_4$ expressed at a very high level. In a typical example, fraction IV with ability to incorporate 1806 pmol $[\text{}^3\text{H}]\text{GMP}$, when resolved as polymerases I, II, III, resulted in total peak incorporating ability of ~4000 pmol i.e. an increase of 120% above fraction IV-value. However, the results obtained from enzymes isolated from equivalent amounts of liver from two preparations passed through same column under identical condition should remain comparable. Another notable feature was that separation as three peaks of enzyme activity tended to change if gradient volume was changed to 70ml or 100 ml (instead of 50 ml). Peaks II and III would split as two adjacent peaks (data not shown) which could have been separate peaks of each class of enzyme shown by other workers (Yu 1977; Liberator and Bresnick 1981a). When at the end of the gradient all the unwanted protein was washed down with 0.5 M $(\text{NH}_4)_2\text{SO}_4$ no enzyme activity was observed (data not shown).

When free enzymes from nuclei of equivalent weight of liver from BP or DMSO treated animals were resolved into three polymerases (Table 2.7) activity of all polymerases showed inhibition due to BP treatment. The extent of inhibition was 42%, 16% and 7% for polymerases II, I and III respectively. Calculated on the basis of specific activity

polymerases II and I were inhibited by 57% and 10% respectively, while polymerase III showed stimulation. The possibility that amount of nonenzymatic protein eluted with each peak plays a role in specific activity is worth considering. In almost all cases we studied, peak I carried nearly 60 - 75% of adsorbed protein while it did not carry more than 20 - 30% of enzyme activity, hence peak I showed poorest specific activity. Peak II showed moderate specific activity having 20 - 30% adsorbed protein and 30 - 50% enzyme activity, while peak III showed maximum specific activity in all preparations. The very high specific activity of polymerase III in the free enzyme preparation is perhaps due to higher purity achieved on chromatography. Since very low amount of protein is eluted, extraneous proteins seem to be completely absent in this fraction. This aspect needs further investigation. Overall, expressed per g liver basis, the free activity of polymerase II was maximally inhibited, I was inhibited less and III showed marginal inhibition.

When engaged enzyme was solubilized from its template-engaged state and resolved into three forms of enzymes (Table 2.8), none of the polymerases was inhibited by BP, whether seen as per g liver basis or on specific activity basis. In fact polymerase I and II showed activity beyond control values by 14% and 55% respectively. Polymerase III was near control. In engaged preparation, however,

the total protein yield in fraction IV from BP preparation was more than control preparation viz. ~12 g liver yielded 8.8 mg protein for DMSO, but 12.2 mg for BP-preparation. However the amount of protein eluted in peak fractions was ~1 mg in both the cases, indicating presence of large amount of non-RNA polymerase protein in BP-preparations. Whether these additional proteins have a role in transcriptional control or not is a question not answered here. Nevertheless, the enzymes obtained from engaged fraction of nuclei were not inhibited due to BP, and if at all polymerases I and II were stimulated above control. Since the engaged fraction of nuclei displayed inhibition in transcription while the enzymes separated and purified from this fraction did not, it is reasonable to assume that BP also affects the chromatin template.

Thus, it appears that BP on entering nucleus, reacts with two of the main components of transcription machinery. Template chromatin is affected because engaged fraction of nucleus is inhibited but enzymes solubilized from that fraction are not inhibited. Total nuclear enzymes are inhibited and this must be due to an effect on free form of the enzyme. However effect of BP on other factors affecting the transcription viz stimulators or inhibitors occurring in the cell or loss of such factors in the method of purification or isolation of individual preparation that might lead to such results cannot be monitored and hence not discussed.

We created a model of BP-induced-inhibition in vitro such that the above observations may be unambiguously clarified. The ultimate carcinogen BPDE I was reacted with nuclei to bring about dose-dependent inhibition in transcriptive ability of the nuclei (Fig. 2.8). This inhibition was due to interaction of BPDE I with one or more nuclear components since increasing amounts of nuclei could competitively reduce the BPDE I induced inhibition (Fig. 2.10). When differentiated with α -amanitin, this inhibition was observed to be mainly due to expressions of polymerases I and II and less so by III. To the best of our knowledge, this is the first report of in vitro model recreation of BP-induced inhibition of nuclear transcription.

Yu and Feigelson (1971) developed a technique to differentiate action of enzyme from that of chromatin by blocking the latter with actinomycin-D and using poly dC to elicit response from the enzyme. In our standardized assay, the final level of inhibition by actinomycin-D in both control or BPDE I inhibited nuclei was same (Table 2.9), indicating similar site of action for BPDE I and actinomycin-D. The actinomycin-D-inhibited nuclei when assayed in presence of exogenous template would reflect ability of enzymes in the preparation, independent of the endogenous template. The enzyme activity assayed in this manner showed 25% less activity in BP preparation (6 v/s 5). In fact when

contribution of actinomycin-D resistant activity was eliminated (7 and 8), the enzyme response was 32% less in BPDE I preparation than control preparation. This confirmed that out of total nuclear inhibition of 70% due to BPDE I, 32% contribution was due to effect on activity of enzymes. When differentiated with α -amanitin, this inhibition was confined to polymerase I and III and not to II (6 v/s 5). However, actinomycin-D in vivo was shown to affect not only the template but also enzyme RNA polymerase II (Yu 1980). Thus the effect of BPDE I on enzyme polymerase II in actinomycin D treated nuclei might not be observed at all, because both control and BPDE I preparations exhibited same low level of polymerase II activity in presence of actinomycin D. This also might result in artificial inflation of value for polymerase III estimated in presence of higher amount of α -amanitin (0.01 mM).

The correlation of such in vitro effects with in vivo observations made by us becomes obvious in many instances. The effect on template inferred in vivo was confirmed in vitro as template inhibition would contribute nearly 38% of inhibition in vitro. The effect on enzyme observed in vivo (inhibition of total enzyme and free enzyme) tallied with 32% inhibition of enzyme observed in vitro.

From all these observations it is concluded that short time after BP administration transcription activity of nuclei is severely inhibited. This inhibition is brought by

the action of BP-metabolite on RNA polymerases as well as on chromatin. Taken together with other published evidences it appears that this effect is a carcinogenic initial response of BP.