C H A P T E R III

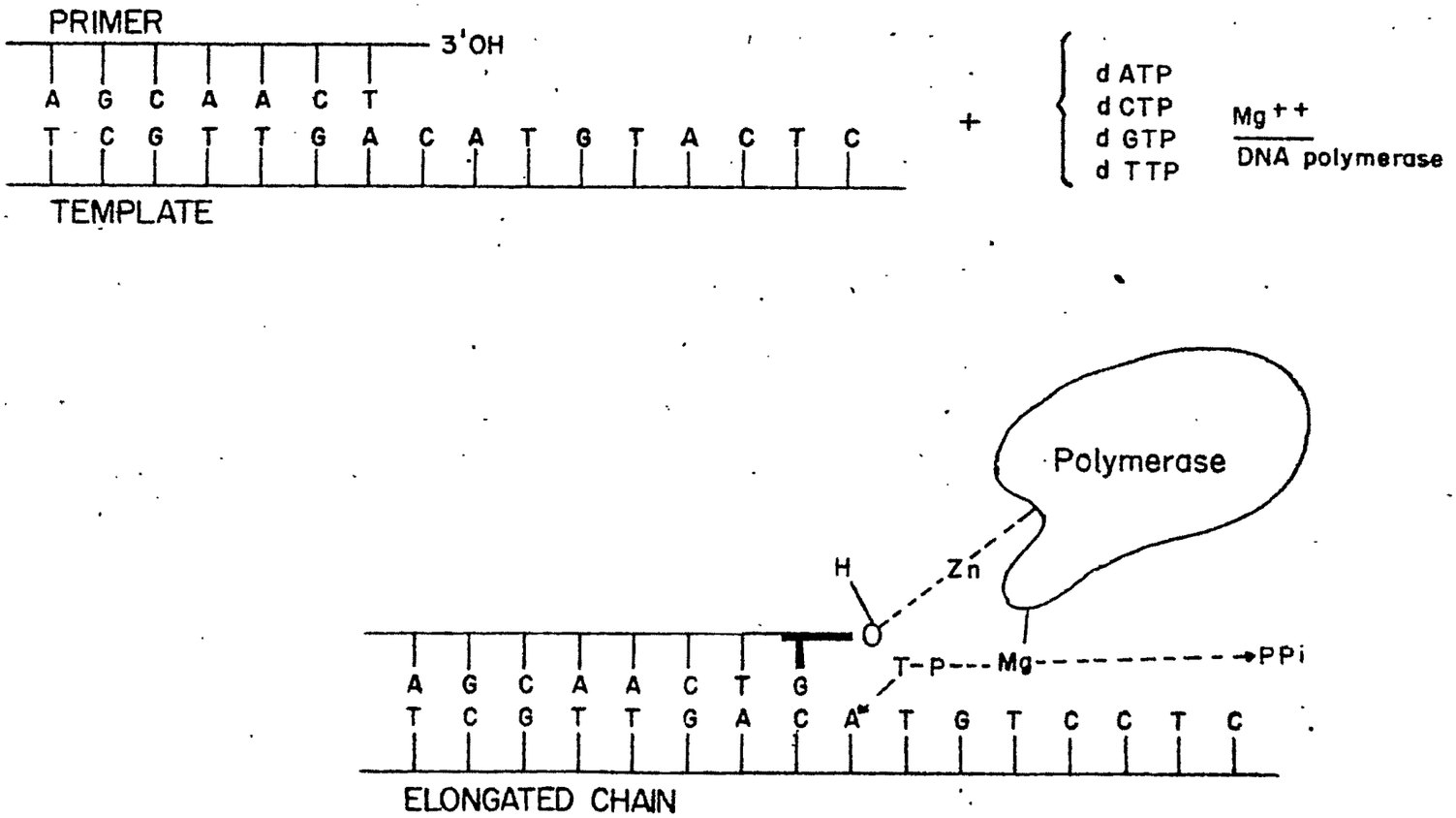
IN VITRO NUCLEIC ACID SYNTHESIS BY PURIFIED DNA POLYMERASES FROM HETEROLOGOUS SOURCES IN PRESENCE OF ENVIRONMENTAL CARCINOGENS

INTRODUCTION

Deoxyribonucleic acid polymerases can be defined as enzymes that catalyze the polymerization of deoxynucleotides upon direction of polynucleotide templates. A diagramatic representation of the current knowledge on the mechanism of DNA polymerization is shown in Figure 19. The general requirements for polymerization are a polynucleotide template, primer with a 3'-OH terminus, a divalent metal cation, and complementary deoxynucleoside triphosphates. The principle reaction involves a nucleophilic attack of the 3'-OH primer terminus upon the α-phosphorus of the deoxynucleoside triphosphate substrate with the displacement of pyrophosphate. Magnetic resonance studies with E. coli DNA polymerase I indicate that Mn^{2+}, as the added divalent cation, coordinates the enzyme to the γ-phosphoryl group of the deoxynucleotide substrate (196). The enzyme in the absence of template changes the conformation of the substrate to one which is a nucleotidyl unit would occupy in the product, double-standard DNA. In addition to a requirement for the added divalent cation, DNA polymerases from animal, bacterial and viral sources have been shown to contain stoichiometric quantities of tightly bound zinc (197).

DNA polymerases have been extensively purified from a variety of animal sources. Multiple forms of DNA polymerases have been isolated from eukaryotic cells. In mammalian cells, there is a general agrement that three classes of DNA polymerases can be delineated in addition to mitochondrial DNA polymerase

REQUIREMENTS FOR DNA POLYMERIZATION



Model of synthesis of DNA based on known properties of DNA polymerases, taken from Loeb et al. (173)

(mt). These enzymes have been designated as DNA polymerase - α, - β and - γ. The enzymes are distinguished on the basis of size, inhibition by sulphydryl inhibitors, template utilization, and sub-cellular location. A number of excellent reviews have dealt in greater details about specific DNA polymerases (57, 198-202).

The high molecular weight DNA polymerase α appears to the most abundant DNA polymerase in dividing mammalian cells (203). DNA polymerase β is a relatively low molecular weight enzyme and is thought to be involved in DNA repair (204). DNA polymerase-γ accounts for only one per cent of the total cellular DNA polymerase activity in some mammalian cells. Although the molecular weight of DNA polymerase-γ is similar to that of DNA polymerase-α, the two enzymes have been shown to be distinct on the basis of immunological criteria (205). The amount of mt DNA polymerase activity also corresponds to only about one per cent of the total DNA polymerase activity in eukaryotic cells. The mitochondrial DNA polymerase purified from yeast is devoid of exonucleolytic activity (206). It has not yet been established by immunological criteria whether the mitochondrial DNA polymerase is distinct from other cellular DNA polymerase nor has it been demonstrated that the enzyme is coded for by mitochondrial DNA (207).

Since the DNA polymerase isolated by Kornberg and his colleagues from E. coli (208) (now referred to as DNA polymerase I) held prominence for more than a decade, a central role for it in replication was often implicitly assumed. The discovery in

1969 by DeLucia and Cairns of a mutant retaining only 0.5 - 1% of assayable DNA polymerase (209) but still capable of DNA replication, led to increased speculation concerning the true function of DNA polymerase I, which at that time was the only well characterised DNA polymerase of *E. coli*. In the event, the major outcome of the discovery of the DeLucia and Cairns mutant, which did not definitively eliminate DNA polymerase I from a replicative role, was the impetus it gave to the scarch for new DNA polymerases. As a result, two further DNA polymerases were isolated, purified and characterized, DNA polymerase II (210) and III (211). While polymerase II is believed to be a repair enzyme (212), polymerase III has been shown to be the product of the dna E gene (213).

Transormation of cells by RNA tumor viruses requires the conversion of viral genetic material into complementary DNA (214, 215). The viriors of RNA tumor viruses contain a DNA polymerase which can transcribe 70s viral RNA into complementary DNA (215-217). This enzyme has been called reverse transcriptase or RNA-dependent DNA polymerase or DNA polymerase of RNA tumor viruses. Homogeneous DNA polymerase isolated from avian myloblastosis virus (AMV) is classified as reverse transcriptase since it can transcribe both RNA and DNA template (215). It has been reported earlier that DNA polymerase (reverse transcriptase) of RNA tumor virus incorporate an unusually large number of non-complementary nucleotides while copying a variety of polynucleotide template (218, 219). The AMV-DNA polymerase lacks any detectable exonuclease activity as a result of which it fails to excise mispaired bases incorporated during synthesis. Animal DNA

polymerases have little, if any, exonuclease activity (57) and seldom incorporate noncomplementary nucleotides. Since AMV-DNA polymerase incorporate and fails to excise noncomplementary nucleotides during polymerisation, the role of DNA polymerase in base selection can be studied with relative ease (220).

As emphasized earlier (see Chapter I, Sec. A.) chemical carcinogens induce modification of the template. Only few investigations have been concentrated on the effects of carcinogen modification on the ability of a template to be copied by a polymerase. β-Propiolactone is one such chemical carcinogen used to modify the polynucleotide template-primer, poly (dA).oligo)dT). It was observed that modification of template by increasing concentration of carcinogen resulted in decreased utilization of the modified templates by AMV-DNA polymerase and by sea urchin nuclear DNA polymerase (221).

Another still enigmatic aspect of alkylation mutagenesis is the tendency of certain agents, notably NTG and occasionally also EMS, to preferentially mutate DNA in the vicinity of replication fork with an intensity that frequently produce multiple closely linked mutations. Although the mechanism of NTG mutagenesis remain obscure, one should consider the possibility that it might also affect one or more of the enzymes of DNA metabolism that are fundamental for error avoidance (85).

It has already been pointed out that NTG selectively mutate replicating region in DNA in bacteria (167, 168), leading to a high frequency of closely linked double mutation (222).

This behaviour occurs only in those cells in which the DNA growing points have been stopped by the mutagen (223), supporting the idea that the action of NTG is on the replication complex itself. The effect of NTG upon DNA replication in vitro was subsequently studied (169). It was found that both the polymerase and exonuclease activities of DNA polymerase III are inactivated by treatment with NTG, although, the DNA template remained unaltered. Having known that the replicating enzyme pol III is sensitive to NTG, we decided to examine the effect of NTG on the in vitro DNA synthesis by homogeneous E. coli DNA polymerase I which is thought to be involved in the DNA repair process in vivo (209) and also on AMV-DNA polymerase which is believed to be the enzyme involved in viral replication (214).

Much less is, however, known regarding the mechanism of dialkylnitrosamine induced mutagenesis at the level of DNA synthesis. In addition to alkylating the template, its possible involvement with DNA synthesizing enzymes should not be ruled out. Experiments were therefore designed to examine such possibility also, in the present investigation.

MATERIALS

Chemicals :

All chemicals used in this section are mentioned either in Chapter I or Chapter II.

Enzyme source :

Homogeneous E. coli DNA polymerase I, Avian Myeloblastosis Virus (AMV) polymerase, Acute Lymphoblastic Leukemic (ALL)-α polymerase, Human β-placental polymerase, homogeneous E. coli RNA polymerase and 'activated' calf thymus DNA were kind gifts of Dr. L.A. Loeb, University of Washington, Seattle, U.S.A. E. coli polymerase I was purified by the method of Jovin et al.(224) as described by Springgate et al.(225), AMV-DNA polymerase was prepared as described by Kacian et al.(226), ALL-α and β-placental polymerase was prepared as described by Dube and Seal (186). E. coli RNA polymerase was purified according to the method described by Springgate and Loeb (227).

METHODS

DNA polymerase assay :

Assay were carried out in a reaction mixture (total volume, 0.1 ml) containing : 0.05 mM Tris-HCl pH 7.4, 5-10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 20 μM one radioactive dNTP ([^{3}H]-dNTP, specific activity was given in respected table) and other three cold dNTP in concentration 25 μM each, 20 μgm 'activated' calf--thymus DNA (115, 186, 209) and 0.4 unit pol I (Kornberg Unit) or 1.2 - 1.5 unit AMV DNA polymerase or 0.02 unit ALL-α DNA polymerase or 0.3 - 0.4 unit Human placental β-polymerase with some special alteration e.g. in case of Pol I and β-polymerase 100 mM KCl was added, AMV-polymerase and α-polymerase 2.5 mM DTT was added to the reaction mixture. β-Polymerase assay were carried

out at pH 7.9. The reaction mixture was incubated at 37°C for 30 min (except 60 min for AMV polymerase). The product was precipitated with 0.5 ml cold 10% TCA using 0.2 ml of DNA (1 mg/ml) as carrier. Then the precipitate dissolved in 0.5 ml of 0.2 M NaOH, reprecipitated with acid, and acid insoluble count was measured according to method described elsewhere (112, 114-115).

Assay of E. coli RNA polymerase :

RNA polymerase activity was measured according to the method of Springgate and Loeb (227). The reaction mixture in a total volume of 0.1 ml contained the following : 50 mM Tris-HCl (pH 8.0), 0.1 mM DTT, 10 mM $MgCl_2$, 5 µg activated calf-thymus DNA, 150 mM KCl, 0.1 mM EDTA, 100 µM ATP, GTP, CTP and [^{3}H]-UTP (specific activity as mentioned in the legend of the corresponding table) and 2.5 µg of enzyme protein. Assay were carried out (in triplicate) at 37°C for 40 minutes. Incorporation of radioactivity into acid insoluble material was determined in a liquid scintillation spectrometer as described by Dube and Loeb (112).

RESULTS AND DISCUSSION

Earlier experiments in the present investigation on the mode of action of NTG on macromolecular synthesis in plant mitochondria have raised two possibilities. The NTG induced inhibition of DNA synthesis may either be due to membrane modification leading to altered permeability of dNTP or due to the inhibition of mitochondrial DNA polymerase. This latter possibility has

therefore been investigated here. Ideally, this investigation would have been more pertinent with purified mitochondrial DNA polymerase. Unfortunately, due to the extreme temperature sensitivity of plant mitochondrial DNA polymerase our repeated efforts to isolate this enzyme have proved futile so far. Considering the universality of DNA synthesis (198), it was therefore decided to examine this possibility with purified DNA polymerases from animal, bacterial and viral sources.

The only report so far available on the effect of NTG on *in vitro* DNA synthesis by Sanchez in 1976 (169) has been carried out with *E. coli* DNA polymerase III and it has been found that both the polymerase and associated exonuclease activities are inactivated by the carcinogen without any effect on template. In view of the fact that a number of potential carcinogens of the alkylating type induce strand breaks in DNA (228-230) which is invariably followed by DNA repair synthesis (231-233), effect of carcinogens on the enzyme responsible for DNA repair synthesis also becomes logical to be analysed. *E. coli* polymerase I, an established repair enzyme (209) was therefore selected as a representative enzyme in this case. In addition, effect of NTG or other carcinogens on eukaryotic and viral DNA polymerases have not been studied in details so far.

The effect of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) on the DNA synthesis catalyzed by homogeneous *E. coli* DNA polymerase I is illustrated in Table 17. It appears that the activity of *E. coli* DNA polymerase I is inhibited about 50% only when the enzyme is preincubated with 5-10 mM NTG for 30 and 60 min at 20°C

in dark. It can thus be seen that fairly high concentration of NTG is required to attain only partial inhibition of DNA polymerase I. Similar concentration of NTG has also been reported to be required to bring about the same order of inhibition of *E. coli* DNA polymerase III, the replicating enzyme (169).

Again, data presented in Table 18 reveals that homogeneous polymerase isolated from a variety of sources are more or less inactivated in presence of high concentration of NTG. It may be interesting to note that the bacterial and viral polymerases differ from each other in their requirement for dithiothreitol (DTT). While DNA polymerase I has no requirement for DTT for its maximal activity, presence of DTT is essential for AMV-DNA polymerase activity (112). From the inhibition data presented in Table 18 it can be noted that AMV-DNA polymerase activity is completely inhibited by the same concentration (15 mM) of NTG. The reason for this inhibition may be attributed to the disappearance (by binding with NTG) of DTT, which is known to be required for maximal AMV DNA polymerase activity (112). It is also known that DTT binds with the NTG and the stoichiometry for binding is 1:4 (169). Naturally, the effect of various concentration of NTG on AMV-DNA polymerase activity in presence and absence of DTT (2 mM) was further examined. The results obtained from such experiment are presented in Table 19. It is revealed that per cent activity of the AMV-DNA polymerase remains almost identical at a particular concentration of NTG (~~[illegible]~~). It may therefore be suggested that inhibition of AMV polymerase activity in presence of NTG might not be only due to the removal of DTT

the inactivation of the polymerase may be the reason that NTG stops progression of the replication point *in vivo*. At the replication point there are a number of proteins with which NTG can react as reported by Sugimura *et al.* (234). This reaction, principally with thiol groups (235) may lead to the production of new products which might enhance the rate of methylation of DNA at the growing points. This greater methylation could result in the increased level of repair at the growing point region which might use a chemically modified DNA polymerase (repair enzyme) induced by NTG (236) similar to the effect shown with methylnitrosourea on *E. coli* DNA polymerase I (237).

The effect of NTG on the *in vitro* RNA synthesis by homogeneous *E. coli* RNA polymerase is illustrated in Table 20. It appears that the activity of *E. coli* RNA polymerase is inhibited considerably when the enzyme is preincubated (for 30 min) with high concentration of NTG (5-15 mM) at 20°C in dark.

The studies on effect of environmental carcinogens on *in vitro* DNA synthesis was further extended to dialkylnitrosamines. Effect of DENA, a prominent member of this group of environmental carcinogen, on *in vitro* DNA synthesis by homogeneous *E. coli* DNA polymerase I was studied. The results presented in Table 21 clearly indicate that DENA has no inhibitory effect on the process. In view of a number of reports related to template modulation by this group of carcinogen (238) effect of pretreatment of DENA on "activated" calf-thymus DNA, which is generally used as template in *E. coli* DNA polymerase I assay, was also investigated. It can be seen, when the template is preincubated with DENA instead of

enzyme, there is however, a stimulation in the E. coli DNA polymerase activity (Table 22).

The effect of DMN, yet another environmental carcinogen, on different homogeneous polymerase activity was also investigated. Table 23 indicates that DMN has stimulatory effect on the in vitro DNA synthesis by homogeneous E. coli DNA polymerase I, and that the stimulation enhances with increasing concentration of the carcinogen. Pretreatment of calf-thymus DNA (template) with DMN also leads to stimulation. This stimulation also increases with increasing time of pretreatment (Table 24). Therefore it can be concluded that dialkylinitrosamines viz. DENA and DMN, modulate "activated" calf-thymus DNA, a template of in vitro DNA synthesis, in such a way that the overall in vitro DNA synthesis is stimulated, when the reaction is catalyzed by homogeneous E. coli DNA polymerase I.

It is therefore clear from the results obtained in Tables 21 and 23 that in presence of DENA and DMN the activity of homogeneous DNA polymerase I is not inhibited but rather stimulated. However, this finding is in contrary to our earlier observation (in Chapter II) that in vitro DNA synthesis by isolated plant mitochondria is inhibited in presence of DENA and DMN. One should therefore consider the possibility of the presence of specific enzyme(s) system within the mitochondria itself, which can convert DENA or DMN into some more potent catabolic products, which in turn can inhibit DNA synthesis in plant mitochondria. To clarify this point "activated" calf-thymus DNA (used as template) was preincubated with lysed mitochondrial extract and DENA or DMN.

This pretreated DNA was now used as template for DNA synthesis catalyzed by *E. coli* DNA polymerase I. The results obtained clearly show that there is no inhibition of DNA synthesis; on the other hand the synthesis is rather stimulated (Table 25). It is therefore tempting to conclude that there is no specific enzyme(s) system in the plant mitochondria which can transform the carcinogen to an "active metabolite". The inhibitory effect of these carcinogens on plant mitochondrial DNA synthesis therefore suggest a systemic peculiarity of plants mitochondria.

Table 17

Effect of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) on the DNA synthesis catalyzed by homogeneous *E. coli* DNA polymerase I

Time of pre-incubation (min)	Concentration of NTG (mM)	pmoles of [^{3}H]--dGMP incorporation per assay	% Activity
30	-	18.5	100
30	5	14	76
30	10	12	65
60	-	23.18	100
60	5	17.8	77
60	10	12	52

E. coli DNA polymerase I was preincubated with NTG (different concentration) at 20°C in the dark for different times as indicated in the Table. Complete assay system and measurement of radioactivity incorporated were same as indicated in 'Methods'. Specific activity of [^{3}H]-dGTP was 100-170 cpm/pmole.

Control was considered as 100% activity.

Table 20

Effect of N-methyl-N'-nitro-N-nitrosoguanidine on *E. coli* RNA polymerase activity

Assay system	Incorporation of [^{3}H]-UMP cpm/assay system	% Inhibition
Complete	3070	0
" + 5 mM NTG	1222	61
" + 10 mM NTG	1136	63
" + 15 mM NTG	876	71

E. coli RNA polymerase I was preincubated, at 20°C for 30 min in dark, with various concentration of NTG as indicated in the Table. Complete assay system, incubation condition and [^{3}H]-UMP incorporation measurement have been described in 'Methods'. Specific activity of [^{3}H]-UTP was 32 cpm/pmole.

Table 21

Homogeneous *E. coli* DNA polymerase I activity in presence of various concentrations of diethylnitrosøamine (DENA)

Assay system	[^{3}H]-dGMP incorporation cpm/assay system
Complete	3685
" + DENA (1 µgm)	3090
" + DENA (3 µgm)	3150
" + DENA (5 µgm)	3052

Complete assay system, incubation condition and measurement radioactivity incorporated were same as described under 'Methods'. Specific activity of [^{3}H]-dGTP was 100-170 cpm/pmole.

Table 22

Effect of template pretreatment by diethylnitrosoamine (DENA) on polymerase activity using homogeneous *E. coli* DNA polymerase I

Assay system for	Time of pretreatment (min)	[^{3}H]-dGMP incorporation cpm/assay system
Untreated template	0	5466
5 µg DENA pretreated template	0	8235
Untreated template	40	7860
5 µg DENA pretreated template	40	9597
Untreated template	60	5211
5 µg DENA pretreated template	60	8476

"Activated" calf-thymus DNA was preincubated with DENA at 20°C in the dark for different times as indicated. Complete incubation system, reaction condition and radioactive processing were same as described under "Methods". Specific activity of [^{3}H]-dGTP was 250 cpm/pmole. Reactions were performed in triplicate and the averages were determined.

Table 23

Homogeneous *E. coli* DNA polymerase I activity in presence of different concentrations of dimethylnitrosamine (DMN)

Assay system	Incorporation of [^{3}H]-dAMP cpm/assay system
Complete	4798
" + DMN (2 µg)	6214
" + DMN (5 µg)	7912
" + DMN (10 µg)	20926

Complete incubation system, reaction condition and measurement of radioactivity incorporated are same as described in 'Methods'.

Specific activity of [^{3}H]-dATP used was 1000 cpm/pmole.

Reactions were performed in triplicate and the averages were determined.

Table 25

In vitro DNA synthesis by *E. coli* DNA polymerase I using 'activated' calf-thymus DNA pretreated with mitochondrial extract and dimethylnitrosoamine (DMN) or diethylnitrosoamine (DENA)

System	pmoles of ^{3}H dAMP incorporated
Pol I + Mit. extract	55
Mit. extract - Pol I	1.6
Pol I + Mit. extract + DMN (5 µg)	143
Pol I + Mit. extract + DENA (10 µg)	136
Pol I + Mit. extract + Antisera against DNA polymerase I (20 µg)	1.02

Mitochondria (10 mg of protein/ml) in a 1 ml suspension was lysed with 0.25% detergent NP-40, 1 M - KCl, 1 mM dithiothreitol, 10 mM-Tris/HCl pH 7.4. The suspension was kept at 4°C for 30 min and was then diluted with an equal volume of 10 mM-Tris/HCl buffer, pH 7.4. The supernatant resulting from centrifugation at 10,000×g for 30 min of the lysed mitochondrial preparation was collected and used as mitochondrial extract.

The "activated" calf-thymus DNA was preincubated with DMN or DENA in presence of above mitochondrial extract at 37°C for 10 min. *E. coli* DNA polymerase I and other constituents of the reaction mixture were then added. The incubation system and reaction condition are same as given in the legend of Table 24.

Figure–20

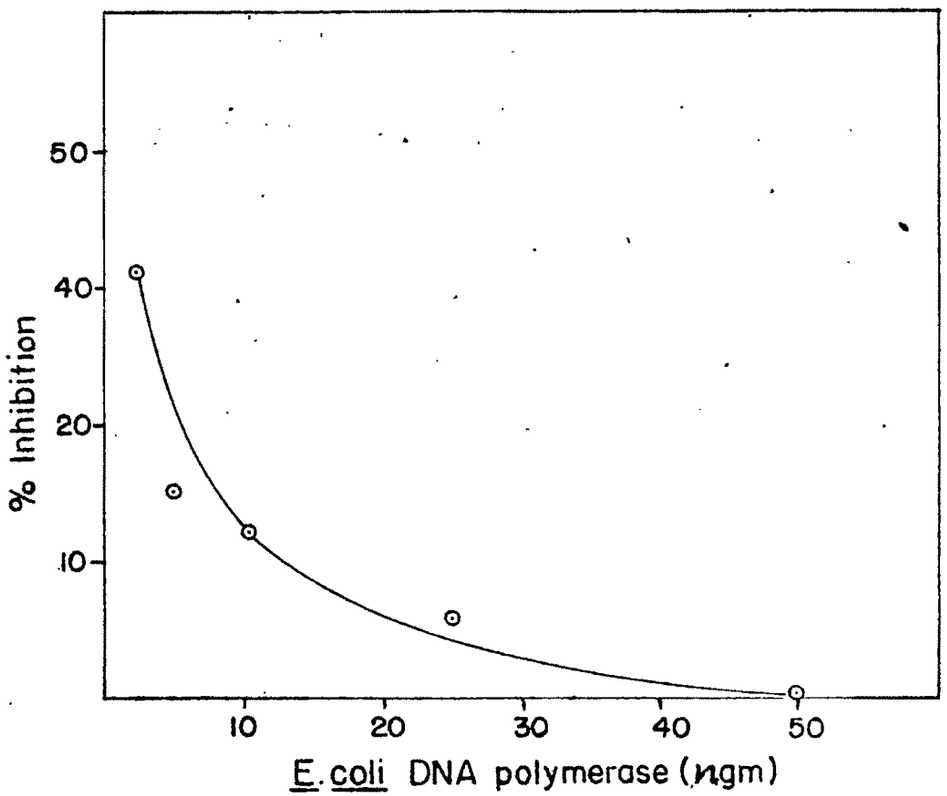
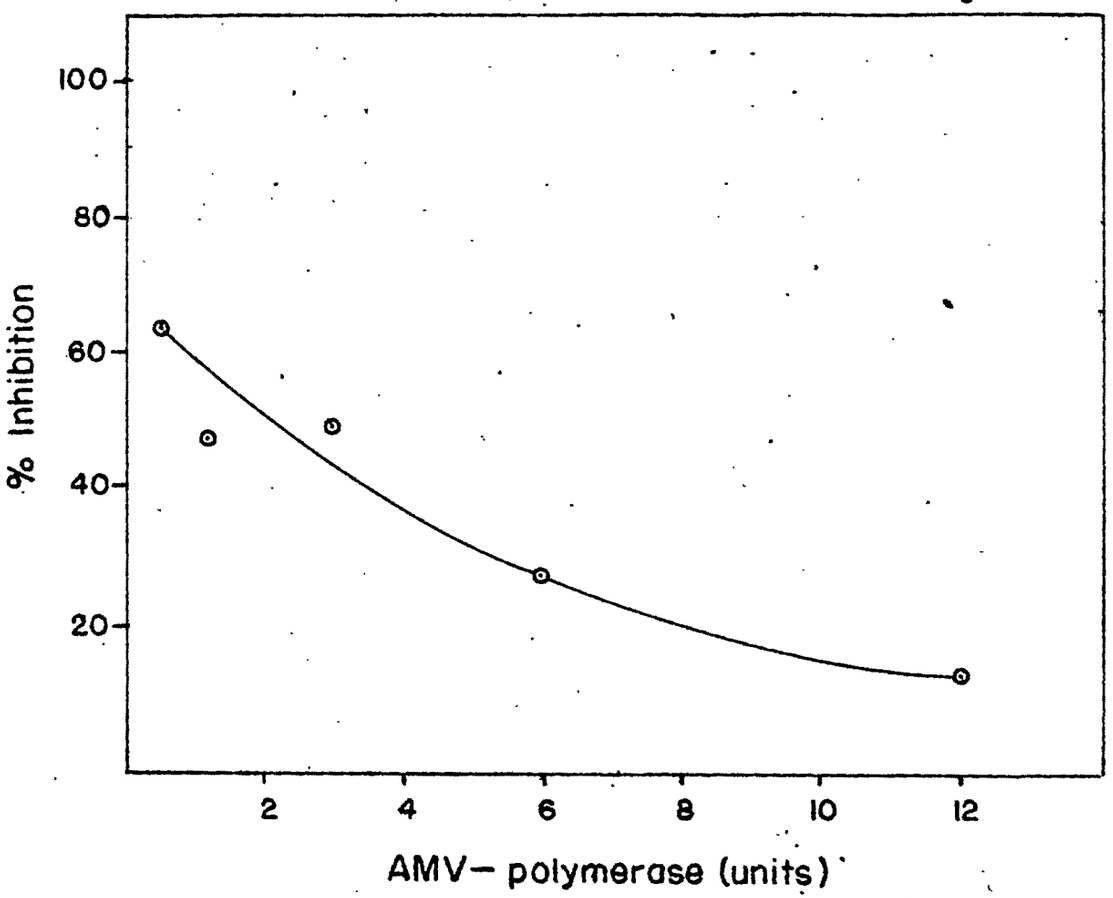


Figure-21



LEGENDS TO FIGURES

Fig. 20 : Effect of increasing amount of E. coli polymerase I on activity of the enzyme itself in presence of NTG (2 mM). The complete assay system is same as described in 'Methods'. Specific activity of [^{3}H]-dGTP was 100 - 170 cpm/pmole.
E. coli polymerase I was preincubated with NTG at 20°C in dark for 30 min.

Fig. 21 : Effect of increasing amount of AMV-DNA polymerase on activity of the enzyme itself in presence of NTG (1 mM). The complete assay system is same as described in 'Methods'. Specific activity of [^{3}H]-dAMP was 1000 cpm/pmole. AMV-DNA polymerase was preincubated with NTG in presence of DTT (2 mM) at 20°C in dark for 30 min.