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CHEMICAL MODIFICATION OF THE SIGMA SUBUNIT OF *Escherichia coli* DNA-DEPENDENT RIBONUCLEIC ACID POLYMERASE.

by

CHITTAMPALLI SHESHA CHAR NARAYANAN

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1983

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

April 8, 1983
Date

Chairman of Examining Committee

April 8, 1983
Date

Executive Officer

Supervisory Committee

ABSTRACT

CHEMICAL MODIFICATION OF THE SIGMA SUBUNIT OF *Escherichia coli* DNA-DEPENDENT RIBONUCLEIC ACID POLYMERASE.

by

Chittampalli Shesha Char Narayanan

Adviser: Joseph S. Krakow, Professor

The function of lysine, arginine, cysteine and carboxylic amino acid (glutamic and aspartic) residues of sigma was studied using chemical modification by group specific reagents. Following modification of 3 arginines with phenylglyoxal or 3 cysteines with N-ethylmaleimide (NEM) or 5 lysines with trinitrobenzene sulfonate sigma activity was lost. Analysis of the kinetic data for inactivation indicated that one lysine or arginine or cysteine residue with estimated pK values of 9, 8 and 8 respectively is essential for sigma activity. At low NEM concentration alkylation was limited to a nonessential cysteine. Modification of lysine or arginine or cysteine residues had no observable effect on the binding affinity of inactivated sigma to the core polymerase but inhibited promoter recognition and initiation effects of sigma on core polymerase. Holoenzyme containing the modified sigma was also able to bind DNA. Modification of aspartic

and/or glutamic acid residues with the water soluble carbodiimides, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) or 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate (CMC) resulted in loss of sigma activity. The inactivation and kinetic data indicated that one carboxylic amino acid is essential for sigma activity. Sigma modified with EDC, CMC or EDC in the presence of glycine was inactive in supporting promoter binding and inactivation by core polymerase. Reaction with EDC plus (^{3}H)glycine resulted in the incorporation of glycine into sigma. The (^{3}H)glycine-sigma is unable to form a stable holoenzyme complex. Limited proteolytic digestion of modified sigma indicated a change in the conformation of sigma following the modification.

Autoradiography of the cyanogen bromide fragments of radiolabelled sigma suggested that the critical lysyl or cysteinyl groups are not in the first 288 amino acids from the N-terminus. The nonessential cysteinyl group was identified as cys132. The critical carboxyl group is in the first 288 amino acids from the N-terminus. It is possible that the N-terminal half of sigma is involved in the interaction of sigma with the core polymerase and the C-terminal half of sigma containing the critical lysyl and cysteinyl groups is involved in the catalytic function of sigma.

This thesis is dedicated to my beloved grandmother

Kutti

I would like to thank Dr. Joseph Krakow for his guidance, patience, encouragement and friendship and for the innumerable things he has taught me. I would like to thank Drs. Kumar and Rudner for teaching me many techniques, for their helpful discussions and for being good friends and teachers. I would like to thank my colleagues Hiroji Aiba, Jesus Angulo and Gopal Nair for helpful discussions and suggestions and for being good friends. Without the enormous love and support of my family especially of my sisters, Alamelu and Padma and my brother, Narasimha Char, this work would have been impossible.

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ABBREVIATIONS

BSA, bovine serum albumin; BTP, 2-bis-(2-hydroxy ethyl) amino-2-(hydroxy mthyl)-1,3-propanediol; CDC, cyclo-heptamylose-5-dimethyl amino naphthalene-1-sulfonyl chloride; CMC, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate; CRP, cyclic AMP receptor protein; $d(A\text{-}T)_n$, poly(dA-dT).poly(dA-dT); $d(I\text{-}C)_n$, poly(dI-dC).poly(dI-dC); DMSO, dimethyl sulfoxide; DTNB, 5,5'-dithiobis(2-nitro benzoic acid); DTT, dithiothreitol; EDC, 1-ethyl-3-(3-dimethyl amino propyl) carbodiimide hydrochloride; EDTA,(ethylene di-nitrilo) tetra acetic acid; Glycine-sigma; sigma subunit with carboxylic amino acid groups modified by EDC plus glycine; ME, 2-mercaptoethanol; NEM, N-ethyl-maleimide; PCMB, p-chloro mercuribenzoate; PG-sigma, sigma with arginyl groups modified by phenylglyoxal; PMSF, phenyl methane sulfonyl fluoride; SAP, *Staphylococcus aureus* V8 protease; SH-sigma, sigma with sulfhydryl groups modified by NEM; TCA, trichloro-acetic acid; TEMED, N,N,N',N'-tetramethylethylene diamine; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNP-, trinitro-phenyl-; WSC, water soluble carbodiimide.

INTRODUCTION

The process of transcription plays an important role in gene expression. In replication all of the DNA is duplicated whereas in transcription there is asymmetric template strand selection. The process of transcription also recognizes the signals from the environment that regulate gene expression. Only portions of DNA are transcribed because of the presence of 'start' and 'stop' signals on DNA. The DNA dependent RNA polymerase (nucleoside triphosphate: RNA nucleotidyltransferase, EC 2.7.7.6) along with various regulatory factors is responsible for the execution of these complex events. This research attempts to study the function and properties of one of the regulatory proteins, the sigma subunit of the E.coli RNA polymerase, in greater detail using chemical modification as a probe.

The importance of RNA in the central dogma of molecular genetics was very obvious in the late 1950's. Several investigators were able to demonstrate, using mammalian or bacterial cell free extracts, the incorporation of radiolabeled nucleotides into an acid insoluble form, an 'RNA like material'. The nucleotide incorporation was into a pre-existing RNA and DNA dependence was not demonstrated. Also the requirement for all 4 ribonucleoside triphosphates was not satisfied. However, in 1960 three investigators independently demonstrated the incorporation of ribonucleoside triphosphates into an acid insoluble form using cell free extracts of E.coli (Hurwitz et al, 1960 and Stevens, 1960) or nuclear extracts of rat liver (Weiss, 1960). The sensitivity of the polymerizing reaction

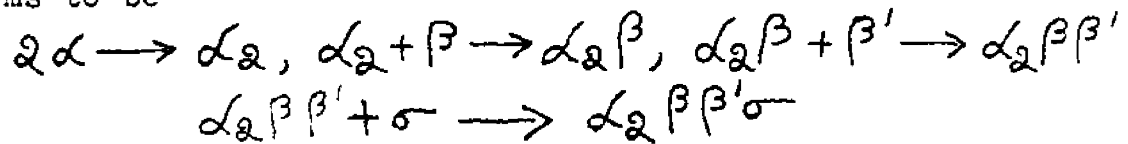
to DNase treatment and the stringent requirement for all 4 ribonucleoside triphosphates indicated that the enzyme was a DNA dependent RNA polymerase. Since then the rapid progress of research in this field has made possible the understanding of the structure and function of this enzyme.

STRUCTURE OF RNA POLYMERASE.

RNA polymerase is a complex enzyme in terms of both structure and function. Of the RNA polymerases from different sources the one from E.coli has been most extensively studied. It is a multi subunit enzyme with an approximate molecular weight of 450,000 and contains 2 Zn^{++} atoms per molecule of enzyme. The enzyme can be isolated in 2 forms; holoenzyme with a subunit composition of β'(160,000), β (150,543), sigma (70,263) and α(36,511) in a molar ratio of 1:1:1:2 and core polymerase which lacks the sigma subunit. The Zn^{++}atoms can be removed from the polymerase by treating the enzyme with 7M urea. The amount of Zn^{++} retained by the individual subunits when dialyzed against Zn^{++} solution (followed by dialysis against EDTA to remove free Zn^{++}) has been determined by atomic absorption spectrophotometry (Wu et al., 1977). The results suggest that 1 atom of Zn^{++} is bound to β' and the other to either β' or β or on the contact region of $\beta\beta'$. Zn^{++} can be replaced by Co^{++} by growing cells in Zn-depleted, Co-enriched medium (Speckhard et al., 1977). The Zn^{++} chelator 1,10-phenanthroline was found to inhibit initiation (McClure et al., 1978).

Several subunit complexes have been found. Free α subunit exists as dimer in solution and β' exists as an oligomer in the absence of urea and is less stable than other subunits (Lill and Hartmann, 1975 and Palm et al., 1975). Mixing β and α subunits gives rise to the complex $\beta\alpha_2$. Such a complex has also been obtained from the flow-through of the phosphocellulose chromatography of RNA polymerase. The fact that this complex can be obtained in the supernatant after sedimenting RNA polymerase in presence of DNA suggests that the complex does not bind DNA. It does bind rifampicin (Zillig et al., 1971). The complex is also indicative of the presence of at least one binding site for α on β subunit. No $\beta\alpha_2\sigma$ complex has been observed. Although $\beta'\sigma$ complex has not been observed in E.coli RNA polymerase subunits, the β' subunit is thought to be involved in sigma binding to core polymerase. Studies using proteolytic digestion as a probe suggest that sigma interacts with β. Evidence for the existence of other complexes such as $\beta\alpha$, $\beta\sigma$, $\beta\beta'$, $\beta'\alpha$ and $\alpha\sigma$ is not convincing. The existence of such intermediate complexes of subunits is not unique to RNA polymerase. Other multi subunit enzymes such as tryptophan synthetase, hemoglobin and aspartate transcarbamylase also show intermediate subunit complexes (Friedman and Beychok, 1979). The sequence of the assembly of the individual subunits to form RNA polymerase has been determined by manipulating the reconstitution conditions. When reconstitution is carried out at 0°C the predominant product seems to be the complex $\beta\alpha_2$ (Zillig et al., 1976).

The subunit complex $\beta\alpha_2$ also accumulates when cyanate treated subunits are used presumably due to the inactivation of β' subunit (Ito and Ishihama, 1973). Core polymerase can be obtained by using unmodified β' subunit. Since DTT prevents the inactivation of β' subunit by cyanate, the carbamylation was suggested as sulfhydryl group specific. Thus the sulfhydryl groups of β' subunit have been implicated in the subunit interaction. The sequence of the subunit assembly seems to be



$$2\alpha \longrightarrow \alpha_2,\ \alpha_2 + \beta \rightarrow \alpha_2\beta,\ \alpha_2\beta + \beta' \rightarrow \alpha_2\beta\beta'$$
$$\alpha_2\beta\beta' + \sigma \longrightarrow \alpha_2\beta\beta'\sigma$$

The core polymerase obtained by reconstitution at 4°C is not active although it has DNA, rifampicin and ATP binding ability. The inactive polymerase can be converted to the active form just by incubation at 37°C for 1 hour (Friedman and Beychok, 1979). A temperature dependent conformational change has been implicated in this activation step. Although the sigma subunit is not a part of core polymerase, the core polymerase reconstituted in presence of sigma is more active as demonstrated by using assays that are not sensitive to sigma (Ishihama et al., 1973).

The quaternary structure of the E.coli RNA polymerase has been studied using cross linking bifunctional reagents or protease digestion. The studies using the latter probe (Lill and Hartmann, 1975) indicate that the presence of sigma subunit protects the β and β' subunits against protease digestion. These subunits are also less susceptible to proteolysis in oligomeric than in monomeric polymerase. No

effect of polymerase aggregation on the cleavage of α or sigma subunits has been observed. Based on these studies it is proposed that the sigma subunit is in close contact with $\beta\beta'$ than with α and $\beta\beta'$ of one polymerase monomer is in contact with those of the other in the dimeric polymerase. Bifunctional crosslinking reagents such as N,N'-bis(2-carboximidoethyl)tartaramide dimethyl ester dihydrochloride or methyl 4-mercaptobutyrimidate crosslink subunits. The products have been analyzed by two dimensional SDS poly-acrylamide gel electrophoresis where the second dimension is performed following periodate oxidation to cleave the linkage. The following complexes have been identified: $\alpha\beta$, $\alpha\beta'$, $\sigma\beta$, $\sigma\beta'$, $\beta\beta'$, $\alpha\sigma\beta$, $\alpha\sigma\beta'$, $\sigma\beta\beta'$ (Coggins et al., 1977); α_2, $\alpha\beta$, $\alpha\beta'$, $\alpha\sigma$, $\beta\sigma$, $\beta'\sigma$, $\beta\beta'$ (Hillel and Wu, 1977). Since no α_2 or $\alpha\sigma$ complexes were detected Coggins et al., suggest that the 4 subunits $\alpha_2\beta\beta'$ are arranged in a linear fashion - $\alpha\beta\beta'\alpha$ with the sigma subunit contacting the $\beta\beta'$ subunits. Hillel and Wu suggest that 2 α and $\beta\beta'$ subunits are in contact with each other and the sigma subunit in contact with the $\beta\beta'$ and one of the α subunits.

Using small angle neutron scattering a three dimensional triangular model for core polymerase has been proposed. The sigma subunit in the holoenzyme was placed such that the subunits are in contact with each other (Stockel et al., 1980).

Depurinated DNA can be crosslinked to the ϵ -amino groups of lysine of RNA polymerase. Following the separation of the

subunits crosslinked to DNA the points of contacts between the subunits and the DNA can be determined. Such experiments have revealed a three dimensional model similar to the triangular model (Chenchick et al., 1981).

MECHANISM OF ACTION OF RNA POLYMERASE.

Promoters are the regions of DNA where RNA polymerase initiates RNA synthesis. RNA polymerase binds to the promoters through an extensive and complex process of site selection. The binary complex (closed complex) undergoes an isomerization resulting in the local melting of the promoter (open complex). This leads to the easy access of the nucleotide substrates to base pair with the codogenic DNA strand bases. Substrate nucleotides complementary to DNA strand bind to the enzyme. RNA chain synthesis is initiated with the formation of a phosphodiester bond between the initiation nucleoside triphosphate (usually a purine nucleoside triphosphate which forms the 5' terminus of the RNA chain) and the elongation nucleoside triphosphate. Elongation of the RNA chain is achieved by the successive formation of phosphodiester bonds between the 3' OH of the growing RNA chain and the elongation nucleoside triphosphates complementary to the bases on the codogenic DNA strand. RNA chain termination is accomplished by the interaction of polymerase with specific termination sequences on DNA either directly or with the aid of termination factors such as rho or nusA gene protein. For the sake of simplicity the process of transcription can be divided into

4 steps; 1) template and promoter binding, 2) RNA chain initiation, 3) RNA chain elongation and 4) RNA chain termination and enzyme release. While the core polymerase is the minimum active form of the enzyme involved in all of the above events the presence of sigma subunit has a remarkable stimulatory effect on the first two events by the core polymerase. Since sigma subunit is released shortly after the initiation (Krakow and von der Helm, 1971; Hansen and McClure, 1980 and DeRiemer and Meares, 1981) it is not involved in elongation or termination. Sigma release seems to be a necessary prerequisite for efficient termination because the binding sites on core polymerase for sigma and nusA gene protein, a protein involved in regulating termination of transcription in vivo, seem to overlap (Greenblatt and Li, 1981).

FUNCTIONAL ROLES OF THE SUBUNITS.

The studies involving RNA polymerase from bacterial mutants have been of immense help in determining the function of the individual subunits. The defect in a mutant RNA polymerase that shows impaired DNA binding property can be traced to the subunit β' (Gross et al., 1976) suggesting that the β' subunit is involved in the template binding function of RNA polymerase. Isolated β' subunit has been found to bind to DNA (Zillig et al., 1976). The catalytic center may be located in β subunit. Evidence for this comes from reconstitution experiments (Rabussay and Zillig, 1969). Mixed reconstitution of polymerase subunits from wild type

and mutants indicates that the resistance of mutants to antibiotics such as rifampicin and streptolydigin that inhibit initiation and elongation can be traced to the β subunit. The function of the α subunit is not clear. Studying the functional role of sigma subunit has been relatively easy due to the ease with which core polymerase and sigma can be reconstituted and also that RNA polymerase can be assayed with (holoenzyme) or without (core polymerase) sigma subunit. Experimental evidence strongly suggests the involvement of the sigma subunit in promoter recognition and RNA chain initiation.

STUDIES ON THE STRUCTURAL GENES OF RNA POLYMERASE SUBUNITS.

Using mutants resistant to antibiotics such as rifampicin, the structural gene for the β subunit has been located at 89 minutes on the *E.coli* genetic map closely linked to the β' subunit gene (Kirschbaum and Scaife, 1974). The gene for α has been mapped at 72 minutes along with the ribosomal protein genes (Jaskunas et al., 1975). The gene for the sigma subunit has been mapped at 66 minutes, close to the dnaG gene. *Salmonella typhymurium* containing the F' plasmid carrying the dnaG gene segment of *E.coli* produces *E.coli* sigma subunit in addition to the host sigma subunit (Nakamura et al., 1977). The genes for α, β, β' and sigma subunits are named respectively rpoA, rpoB, rpoC and rpoD. Since mapping the sigma subunit gene, several temperature sensitive mutants affecting the gene have been studied. This has formed another approach to study the properties of sigma. In general these mutants behave normally at low temperatures but do not survive at

higher temperatures. Sigma subunits from these mutants are thermolabile; they all lose activity at higher temperatures (42 to 52°C). Some of the sigma subunits exhibit different salt sensitivity. rpoD2 is more sensitive to inactivation by salt (Travers et al., 1978). Optimal RNA synthesis occurs at slightly higher ionic strength for some mutant sigmas compared to the wild type sigma. rpoD2 has an altered chemical property; the tryptic digest has an extra lysine peptide (Burgess et al., 1979). This mutation also seems to result in the transcription of lac mRNA in the absence of cyclic AMP and catabolite activator protein more efficiently than the wild type sigma (Travers et al., 1978). Another mutant sigma shows lower mobility than the wild type sigma in SDS polyacrylamide gel electrophoresis (Harris et al., 1978). rpoD800 in addition to exhibiting thermolability, salt sensitivity and an altered chemical property seems to be more acidic (Burgess et al., 1979). The inactivation at high temperature seems to be due to the aggregation of rpoD800 sigma to at least hexamers (Lowe et al., 1981). In the case of rpoD40, an amber mutation, at higher temperatures sigma synthesis is reduced with the appearance of an amber fragment (Osawa and Yura, 1980). The rpoD gene wild type has been cloned and sequenced recently (Burton et al., 1981). Sequencing the mutant genes could lead to additional valuable information on sigma subunit.

OTHER SIGMA-LIKE SUBUNITS.

It is pertinent to mention here that several sigma-like

polypeptides have been found to be involved in the sequential gene expression in the sporulating B.subtilis and phage infected E.coli and B.subtilis. Some of these sigma subunits are needed to transcribe the genes for other sigma subunits which are in turn needed to express the genes of yet other sigma subunits. These sigma subunits have been termed 'cascades of sigma factors' (Losick and Pero, 1981). T4, T5, T7 of E.coli and SPO1, SPO82 of B.subtilis have been studied in detail. T4 infection of E.coli induces the synthesis of proteins P10, P12, P15 and P22 (the numbers refer to the approximate molecular weight in thousands). P10 is involved in the inhibition of the host sigma subunit (Stevens, 1976). The other three proteins are involved in the expression of late T4 genes (Wu et al., 1975a). In the case of T5 E.coli phage, peptides P11, P15 and P95 are synthesized soon after the infection which are involved in the regulation of T5 gene expression (Szabo et al., 1975). In the formation of SPO1 virions in B.subtilis, soon after infection the early phage genes are expressed leading to the synthesis of P26. This protein replaces the host sigma from the polymerase and confers on the enzyme the ability to express the middle genes. The products of the middle genes namely P13 and P24 turn on the expression of the late genes (Losick and Pero, 1981). In sporulating cells, the sporulation specific genes are transcribed only by a sigma-like peptide, sigma 37, which is synthesized at the onset of sporulation. Late in sporulation, sigma 37 is replaced by sigma 29 (Losick and

Pero, 1981). A model has been proposed to explain the action of these promoter specific sigma-like subunits.

The effects of sigma on promoter recognition and initiation will be discussed in detail.

PROMOTER RECOGNITION.

The effect of the sigma subunit on core polymerase binding to DNA has been investigated by variety of techniques. This is complex to study due to various factors such as the complexity of the ligand (RNA polymerase), availability of numerous sites on DNA for the ligand and discriminatory behavior in binding of the two types of the ligand (core polymerase and holoenzyme) towards different binding sites. Various techniques employed to study the kinetics of interaction of RNA polymerase with DNA include sedimentation velocity (von Hippel et al.,1974), nitrocellulose filter binding assays (Hinkle and Chamberlin, 1972), difference boundary sedimentation velocity (Lohman et al., 1980), DNA cellulose chromatography (deHaseth et al., 1978), electron microscopy (Kadesch et al., 1980) and single step initiation assay (Hansen and McClure, 1980).

In general, the binding of RNA polymerase to DNA can be represented by 2 systems; $R + D \rightleftharpoons RD$ and $R + P \rightleftharpoons RP$ where R is the polymerase in molar concentration and P and D are respectively molar concentration of promoter and non-specific sites(will be refered to as DNA). The molar concentration of DNA in terms of binding sites, equal to the molar

concentration of nucleotides, remains practically unchanged after complex formation and hence the reactions follow pseudo first order kinetics. The apparent second order rate constant, k2, for DNA-RNA polymerase (core polymerase or holoenzyme) association has been estimated as 10^8 M^{-1} S^{-1} (Hinkle and Chamberlin, 1972). Knowing the dissociation constant, kd, of complexes: holoenzyme-DNA, holoenzyme-promoter or core polymerase-DNA (or promoter) the respective binding constants, K, can be calculated by using the relationship:

$$K(M^{-1}) = \frac{k2\ (M^{-1})\ (S^{-1})}{kd\ (S^{-1})}$$

The dissociation constant for a particular complex is estimated by taking the reciprocal of the half time of the release of the enzyme from the binary complex. The half time for dissociation of polymerase from the binary complex can be measured by competing the polymerase bound to labeled or unlabeled T7 DNA with respectively unlabeled or labeled T7 DNA. Based on these studies the binding constants have been estimated as 10^{11} M^{-1} for core polymerase-DNA complex, 10^8 M^{-1} for holoenzyme-DNA complex and 10^{12} to 10^{14} M^{-1} for holoenzyme-promoter complex (Hinkle and Chamberlin, 1972a).

The nonspecific interactions of core polymerase or holoenzyme with DNA have also been investigated by deHaseth et al., (1978) using DNA cellulose chromatography where the polymerase bound to DNA cellulose is eluted and the slope of the elution

profile is related to the binding constant. The binding constants reported were 10^{10} M^{-1} for core polymerase-DNA complex and 10^{6} M^{-1} for holoenzyme-DNA complex. Using a difference boundary sedimentation technique similar values have been obtained for nonspecific binding using a formula

$$K = v/L$$

where v is the binding density in moles of polymerase bound per mole of nucleotide DNA and related to the sedimentation coefficient ratio obtained experimentally and L is the moles of free polymerase. In the above experiments the calf thymus DNA used is not entirely free of nicks and the binding behavior of the polymerase is very different towards the nicked DNA. More accurate estimates of the binding constants have been made using restriction endonuclease fragments containing promoters of phage DNAs where the nonspecific binding sites are relatively minimal or fragments containing no promoter to minimize the promoter binding effects in studying nonspecific binding. By employing probability statistics and filter binding assays, Strauss et al., (1980) have determined the binding constant for the T7 A1 and D promoter-containing fragments to be 3×10^{9} M^{-1}. The binding density for a fragment containing the A1 and A3 promoters of T7 DNA was measured using electron microscopy and the binding constant thus obtained ranges between 10^{8} and 10^{9} M^{-1} (Kadesch et al., 1980). Similar studies on a fragment containing no major promoters of T7 DNA give a binding constant of 10^{4} M^{-1}.

In studies involving the filter binding assay combined with gel electrophoresis, binding constants of 10^8 to 10^{11} M^{-1} have been obtained for fd RF DNA (Seeburg et al., 1977) or T5 DNA (von Gabain and Bujard, 1979) promoters.

Despite the discrepancy in the values for the binding constants, the binding constant for holoenzyme-promoter complex is several orders of magnitude greater than the binding constant for core polymerase-DNA complex and the binding constant for holoenzyme-DNA complex is several orders of magnitude less than that for the core polymerase-DNA complex. Thus the effect of sigma in holoenzyme seems to be to increase the affinity for promoter sites and decrease the affinity for nonspecific DNA sites.

The holoenzyme-promoter complex is more sensitive to temperature than the core polymerase-DNA complex and depending on the temperature and ionic strength two types of holoenzyme-promoter complexes can be obtained. At high ionic strength (u = 0.2) and low temperature (0°C) stable complexes are formed as demonstrated by electron microscopy which are unable to initiate synthesis of RNA. The stability of the complex increases progressively with increasing temperature and at low ionic strength (u less than 0.1). These results have been interpreted as due to the existence of two types of holoenzyme-promoter complex; closed promoter complex with relatively low binding constant and not primed for initiation which undergoes a temperature dependent isomerization that involves a local unwinding of the DNA to form open promoter

complex (Chamberlin, 1976). This can be represented by the following scheme:

$$R + P \underset{k_{-1}}{\overset{k1}{\rightleftharpoons}} RPc \underset{k_{-2}}{\overset{k2}{\rightleftharpoons}} RPo$$

where R is RNA polymerase, P is promoter, RPc is closed promoter complex, RPo is open promoter complex and k's are the respective reaction rate constants.

The binding constant for holoenzyme promoter interaction can be resolved into the components contributed due to the closed promoter complex formation and the isomerization steps. The overall binding constant for open promoter complex formation, K, is the product of the bimolecular association constant, K_I, and the equilibrium constant for isomerization, K_{II}. These constants have been evaluated for individual promoters using abortive initiation reactions with initiation nucleoside monophosphates (GMP or AMP) and the elongation nucleoside triphosphate complementary to the promoter sequence (McClure, 1980 and Hawley and McClure, 1980). A lag in the approach to the steady-state rate of abortive initiation can be seen if the reaction is initiated by adding the enzyme + nucleotide mix to DNA as opposed to adding nucleotide mix to enzyme + DNA. The product of the intercept and the reciprocal slope of the lag - 1/enzyme plot gives K_I. In the presence of heparin or $d(A\text{-}T)_n$ the decay in the open complex with time can be measured using the abortive initiation assay (Cech and McClure, 1980). The dissociation

constant thus obtained and the intercept of the lag - 1/enzyme plot have been related to K_{II}. The overall binding constant for a lambda promoter, P_R, is about 2×10^{11} M^{-1}. The contribution due to binary complex formation is about 8×10^8 M^{-1} and the isomerization equilibrium constant is 3×10^2 (Hawley and McClure, 1980). A comparison of such values for the A2 and D promoters of T7 DNA indicates that the weak promoter, D, has a lower association constant for closed complex formation than the strong promoter, A2. But the rate of isomerization for D is 3 times that of the A2 promoter (McClure, 1980). The DNA sequence seems to be an important factor in determining the extent of binding and the isomerization steps.

These kinetic studies have helped develop a model for promoter recognition by RNA polymerase in which the polymerase diffuses through the general sites by a series of associations and dissociations till it comes across the promoter. This is in accordance with the theoretical considerations; taking T7 DNA as an example it has about 37,000 base pairs which make 74,000 possible general sites for RNA polymerase. A binding constant of 2×10^4 M^{-1} for holoenzyme DNA interaction and a second order rate constant of 10^8 M^{-1} S^{-1} would give a dissociation constant of 10^4 S^{-1} with a half life of dissociation of 0.1 mS. This would lead to a prediction that the holoenzyme would locate the promoter in well below 30 seconds, the time determined experimentally for promoter location with T7 DNA.

INITIATION.

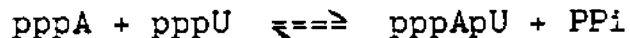
The process of initiation is the formation of the first phosphodiester bond between the initiation nucleoside triphosphate (usually a purine nucleoside triphosphate) which forms the 5' terminus of the RNA chain and the elongation nucleoside triphosphate. During transcription RNA polymerase undergoes a transition from holoenzyme-DNA-open complex to core polymerase-DNA-RNA complex. These complexes have been partially characterized; the former complex is promoter specific whereas in the core polymerase complex interactions between core polymerase and DNA are non-specific and core polymerase protects about 12 nucleotides of nascent RNA chain against RNAse digestion (Kumar and Krakow, 1975). During the transition sigma subunit is released. The release of sigma facilitates the strong interaction between RNA polymerase and DNA which is needed for efficient transcription and also the interaction of a transcription termination regulatory protein (nusA gene protein) with core polymerase (Greenblatt and Li, 1981). Recently it has been proposed that initiation involves all the steps upto and including sigma release (Hansen and McClure, 1980). Several methods have been used to demonstrate sigma release. They include gel electrophoresis under native conditions (Krakow and von der Helm, 1971), sedimentation analysis (Gerard et al., 1972), Fluorescence spectroscopy (Wu et al., 1975), abortive initiation (Hansen and McClure, 1980) and photoaffinity labelling (DeRiemer and Meares, 1981).

Krakow and von der Helm and Hansen and McClure have shown, using $d(A-T)_n$ or lambda promoter P_R, as template, that sigma is released when 8 to 10 nucleotides have been incorporated. Using photoaffinity labelling the sigma subunit can be labelled when the first 4 nucleotides have been incorporated. It is possible that after 4 nucleotides have been incorporated sigma is not released but may not be accessible for labelling. The polymerase has a broad specificity for initiation nucleotide in the sense that purine nucleoside mono or diphosphates or dinucleotides can be used in vitro for initiation whereas only nucleoside triphosphates (as Mg^{++} or Mn^{++} chelate) are used for elongation.

In the presence of sigma initiation using natural templates is restricted to the sites on DNA called initiation points located about 10 bases downstream of the heptablock in the promoters (Gilbert, 1976). Core polymerase can also initiate as efficiently as holoenzyme by employing the 3' OH of the nicked regions of DNA as primer (Hinkle et al., 1972). However, the presence of sigma lowers such nonspecific initiation by core polymerase. Also core polymerase seems to prefer initiation with GTP and the presence of sigma abolishes this discrimination (Hoffman and Niyogi, 1973).

Several assays have been developed for initiation that use natural or synthetic DNA template. Using a natural template such as T7 DNA, initiation can be studied by single round transcription (Chamberlin et al., 1979) or abortive initiation (Johnston and McClure, 1976). The incorporation of

γ(^{32}P)ATP or GTP in a RNA polymerizing reaction can be taken as a direct measure of initiation. Transcription can be restricted to one round by employing a template competitor such as heparin. In abortive initiation, by employing one of the initiation nucleoside triphosphates (ATP or GTP) and UTP or CTP elongation can be prevented because for continued elongation all 4 nucleoside triphosphates are necessary. Dinucleoside tetraphosphates are the predominant product formed although tri and tetra nucleotides are also formed depending on the template sequence. Many assays have been developed using synthetic DNAs such as $d(A-T)_n$ where the polymerase does not go through extensive site selection process. These include pyrophosphate exchange assay (Krakow and Fronk, 1969) and abortive initiation assay (Hansen and McClure, 1979). The initiation reaction



$$pppA + pppU \rightleftharpoons pppApU + PPi$$

directed by $d(A-T)_n$ can be reversed in the presence of excess of PPi and by employing (^{32}P)PPi the label can be incorporated predominantly into UTP. By using charcoal binding where only nucleotides (but not PPi) are adsorbed to the charcoal the amount of (^{32}P)UTP formed can be measured and used as an assay. By using AMP and UTP in the $d(A-T)_n$ directed assay the synthesis can be restricted to pApU because AMP cannot serve as a substrate for elongation. The product can be separated from the substrate by paper chromatography and the quantity of pApU formed is a measure of initiation. All these

assays are stimulated by addition of sigma.

It is obvious that distinct structural domains or sites should exist on RNA polymerase to facilitate the interaction of polymerase with substrates, product and template. Four sites have been proposed (Krakow et al., 1976).

1) DNA binding site.
2) Product site, to which a portion of the growing RNA chain is associated in the ternary complex.
3) Elongation nucleotide site, to which nucleoside triphosphate as the Mg^{++} (or Mn^{++}) chelate is bound.
4) Initiation nucleotide site or 3' OH binding site which accomodates the initiation nucleotide or the 3' OH end of the growing RNA chain.

Dissociation constants of nucleotides for the elongation and initiation sites have been determined in the absence of template using techniques such as equilibrium dialysis (Wu and Goldthwait, 1969) and fluorescence quenching (Wu and Goldthwait, 1969). For ATP or GTP, the Kd value for the initiation site is about 150 uM and the Kd value for the elongation site with Mg-ATP or Mg-GTP is 15 uM. The values determined using abortive initiation reaction are 1800 and 30 uM for the initiation and elongation sites respectively (McClure et al., 1978). The order of binding of the nucleotides is not clear. According to rifampicin challenge experiments it seems to be elongation nucleotide followed by initiation nucleotide. Based on abortive initiation the order is reverse (Rhodes and Chamberlin, 1975 and McClure et al.,

1978). It has also been suggested that the weakly bound initiation nucleotide is trapped by the elongation nucleotide. The apparent Michaelis constant, K, for UTP varies depending on the type of polymerase (core polymerase or holoenzyme) used. In the $d(A-T)_n$ directed assay K_{UTP} is 70 uM for holoenzyme and 4 mM for core polymerase (Hansen and McClure, 1979 and 1980). K initiation nucleotide varies depending on the nucleotides; K_{AMP} = 2.5 mM, K_{ADP} = 0.7 mM and K_{ATP} = 0.5 mM (McClure et al., 1978 and Hansen and McClure, 1979). Thus the effect of sigma seems to decrease K_{UTP} by 60 fold. Although $d(A-T)_n$ has no promoters the binding constant for core polymerase is about 10^5 M^{-1} and that of holoenzyme is 10^9 M^{-1}. However, core polymerase does form the open complex with $d(A-T)_n$ and is able to initiate (Hansen and McClure, 1980). The marked difference in the pApU synthesis between core polymerase and holoenzyme (Hansen and McClure, 1979) seems to be due to the decrease in K_{UTP} by 60 fold due to the presence of sigma. A possible effect of sigma on initiation site of RNA polymerase is also implicated (Kumar and Krakow, 1976 and Hoffman and Niyogi, 1973).

The effect of sigma subunit on transcription can be summarized as follows:

1) The binding of the polymerase to the general DNA sites is markedly reduced and the binding to the promoter sites is markedly increased.
2) The rate of chain initiation is increased.
3) The initiation at nonspecific sites such as nicks is

markedly reduced.

These effects have been suggested as due to a possible interaction of sigma subunit with DNA directly or core polymerase. The former possibility is considered based on the following observations: The polymerase-promoter complex obtained after digesting the remainder of the DNA by DNAse is able to initiate and transcribe a short RNA chain but the free DNA fragment (about 40 base pairs in length) does not productively rebind to the enzyme. This suggests that DNA regions in addition to the 40 base pairs surrounding the initiation point are necessary for the polymerase binding. These regions are called recognition sequences. Based on this a model was proposed (Pribnow, 1975) where the binding of holoenzyme to the recognition site is mediated by sigma subunit through a possible direct interaction with DNA. Many sigma-like proteins that regulate transcription in B.subtilis and its phages could function through direct interaction with DNA (Losick and Pero, 1976). Sequence analysis of various promoters has revealed interesting information. In general, a 6 to 7 nucleotide sequence around 10 and 35 bases upstream from initiation point is conserved in all of the promoters. As discussed earlier several sigma-like proteins of B.subtilis and phage SP01 recognize only certain promoters of the host and phage. The sequence in the '-35' and '-10' regions in these promoters differs from the sequence of the sigma-55 specific promoter. It is proposed that sigma is responsible for promoter recognition by making contacts with critical

nucleotides in the promoters (Losick and Pero, 1981). This is supported by the finding that sigma interacts directly with polynucleotides; it has been reported that E.coli sigma binds to heparin (Sternbach et al., 1975) and supercoiled plasmid DNA (Kudo and Doi, 1981) and B.subtilis sigma binds to poly (C) (Pero et al., 1975).

The conformational change in core polymerase upon association with sigma is well documented. Using low angle X-ray diffraction studies it was determined that the holoenzyme is more compact than the core polymerase (Pilz et al., 1972). The proteolytic pattern of core polymerase and holoenzyme are different (Lill and Hartmann, 1975 and Fisher and Blumenthal, 1980). The aggregation of core polymerase at low ionic strength is reduced from hexamer to monomer or dimer in presence of sigma (Berg and Chamberlin, 1970). There are also differences in the interaction with DNA between core polymerase and holoenzyme as shown by various techniques (Krakow et al., 1976). The number of ions released during core polymerase-DNA interaction is twice the number observed for the holoenzyme-DNA interaction suggesting that more sites are available for DNA interaction on core polymerase than on holoenzyme (deHaseth et al., 1978). The sigmoidal relationship between the rate of abortive initiation directed by $d(A-T)_n$ and core polymerase concentration has been interpreted as core polymerase being active as a dimer as opposed to active holoenzyme monomers (Hansen and McClure, 1980). The ion release data seem to support this finding.

With appropriate templates core polymerase prefers to start initiation with GTP and this can be switched to ATP upon addition of sigma subunit (Hoffman and Niyogi, 1973) and this has been suggested as due to the difference in conformation between core polymerase and holoenzyme.

Any interrelationship of these effects of sigma subunit is not known. For example, it is not known to what extent the promoter recognizing effect of sigma and the lowering of affinity for nonspecific site are interrelated. Also not known is the extent of interrelationship between the effects on initiation at the promoter sites and at the single strand nicks. This research was undertaken to attempt to unravel these different effects of sigma subunit.

Two probes were used in the study - chemical modification and limited proteolytic digestion. Peptide mapping with respect to the activity of sigma subunit was attempted. Although stable fragments were obtained by limited proteolytic digestion (using trypsin, SAP, chymotrypsin or subtilisin) of sigma under native conditions, purification of these stable fragments for detailed studies was very difficult mainly due to the difficulty in preparing adequate amounts of sigma.

Chemical modification of proteins has been extensively used as a probe in studying function-structure relationships. Of the methods available affinity labelling and modification of amino acid side groups using specific reagents have been of help in understanding the structure and function of RNA polymerase. The four binding sites on RNA polymerase namely

the template, the initiation and elongation nucleotide and the product binding sites could be considered as active centers of the enzyme. In addition, the enzyme also has a rifampicin binding site (Krakow et al., 1976). These sites interact with the ligands to form noncovalent complexes. These sites can be labelled with chemical reagents or with labelled natural ligands. An alkylating derivative of rifamycin SV, 3-(2-bromoacetamidoethyl)-thio-rifamycin SV, modifies β and sigma subunits (Stender et al., 1975). A fluorescent derivative of 6-methyl thioinosinedicarboxaldehyde inhibits polymerase by modifying a lysyl group in the β subunit (Nixon et al., 1972). The reagent 5-formyl uridine 5' triphosphate reacts with amino groups to form a Schiff base which can be reduced to a covalent complex. The label was found associated with β and β' subunits (Armstrong et al., 1979). These studies strongly suggest the involvement of the β subunit in catalytic functions of the enzyme. RNA polymerase also binds poly-deoxy-4-thiothymidylic acid. Upon uv irradiation covalent complexes are formed between this poly nucleotide and predominantly the β' subunit (Frischauf and Scheit, 1973). Photocrosslinking studies of T7 DNA-holoenzyme complex at 254 nm have revealed the following information: α subunits are not involved in DNA-enzyme interactions. Subunits β', β and sigma are labelled in the non-specific interactions and subunits β and sigma are labelled in promoter specific and initiation complexes suggesting that sigma participates directly in promoter recognition (Hillel

and Wu, 1978). Studies using group specific reagents suggest that arginine and tryptophan residues are critical for the activity of the enzyme (Wasylyk and Malcolm, 1975 and Armstrong et al., 1976a). Cysteinyl groups have been implicated in promoter binding and recognition (Yarbrough and Wu, 1974 and Krakow, 1975), initiation and subunit-subunit interaction (Ito and Ishihama, 1973). The template binding and the catalytic functions also seem to depend on lysyl groups (Krakow, 1973; Armstrong et al., 1974; Bull et al., 1975 and Makoff and Malcolm, 1980). No such studies have been done on sigma subunit.

CHEMICAL MODIFICATION OF SIGMA SUBUNIT.

Lysyl, arginyl, cysteinyl and carboxyl groups of aspartic and glutamic acids of sigma were modified.

Several reagents are availabe for specific modification of lysyl groups. They are TNBS, KCNO, succinic anhydride, acetic anhydride and reductive methylation using HCHO and $NaBH_4$. Acetylation in a microscale requires that acetic anhydride be dissolved in solvents such as dioxane or DMSO (Montelaro and Rueckerts, 1975) and these solvents have side effects on the activity of sigma; dioxane was found to inhibit core polymerase as well as sigma. The reducing agents, $NaBH_4$ or $NaCNBH_3$, also were found to have inhibitory effect on the activity of sigma. For these reasons trinitrophenylation, carbamylation and succinylation of lysyl residues were carried out. TNBS reacts primarily with amine groups at alkaline

pH (1) (the numbers in the parenthesis refer to the reactions in Figure A1). In proteins the epsilon amino group of lysyl residues and the amino terminal form targets for the reaction. TNBS also reacts with sulfhydryl groups but to a lesser extent. At alkaline pH or in the presence of ME the trinitrophenyl derivative of sulfhydryl group is labile.

Cyanate ion reacts with amine to form stable carbamyl derivative of the amine (2). Reaction with sulfhydryl, phenolic, carboxyl and imidazole groups also occurs. However, the adducts are unstable at or above pH 7 and the reaction is easily reversible upon removal of the cyanate ion (Means and Feeney, 1971).

Succinylation is highly specific for amines (3). As seen in the reaction, unlike trinitrophenylation or carbamylation the product of succinylation has a negative charge.

These reactions are specific to unprotonated amine. By manipulating the pH of the reaction the epsilon amino group of lysyl residues can be maintained unprotonated and thus can be modified by the reagents.

Guanidino groups of arginyl residues were modified using phenylglyoxal at pH 8. The derivative contains 2 phenylglyoxal molecules per guanidino group (4).

Cysteinyl groups were modified using PCMB, NEM or DTNB at neutral pH (5 and 6). The reaction with DTNB or PCMB is reversible in the presence of ME. NEM also seems to react with amine, proline and histidine. However, the reaction is very slow at neutral pH (Means and Feeney, 1971).

Water-soluble carbodiimides were used to modify carboxyl groups. Although amines, phenolic and sulfhydryl groups also react, at acidic pH predominantly carboxyl groups react (7). Two reagents were used - CMC and EDC. EDC is relatively less bulky compared to CMC.

The current research attempts to study any possible involvement of lysyl, arginyl, cysteinyl and carboxyl groups in the following properties or effects of the sigma subunit:

1) Reconstitution of sigma with core polymerase.
2) Binding of sigma to core polymerase bound to DNA.
3) Effect on promoter recognition by RNA polymerase.
4) Effect on core polymerase-general DNA site interaction.
5) Effect on binding of core polymerase to single strand nicks.
6) Effect on initiation at promoters.
7) Effect on initiation at single strand nicks.

MATERIALS AND METHODS

I SOURCE OF MATERIALS.

A. General reagents. All chemicals were reagent grade. Tris, ME, mercaptoethylamine, BTP, DMSO, polyethyleneglycol (Mol. Wt. 6000), BSA, lysine and glutathione were purchased from Sigma Chemical Co. DTT was obtained from P-L Biochemicals. Phenol was purchased from Fisher Scientific Co. and redistilled twice prior to use. EDTA was purchased from Baker Chemical Co. Amido Black was a product of Hartman-Leddon. Polyethyleneimine (50%) was obtained from BDH. Brij-58, PMSF and ammonium sulfate were products of Pierce Chemical Co. Bio-Rex 70, Cellex N-1 and charcoal were obtained from Bio-Rad Labs. DEAE-sephacel was obtained from Pharmacia. Ultrogel ACA-34 was a product of LKB. Cesium chloride was purchased from Schwarz/Mann. Yeast extract and tryptic peptone were obtained from US Biochemical Co. Dialysis bags were obtained from Arthur Thomas Co. and boiled twice for 10 minutes in 0.1% sodium carbonate, rinsed in water and stored in 10 mM EDTA. Reactions were performed in disposable polystyrene tubes purchased from Sarstedt. Cellulose nitrate tubes were purchased from Beckman. Liquifluor obtained from New England Nuclear was diluted in toluene.

B. Electrophoresis reagents. Acrylamide, Bis-acrylamide, TEMED, ammonium persulfate and glycine were products of BRL or Bio-Rad Labs. Agarose and low temperature melting agarose were obtained from BRL. Bromphenol blue was obtained from

Allied Chemicals. Coomassie brilliant blue R250 was obtained from Pierce Chemical Co. Ethidium bromide was a product of Sigma Chemical Co. SDS was purchased from BDH or Bio-Rad Labs.

C. Chemical modification reagents. TNBS, DTNB, PCMB and NEM were products of Pierce Chemical Co. Phenylglyoxal was a product of Aldrich. KCNO was obtained from BDH. Succinic anhydride, CMC and EDC were purchased from Sigma Chemical Co.

D. Enzymes. Lysozyme, chymotrypsin, trypsin, pancreatic DNase I and subtilisin were purchased from Sigma Chemical Co. Staphylococcus aureus V8 protease was obtained from Miles. Restriction endonucleases were purchased from BRL.

E. Nucleotides and poly deoxynucleotides. UTP, ATP, CTP, GTP, 5'AMP, ApU, UpA, $d(A-T)_n$, $d(I-C)_n$ were purchased from P-L Biochemicals. ATP and UTP purified by high pressure liquid chromatography were purchased from ICN. Calf thymus DNA was obtained from Sigma Chemical Co.

F. Radioisotope labelled reagents. (^{3}H)UTP, (^{3}H)ATP, (^{3}H)thymidine, (^{3}H)dCTP and (^{3}H)dTTP were obtained from ICN. (^{3}H) $d(I-C)_n$ (2000 cpm/nmol) and (^{3}H) $d(A-T)_n$ (2250 cpm/nmol) were prepared using E.coli DNA polymerase according to the procedure described by Jovin et al. (1969). (^{3}H)TNBS was obtained from NEN or Amersham. (^{32}P) Na pyrophosphate and (^{3}H)NEM were products of NEN. (^{14}C)phenylglyoxal (7000 cpm per nmol) was prepared from (^{14}C)acetophenone (ICN) by selenium oxidation (Riley and Gray, 1943).

G. Bacteria. E.coli K12 cells (half log phase) were purchased from Grain Processing Corporation.

II SOLUTIONS.

A. Stock solutions. Brij-58 (5%) was prepared by dissolving Brij in water at 90°C. Polyethyleneimine (5%) was prepared by dilution in water from a 50% solution and the pH adjusted to 7.6 with HCl. PMSF solution was prepared by dissolving in DMSO and adjusting to 50% DMSO with water. The final concentration of PMSF was 0.04 M.

B. RNA polymerase purification buffers. TGED - 10 mM Tris-HCl (pH 8), 5% (W/V) glycerol, 0.1 mM EDTA, 0.1 mM DTT; KP - 40 mM potassium phosphate (pH 7), 5% glycerol, 10 mM ME; TED - TGED lacking glycerol and storage buffer - 50% glycerol, 10 mM potassium phosphate (pH 7).

C. Gel elctrophoresis buffers. TBE - 80 mM Tris, 80 mM boric acid, 2.5 mM EDTA, final pH 8; Tris-glycine - 30 mM Tris, 192 mM glycine, 0.1% SDS, final pH 8.8.

D. Protease solutions. Stock solutions of proteases were prepared by dissolving 0.5 mg protease in 0.5 ml of 10 mM sodium phosphate (pH 7) at 4°C a few minutes prior to use. Protein content was determined according to Schaffner and Weissmann (1973).

E. T7 DNA binding buffers. Binding buffer A - 10 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 10 mM ME, 1 mM EDTA, 50 mM NaCl, 500 ug/ml BSA; Binding buffer B - 20 mM Tris-HCl (pH 8), 0.1 mM EDTA, 0.1 mM DTT, 10 mM $MgCl_2$, 5% glycerol, 40 mM KCl, 500 ug/ml BSA; Elution buffer - 10 mM Tris-HCl (pH 8), 1 mM EDTA, 0.1% SDS.

F. Restriction endonuclease buffers. HaeIII buffer - 50 mM

Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM DTT, 500 ug/ml BSA, 37°C; HindIII - 20 mM Tris-HCl (pH 7.4), 7 mM $MgCl_2$, 60 mM NaCl, 500 ug/ml BSA, 37°C; BclI - 12 mM Tris-HCl (pH 7.4), 12 mM $MgCl_2$, 12 mM NaCl, 0.5 mM DTT, 50°C. These buffers were prepared at 10 times the required concentration and diluted 1:10. BSA was added separately.

G. Chemical modification reaction solutions. TNBS, KCNO, succinic anhydride, phenylglyoxal and NEM solutions were prepared in water. The latter three solutions were prepared with vigorous vortexing a few minutes prior to use. DTNB solution was prepared in 100 mM potassium phosphate (pH 7). PCMB solution was prepared in water by adjusting the pH to 10 using 0.1 M NaOH. EDC and CMC were prepared as aqueous solutions. The following molar extinction coefficients (M^{-1} cm^{-1}) were used (the absorbance maxima in nm are indicated in the parenthesis): TNBS (340) - 600, NEM (300) - 600, PCMB (232) - 16900, phenylglyoxal (250) - 11300.

H. Miscellaneous buffers. TMS - 10 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 2 mM ME; TE - 10 mM Tris-HCl (pH 8), 1 mM EDTA.

III PREPARATION OF CHROMATOGRAPHIC MATERIALS.

A. DEAE-sephacel and Bio-Rex 70. These were suspended in 0.5 M NaOH for 30 minutes, washed thoroughly with deionized water till the pH was neutral, suspended in 0.5 M HCl and washed with water till the pH was neutral. DEAE-sephacel was suspended in KP buffer and Bio-Rex 70 was suspended in TGED + 0.15 M NaCl.

B. Ultrogel ACA-34. This resin was provided as preswollen beads in 70% ethanol. The resin was thoroughly washed with water and suspended in TGED + 0.5 M NaCl.

C. DNA cellulose. DNA cellulose was prepared according to the procedure described by Alberts and Herrick (1971) using Cellex N-1 and 5 mg/ml calf thymus DNA solution.

IV PREPARATION OF FILTERS.

A. Glass-fiber filters. GF/C glass-fiber filters (Enzo Biochem) were soaked in 10 mM ATP (pH 7) for 20 minutes prior to use.

B. Nitrocellulose filters. The filters (0.45 uM pore size) (Millipore Corp.) were soaked in 0.01 M KOH for 30 minutes, rinsed thoroughly with deionized water and stored in 20 mM Tris-HCl (pH 8), 50 mM KCl.

V RNA POLYMERASE ASSAY.

The assay mix (250 ul) contained 20 ul of 2 mg/ml calf thymus DNA, 10 ul of fractions, 80 mM Tris-HCl (pH 7.6), 20 mM $MgCl_2$, 40 mM mercaptoethylamine, 1 mM each of ATP, CTP, GTP and (^{3}H)UTP (5000 cpm/nmol). The mix was incubated at 37°C for 10 minutes and TCA precipitable radioactivity was collected on a glass-fiber filter, dried and counted in 5 ml Liquifluor-toluene.

VI PURIFICATION OF RNA POLYMERASE.

The method was a modification of the procedure described by Burgess and Jendrisak (1975).

A. Cell lysis. E.coli K12 cells (250 g) were suspended

in 600 ml of 0.2 M NaCl solution containing 12 mM PMSF using a Waring blender. The pH was adjusted to 8 using 1M Tris base. With stirring (using a Sorvall Omnimix with rubber blades) 15 ml of 0.5 M EDTA (pH 7.6) was added. Cell lysis was initiated at 20°C by adding 10 ml of a freshly prepared solution of 2.4% lysozyme in 10 mM Tris-HCl (pH 8). The lysate was stirred for 30 minutes. During the subsequent steps a temperature of 0° to 4°C was maintained. To the lysate was added 1 ml of 1 M ME, 30 ml of 5% Brij-58 and 25 ml of 1M Tris-HCl (pH 8) and stirred for 20 minutes.

B. PEI precipitation. To 6 tubes containing 1 ml each of lysate 100 to 200 ul of 5% polyethyleneimine in 20 ul increments was added, vortexed and centrifuged for 10 minutes at 2000 RPM on a table top centrifuge. The first tube with a clear supernatant was taken as the end point of titration. In general, 120 to 140 ul of polyethyleneimine per ml of lysate was added with slow stirring. The mixture was stirred for 5 minutes with a non-aerating stirrer (Kraft Apparatus Inc) and centrifuged at 12000 RPM for 20 minutes using Beckman J-21 centrifuge and JA 14 rotor. The pellet was used for RNA polymerase extraction.

C. RNA polymerase extraction. The polyethyleneimine pellet was suspended in 1000 ml of TGED + 0.5 M NaCl, stirred with the non-aerating stirrer for 20 minutes and centrifuged at 12000 RPM for 20 minutes. The pellet was suspended in 1000 ml of TGED + 1 M NaCl, stirred and centrifuged as described above. The supernatant was brought to 55% saturated ammonium

sulfate by adding 32.6 g of solid ammonium sulfate per 100 ml of supernatant. The solution was stirred for 30 minutes and centrifuged at 12000 RPM for 30 minutes. The pellet was dissolved in KP buffer and the conductivity was adjusted (using CDM 2d conductivity meter) to less than 9 milli MHO by dilution with KP buffer.

D. DEAE-sephacel chromatography. The protein solution in KP buffer was loaded on a DEAE-sephacel column (5.5 cm x 30 cm) at a flow rate of 60 ml/hour. The column was washed with KP buffer till the A_{280} was zero. The column was eluted with 1200 ml of linear gradient of KP buffer and KP buffer containing 0.7 M KCl and 0.06 M K_2HPO_4. Fractions of 15 ml were collected and assayed for RNA polymerase activity.

E. DNA cellulose chromatography. The active fractions from the DEAE chromatography were pooled and diluted with TGED buffer till the conductivity was less than 5 milli MHO. The solution was loaded on a DNA cellulose column (5.5 cm x 10 cm) at a flow rate of 40 ml/hour, washed with TGED + 0.15 M NaCl and the column was eluted with 1000 ml of linear gradient of TGED + 0.15 M NaCl and TGED + 1 M NaCl. The content of glycerol in the loading, washing and elution steps was maintained at 10% (W/V). Fractions of 15 ml were collected and assayed.

F. Ultrogel ACA-34 chromatography. Active fractions from the DNA cellulose chromatography were pooled, precipitated with ammonium sulfate and centrifuged. The pellet was dissolved in less than 5 ml of TGED + 0.5 M NaCl, layered on

the column (5.5 cm x 60 cm). The column was developed with TGED + 0.5 M NaCl at a flow rate of 30 ml per hour. Purity of the active fractions (10 ml volume) was assessed by SDS polyacrylamide gel electrophoresis. A summary of the purification is given in Table 1.

VII PURIFICATION OF SIGMA SUBUNIT.

The purified polymerase in TGED + 0.1 M NaCl was passed through the Bio-Rex 70 column (3 cm x 25 cm) and the flow through of which through the DEAE column (1.5 cm x 10 cm). The Bio-Rex 70 column was washed with TGED + 0.1 M NaCl and the wash was passed through the DEAE column. The DEAE column was eluted with 200 ml of linear gradient of TED + 0.1 M NaCl and TED + 0.35 M NaCl. Protein containing fractions (8 ml volume) were analyzed by SDS gel electrophoresis. Sigma containing fractions were precipitated with ammonium sulfate. The pellet was dissolved in 0.5 ml of TGED + 0.5 M NaCl and layered on an Ultrogel ACA-34 column (1.5 cm x 60 cm). The column was developed with TGED + 0.5 M NaCl at a flow rate of 5 ml per hour. Fractions (5 ml) were analyzed by electrophoresis. Sigma containing fractions were precipitated with ammonium sulfate. The pellet was dissolved in the storage buffer. The concentration of sigma, in general, was maintained between 5 and 10 mg/ml. Figure 1 shows the electrophoretic pattern of the sigma fractions after DEAE and Ultrogel chromatography.

The specific activity of RNA polymerase ranged from 710 to 820 units per mg. The yield was from 35 to 50% (based on

total activity). The amount of sigma in RNA polymerase (quantitated by scanning the SDS polyacrylamide gel) ranged between 40 to 60%. The values of percentage saturation of sigma were not very different between stationary phase cells and half log cells. Based on these values the percentage yield of pure sigma was 30 to 45%. For example, a preparation of 55 mg of RNA polymerase with 50% sigma saturation gave 2.75 mg of pure sigma which corresponds to a 40% yield.

VIII GROWTH AND PURIFICATION OF BACTERIOPHAGES.

A. Solutions. T-Broth - 1000 ml water, 10 g tryptone, 5 g NaCl, 1 g NH_4Cl, 3 g KH_2PO_4, 30 ml glycerol, 25 mg thymine and 1 ml 1 M $MgSO_4$ (autoclaved separately); CsCl A - 25 g CsCl in 15 ml water; CsCl B - 2 vol of CsCl A + 1 vol of TE; CsCl C - 1 vol of CsCl A + 1 vol of TE; CsCl D - 1 vol of CsCl A + 2 vol TE.

B. Growth. *E.coli* B thy$^-$ was grown at 37°C overnight in T-Broth with aeration. An equal volume of T-Broth was then added to the culture. T5 or T7 phage (10^{10} phage particles per 500 ml culture) was added to the culture and incubation continued with aeration till cell lysis (4 to 6 hours). To the T5 phage culture pancreatic DNase I was added to a final concentration of 0.2 ug/ml and incubated at 37°C for 4 to 5 hours. T7 DNA was labelled *in vivo* with (^3H) thymidine as follows: *E.coli* B thy$^-$ was grown overnight in 200 ml T-Broth. Following dilution with 200 ml T-Broth 1 mCi of (^3H) thymidine was added and incubation continued for 1 hour. The culture was innoculated with 10^{10} phage particles and incubation

continued until lysis.

C. Purification of phage particles. The lysate was centrifuged at 8000 RPM for 10 minutes using Beckman JA-14 rotor. Sodium chloride (12.5 g/500 ml) and polyethylene glycol-6000 (50 g/500 ml) were added to the supernatant and stirred for 30 minutes. The suspension was centrifuged at 10000 RPM for 10 minutes using JA-14 rotor. The pellet was suspended in TE + 1 M NaCl and centrifuged at 10000 RPM for 10 minutes. The supernatant was saved. The pellet was extracted with TE + 1 M NaCl several times. The supernatants were pooled and centrifuged for 30 minutes at 30000 RPM (Beckman Ti 35 rotor). The pellet was suspended in 4 ml of TE buffer and 1 to 2 ml was layered on a 5 ml cellulose nitrate tube containing from bottom to top the following solutions: 0.5 ml CsCl A, 1 ml each of CsCl B, C and D. Following centrifugation at 40000 RPM (Beckman SW 50.1 rotor) for 35 minutes at 25°C, the phage band (usually on the top of solution B) was collected by aspiration with a Pasteur pipet.

IX PURIFICATION OF PHAGE DNA.

The phage solution in CsCl was dialyzed against TE buffer for 3 to 4 hours and extracted with TE buffer saturated with phenol. To avoid DNA shearing the extraction was carried out in a test tube using a mechanical roller at 60 revolutions per minute for 1 hour. The tube was chilled in an ice bucket for 5 minutes and centrifuged at 2000 RPM for 10 minutes using a table top centrifuge. The aqueous phase was dialyzed

against TE buffer over two nights with two buffer changes. The final buffer contained 0.1 mM EDTA instead of 1 mM EDTA. DNA was stored at $0^{o}C$ after adding a drop of chloroform. DNA solutions thus obtained had a 260:280 ratio between 1.8 and 2. DNA content was expressed as equivalents of nucleotide phosphate using a value of 6750 as molar extinction coefficient at 260 nm. The specific activity of the (^{3}H) T7 DNA was 5000 to 8000 cpm/nmol.

X PURIFICATION OF RESTRICTION ENDONUCLEASE FRAGMENTS.

Phage DNA was digested with restriction endonuclease. One unit of enzyme per ug of DNA was used. Half of the total enzyme required was added. Following incubation for 4 to 5 hours the other half was added and incubation continued for another 4 hours. Electrophoretic analysis of the digest on agarose gels showed that the digestion was complete under these conditions. The fragments were separated by electrophoresis on 1% low temperature melting agarose gel and isolated from the gel as described by Weislander (1981). The fragments were found to be homogeneously pure as judged by agarose gel electrophoresis (Figure A2).

XI GEL ELECTROPHORESIS.

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) using Tris-glycine buffer system. Running gels (7.5% polyacrylamide concentration) with the dimensions 14.5 x 9.5 x 0.15 cm containing a 2 cm stacking gel (4% polyacrylamide) were run at 25 mamps

through the stacking gel and 30 mamps through the running gel. When the tracking dye was 0.5 to 1 cm from the bottom the gels were removed and stained in 0.2% coomassie brilliant blue in 50% methanol and 10% acetic acid for 30 minutes at 60°C and destained in 10% 2-propanol and 10% acetic acid. Slab agarose gel electrophoresis was performed in a horizontal apparatus according to the method described by McDonell et al. (1977) using TBE buffer system. For T5 DNA 0.7% and for T7 DNA 1% agarose gels (in TBE) were used. The dimensions of the gels were 14.5 x 13.5 x 0.5 cm. The gels were run at 15 mamps for 18 hours, stained with 2 ug/ml ethidium bromide in water for 30 minutes at room temperature and destained in water overnight before photographing.

XII PROTEIN DETERMINATION.

The values for protein determination using the method of Schaffner and Weissmann (1973) with BSA as standard ($\epsilon^{1\%}_{280\ nm}$ = 6.6) agreed well with the known extinction coefficients (1% solutions) for core polymerase (5.8), holoenzyme (6.7) and sigma (5.6) (Levine et al., 1980 and Burton et al., 1981). For example, a 1:10 dilution of homogeneously pure sigma preparation had an A_{280} = 0.640 with 280:260 ratio of 1.8. Based on $\epsilon^{1\%}_{280}$ = 5.6 the above value corresponds to a protein content of 1.16 mg/ml. Using the method of Schaffner and Weissmann (1973) a value of 1.11 mg/ml was obtained. Throughout this study protein determinations were carried out using the method of Schaffner and Weissmann (1973).

XIII INITIATION ASSAYS.

A. Single round transcription. Single round transcription of T7 DNA in presence of heparin was carried out as described by Chamberlin et al. (1979).

B. Abortive initiation assays. The assays directed by T7 DNA or $d(A-T)_n$ were done according to the procedure described by Hansen and McClure (1979). The conditions are described under the individual legends. The nucleotide products were separated from (^3H)UTP by chromatography on Whatman 3M paper using the WASP solvent system (water-17, saturated ammonium sulfate-80, isopropanol-2, 0.5M EDTA-1 and pH adjusted to 8.3 with ammonium hydroxide).

C. Pyrophosphate exchange assay. The assay was done as described by Krakow and Fronk (1969).

D. Nonspecific initiation. Initiation at single-strand breaks in T7 DNA was studied as described by Hinkle et al. (1972).

XIV FILTER BINDING STUDIES.

These studies were carried out as described by Hinkle and Chamberlin (1972). Binding studies involving (^3H) $d(A-T)_n$ or (^3H) $d(I-C)_n$ were done by forming enzyme-DNA binary complex at 37^oC for 10 minutes. Following the addition of heparin the incubation was continued for 10 minutes. The assay mix was filtered, washed with 1 ml of binding buffer A and the amount of labelled DNA bound to the filter was quantitated.

XV PROMOTER RECOGNITION STUDIES.

These studies were done using HaeIII T7 DNA digest or HindIII T5 DNA digest according to the method described by Gabain and Bujard (1977).

XVI CHEMICAL MODIFICATION OF SIGMA SUBUNIT.

In order to remove the free reagent from the sigma bound reagent following modification two methods were available; filtration of the reaction mix through a 0.5 x 2 cm G-50 column and overnight dialysis against a suitable buffer. Although both these methods gave comparable results overnight dialysis was chosen because of the 95 to 100% recovery of protein and relatively unchanged protein concentration. The sigma preparations for control experiments were prepared the same way as for the experimental but with no reagent added and dialyzed overnight.

A. Trinitrophenylation. Analysis of the mol TNP bound per mol sigma indicated that the reaction with (^{3}H)TNBS plateaus at about 20 minutes irrespective of the concentration of (^{3}H)TNBS. Unless otherwise noted the modification reactions with TNBS were carried out at 37°C for 30 minutes. Trinitrophenylation was carried out in the presence of 40 mM BTP buffer (pH 9) and varying concentrations of TNBS. Mol TNP incorporated per mol sigma was calculated using a molecular weight of 70263 for sigma (Burton et al., 1981).

B. Carbamylation. The reaction was carried out in 200 mM borate buffer (pH 9) and varying concentrations of KCNO at 37°C for 90 to 120 minutes.

C. Succinylation. The reaction was carried out in 50 mM sodium phosphate (pH 9) and varying concentrations of succinic anhydride at 37°C for 30 minutes. Unlike carbamylation and trinitrophenylation the activity of sigma following succinylation could be recovered by dialysis; 40 to 50% after 5 hours and 90% after overnight dialysis.

D. Reaction with phenylglyoxal. The reaction was carried out at 37°C in 50 mM potassium phosphate (pH 8) and varying concentrations of phenylglyoxal. The duration of incubation depended on the concentration of phenylglyoxal. At the end of the incubation period 50 ul of 0.5 M arginine in water was added and incubation continued for 5 minutes. The mix was dialyzed overnight against 40 mM potassium phosphate (pH 7).

E. Reaction with NEM, PCMB or DTNB. The reaction was carried out in 100 mM triethanolamine-H_2SO_4 (pH 7) at 37°C.

F. Reaction with CMC or EDC. The reaction was carried out in 100 mM triethanolamine-H_2SO_4 (pH 7) at 37°C for 60 minutes and the mix was dialyzed against 20 mM Tris-HCl (pH 6.8) overnight.

XVII IDENTIFICATION OF TNP-AMINO ACID.

A. Synthesis of TNP-amino acid standards. ϵ-TNP-lysine and L-TNP-methionine were synthesized as described by Okuyama and Satake (1960). S-TNP-cysteine was synthesized using reduced glutathione according to the procedure described by Hollenberg et al. (1971).

B. Identification of TNP-amino acid in TNP-sigma.

(^3H) TNP-sigma (100 ug with varying mol TNP per mol sigma) was dialyzed against water overnight and hydrolyzed for 16 hours at 105°C in 6 N HCl in a sealed tube. The hydrolysate was brought to 1 N HCl with water and extracted with ether several times. The aqueous phase was dried under reduced pressure in a rotary evaporator and the residue was washed 3 times with water. The TNP-amino acids were extracted using ethyl acetate: 1-butanol:acetic acid (100:100:1) and chromatographed on Whatman No 1 filter paper as described by Hollenberg et al. (1971) using 1.5 M sodium phosphate buffer (pH 6) system.

XVIII RECONSTITUTION.

A. Reconstitution with core polymerase. (^3H) TNP-sigma (with varying mol TNP per mol sigma) was incubated at 37°C for 5 minutes with various amounts of core polymerase in TMS buffer containing 0.5 M NaCl and 5% (W/V) glycerol. The final volume of 50 ul was layered on a 15 to 35% linear glycerol gradient (total volume 4.4 ml) in TMS buffer with 0.5 M NaCl layered on 0.5 ml of 35% glycerol in TMS buffer with 0.5 M NaCl in 5 ml cellulose nitrate tubes. The tubes were centrifuged at 45000 RPM for 22 hours at 4°C in a SW 50.1 rotor. Fractions of 0.4 ml were collected and aliquots were used for protein determination and for analysis by SDS polyacrylamide gel electrophoresis. An aliquot was TCA precipitated, filtered and counted in 5 ml Liquifluor-toluene.

B. Reconstitution with DNA-core polymerase complex. Core polymerase-DNA (T7 DNA or $d(A-T)_n$) complex was formed

in TMS buffer containing 5% glycerol at 37°C for 10 minutes. Following the addition of an equimolar amount of (^3H) TNP-sigma (7 TNP per sigma) the incubation was continued for 5 minutes. The mix (50 ul) was layered on a 1 cm x 60 cm column of Ultrogel ACA-44 equilibrated with TMS buffer containing 5% glycerol with or without 0.5 M NaCl. The column was developed with the appropriate TMS buffer at 3 ml per hour. One ml fractions were collected. Protein content, A_{260} and radioactivity were determined for each fraction.

XIX A POSSIBLE CHANGE IN THE CONFORMATION FOLLOWING MODIFICATION.

Sigma or TNP-sigma (15 ug) in 20 mM Tris-HCl (pH 8), 1 mM DTT was digested with 0.4% SAP in 0.01 M sodium phosphate (pH 7) at 37°C for varying time. The digest was electrophoresed on 7.5% SDS polyacrylamide gel.

XX PEPTIDE MAPPING.

A. CNBr fragments. (^3H) TNP-sigma was digested overnight with 0.2 to 1 M CNBr (J.T. Baker Chemical Co.) in 70% formic acid in dark at 25°C. The digest was frozen at -70°C for 1 hour, lyophilized and dissolved in 10 mM Tris-HCl (pH 8), 1 mM DTT. The digest (10 to 15 ug) was electrophoresed on 15% SDS polyacrylamide gel.

B. Autoradiography. The destained gel was soaked in Enhance (New England Nuclear) for 1 hour, washed with water for 1 hour and dried. Autoradiography was carried out using Kodak XAR-5 film (13 cm x 18 cm) at -70°C for 7 days.

XXI SPECIFIC ACTIVITY OF (^3H) NEM.

A stock solution of 60 ul (60 uCi) of (^3H) NEM (New England Nuclear, 55.8 Ci/mmol) in pentane was mixed with 150 ul of 30 mM unlabelled NEM. The pentane was evaporated under a stream of nitrogen. 5 ul aliqots were added to 1.5 ml of 100 mM TEA-H_2SO_4 (pH 7) or 1.5 ml of freshly prepared 1 M cysteine in 100 mM TEA-H_2SO_4 (pH 7) and incubated for 5 minutes at 37^oC. 10 ul aliquots were used for counting.

If NEM is dried alone, only 20% of the radioactivity can be recovered. If the NEM-cysteine adduct is dried, better than 95% of the radioactivity can be recovered. The NEM-sigma precipitate does not disperse into Liquifluor; less than 5% of the radioactivity is found in the Liquifluor. The NEM-cysteine adduct count comparable to this procedure was taken as the specific activity of NEM (Table 8).

RESULTS

The procedure for the purification of sigma subunit described here was developed under the supervision of Dr. S. A. Kumar, in 1978. The procedure differed from the one described by Lowe et al. (1979) in few aspects. The polymerase obtained after Ultrogel ACA-34 filtration was used without separation into core polymerase and holoenzyme components. By doing so the majority of the contaminating proteins in the flowthrough of the Bio-Rex 70 chromatography were β, β' and α subunits. The amount of these contaminants varied from one polymerase preparation to another. Most of these contaminants were removed by eluting the DEAE column with a NaCl gradient. Any traces of the contaminants still remaining were removed by gel filtration on Ultrogel ACA-34.

In general the modification of sigma using different reagents seems to affect various properties of sigma. It was necessary to examine any effect of the products of the reactions (for example, sulfite in trinitrophenylation or H^+ in succinylation) on the activity of sigma subunit. Figure A3 shows the results of such a study. Sodium sulfite upto 20 mM has no effect on sigma activity. The activity of sigma is not changed at pH as low as 6.5. Also any pH changes in the succinylation reaction are compensated by the buffer. Since carbamylation reaction is carried out at 1 M

KCNO the effect of high ionic strength on the activity of sigma was tested. KCl upto 2 M has no effect on the sigma activity.

In the modification reactions, the volume of the sigma solution (50% glycerol in 10 mM KP buffer, pH 7) varied from one reaction to another thus changing the concentration of glycerol in the reaction mix. Any effect of glycerol concentration on trinitrophenylation was examined. Glycerol concentration upto 45% has no effect on the extent of modification (Figure A4-A).

Although ^{3}H TNBS (from Amersham or NEN) and unlabelled TNBS were identical in spectral properties it was necessary to examine if the labelled and the unlabelled TNBS are identical in reactivity. Keeping the total concentration of TNBS in the reaction mix constant the percentage of ^{3}H TNBS was varied from 10 to 90%. Based on the experimental value for each point the expected values were calculated for the other points and the average value is used in the graph. As seen in Figure A4-B the experimental curve fits well with the expected one suggesting that the reactivity of the labelled and unlabelled TNBS is identical.

TRINITROPHENYLATION OF SIGMA.

The concentration dependent trinitrophenylation of sigma is shown in Figure 2. In the case where nonspecifically bound TNBS is not removed the mol TNP bound per mol sigma reaches a plateau of approximately 35 at about 5 mM TNBS. Based on the molecular weight of 70263 (Burton et al., 1981) this

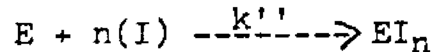
corresponds to 100% of the lysyl groups of sigma. In the experiments where the free and nonspecifically bound TNBS is removed by dialysis an obvious biphasic response was observed. At 1.5 mM TNBS approximately 10 TNP groups are bound per sigma. Raising the concentration of TNBS to 5 mM results in approximately 20 TNP groups bound per sigma. At 10 mM TNBS a maximum of about 30 TNP groups are bound per sigma. This corresponds to about 90% of the total lysyl groups of sigma. Under the reaction conditions adopted the possible aminoacyl residues that are modified are lysyl, cysteinyl or the N terminal methionyl groups. Figure 3 shows the absorption spectra (between 250 and 500 nm) of TNP-sigma, authentic ϵ-TNP-lysine and authentic S-TNP-cysteine. TNP-sigma was estimated to have 7 mol TNP per mol sigma and was completely inactive in stimulating the T7 DNA directed transcription by core polymerase. As seen in the figure TNP-sigma shows the spectrum characteristic of authentic ϵ-TNP-lysine with an absorbance maximum at around 345 nm and a shoulder at 425 nm suggesting the modification of lysyl and not cysteinyl residues of sigma. This was further confirmed by paper chromatography of the TNP-aminoacids obtained from the acid hydrolysate of ^{3}H TNP-sigma using authentic standards. The ^{3}H TNP-aminoacids isolated from the 10 mol TNP per mol sigma or 28 mol TNP per mol sigma cochromatographed with the authentic ϵ-TNP-lysine with an Rf value of approximately 0.65. Theoritically a tenth of the total amount of radioactivity in the 10 mol TNP per mol sigma

should be found at the area corresponding to the Rf value of TNP-methionine if the N terminal methionyl residue is also modified along with lysyl residues. No detectable radioactivity could be recovered from the areas corresponding to the Rf values of either S-TNP-cysteine (0.17) or TNP-methionine (0.5) (Table 2). The biphasic curve in Figure 3 suggests that there are three different classes of lysyl groups with respect to reactivity with TNBS; two sets of readily accessible (between 0 and 1.5 mM and 4.5 and 7 mM TNBS) and about 10 relatively not easily accessible lysyl residues (between 1.5 and 4.5 mM TNBS). The apparently higher level of incorporation of ^{3}H TNBS in the undialyzed sample could probably be due to the nonspecific and/or non-covalent binding of TNBS. The time course at three concentrations of TNBS (Figure 4) shows an initial rapid rate followed by a slower rate approximating a plateau. Using low concentrations of TNBS it is possible to limit the reaction to only a few lysyl groups per sigma. A plot of the percent residual activity versus the mol TNP incorporated per sigma extrapolates to a value of 5 lysyl groups modified per sigma at 100% inactivation (Figure 5). Kinetic studies of the inactivation were performed in order to determine the number of critical lysyl residues.

KINETICS OF THE INACTIVATION OF SIGMA.

The inactivation of sigma by TNBS follows pseudo first order kinetics at 1.4 or 1 mM TNBS (Figure 6). The reaction

of TNBS with sigma can be represented by the equation



$$E + n(I) \xrightarrow{k''} EI_n$$

where k'' is the apparent second order rate constant, E is the free enzyme, n is the number of molecules of inhibitor I. Since the inactivation follows pseudo first order kinetics, the pseudo first order rate constant $k' = k''(I)^n$. The logarithmic conversion of this relation namely $\log k' = \log k'' + n \log I$ is an equation for a straight line and a plot of log k' versus the log of TNBS concentration should give a straight line with slope equal to n, the number of inhibitor molecules needed to inactivate a single subunit of sigma. The values for k' were calculated by taking the reciprocal of $t\frac{1}{2}$, the time required to inactivate 50% of sigma activity. Figure 7 shows such a plot with the slope of the straight line equal to 1.2 suggesting that the modification of 1 lysyl group by TNBS results in complete inactivation of sigma.

INACTIVATION AS A FUNCTION OF pH.

The effect of pH on the rate of inactivation by TNBS is shown in Figure 8. Since the activity of sigma was 30 to 50% lower after incubation at pHs below 6 the reactions were studied at pHs above 6.5. k'' was obtained by taking the ratio of k' to the TNBS concentration (Figure 6). Two assay systems were used to follow the activity of sigma. The k'' values using the T5 DNA assay system were slightly higher

than those using the pApU system with $d(A-T)_n$ as template. In both the systems the rate of inactivation increases with pH. The rate constant is approximately 50 M^{-1} Min^{-1} at pH 6.5 and increases by 10 to 15 fold at pH 11. The apparent pK of the critical lysyl residue was estimated to be 8.8 using the T5 DNA assay system and 9.3 using the pApU assay system.

RECONSTITUTION OF TNP-SIGMA WITH CORE POLYMERASE.

The conditions employed in the ultracentrifugation experiments are such as to get a good separation between free sigma and core polymerase; the free sigma can be found between fractions 3 and 6 with the peak at fraction 4 or 5 and core polymerase or holoenzyme peaks at fraction 9 or 10. By mixing core polymerase and sigma in a molar ratio of 0.25:1 (Figure 9b), 0.5:1 (Figure 9c) and 0.75:1 (Figure 9d) respectively 25, 50 and 75% of the free ^{3}H TNP-sigma (8 TNP per sigma) can be titrated out by core polymerase and TNP-sigma can be titrated out completely using full equivalence of core polymerase (Figure 9e). The affinity of TNP-sigma for binding to the core polymerase was studied by mixing TNP-sigma (in stoichiometric amounts to core polymerase) with sigma in ½, 1 or 2 times the amount of TNP-sigma (8 TNP per sigma) and studying the reconstitution with core polymerase (Figure 10). If the binding of TNP-sigma to core polymerase is comparable to that of sigma it is possible to predict the amount of free TNP-sigma and TNP-sigma bound to core polymerase in each of the three experiments. The

values calculated based on the experiments agree well with the predicted ones suggesting that TNP-sigma binds to the core polymerase with an affinity comparable to sigma. TNP-sigma is also able to bind to the core polymerase bound to DNA (T7 or $d(A-T)_n$). Figure 11 shows that a mixture of ^3H TNP-sigma (10 TNP per sigma) and DNA when passed through the Ultrogel ACA-34 filtration column resolves into free TNP-sigma (broken line) and DNA (solid line). TNP-sigma when mixed with the core polymerase-DNA complex is retained with the DNA following filtration (Figure 11b). If such a mixture is passed through a column containing 0.5 M NaCl the core polymerase and hence the TNP-sigma bound to it dissociate from DNA (Figure 11c).

TEMPLATE BINDING STUDIES.

Figure 12 shows the effect of trinitrophenylation on the binary complex formation between core polymerase and $d(A-T)_n$ or $d(I-C)_n$. In the absence of any template competitor, very little difference in DNA binding can be observed between core polymerase and holoenzyme. However, in the presence of competitor such as heparin added after the binary complex has been formed, the free and loosely bound polymerase can be removed by heparin. The amount of $d(A-T)_n$-holoenzyme complex refractory to heparin attack is about twice the amount of $d(I-C)_n$-holoenzyme complex; approximately 60% of the $d(A-T)_n$ and 30% of the $d(I-C)_n$ is bound to the holoenzyme in presence of heparin. In either case, as seen in

the figure, there is a 3 fold difference in the amount of DNA bound between core polymerase and holoenzyme and trinitrophenylation completely inactivates sigma.

EFFECT ON CORE POLYMERASE-T7 DNA BINARY COMPLEX FORMATION.

RNA polymerase holoenzyme is known to be involved in the formation of tight binary promoter complex (Hinkle and Chamberlin, 1972). The data presented in Figure 13 confirm these findings. Only 20% of the ^{3}H T7 DNA from the preformed T7 DNA-holoenzyme complex can be displaced by a 10 fold excess of unlabelled T7 DNA and 85% of the ^{3}H T7 DNA initially bound to the core polymerase can be competed out. Such a marked difference between core polymerase and holoenzyme cannot be observed between core polymerase and core polymerase with TNP-sigma.

Such binding experiments were also done using restriction endonuclease fragments of T7 DNA. BclI B fragment of T7 DNA (Rosenberg et al., 1979) containing the 21% of the T7 DNA from the left genetic end has the major 'A' promoters. The results from the binding experiments using the promoter containing fragment (Figure 14) are similar to the results represented in Figure 13 where the conditions employed allow the formation of tight binary complex (promoter complex).

PROMOTER RECOGNITION STUDIES.

T5 DNA. Studies done on T5 DNA (Gabain et al., 1976 and Gabain and Bujard, 1977) indicate that of the 16 HindIII fragments of T5 DNA, fragments ILMO have no promoters for

E.coli RNA polymerase and others have from 1 to 4 promoters and therefore form tight complexes with holoenzyme. As seen in Figure 15 (E,F,G,H) fragments ILMO can be titrated out of the holoenzyme-DNA complex with single stranded calf thymus DNA over a period of 6 hours while the promoter-containing fragments in the holoenzyme-DNA complex are refractory to competition. The core polymerase (Figure 15 B,C,D) while binding to the promoter fragments also seems to show a nonspecific binding for the fragments containing no promoter. In either case the fragments, with or without promoters, decay from the core polymerase-DNA complex within 3 hours of competition. This effect of sigma seems to be completely abolished upon modification. As shown in Figure 15 (I,J,K,L) the band pattern with core polymerase + TNP-sigma is similar to the band pattern with core polymerase alone. The effect of progressive modification of sigma on promoter recognition is shown in Figure 16. With 1 lysyl group of sigma modified (see discussion) (Figure 16 A,B,C) the sigma is still able to recognize promoter and the binary complex formed between DNA and core polymerase-TNP-sigma is as tight as the complex formed between DNA and holoenzyme. However, the fragments lacking promoters are also retained which seems to suggest the loss of the ability of sigma to discriminate between nonpromoter sites and promoter sites. Upon modification of 3 lysyl groups (Figure 16 D, E, F) the binary complex between DNA and core polymerase-TNP-sigma seems to be loose. In the case where 5 or more lysyl groups of

sigma are modified the DNA fragments can be displaced from the binary complex completely within 6 hours of competition (Figure 16 G,H,I and J,K,L). These experiments were done with preincubation of 2 minutes. With prolonged preincubation (30 minutes) the modified sigma (with 1 or 3 lysyl groups modified) does seem to not only recognize promoters but also discriminate the nonpromoter sites. Thus the effect of sigma on the core polymerase-nonpromoter site interaction seems to be refractory to the trinitrophenylation. This was confirmed by the binding studies carried out using HaeIII A fragment of T7 DNA (Figure 17). The fragment covers between 19 and 26 percent of T7 DNA from the left genetic end and has no major promoters (Studier et al., 1979). TNP-sigma or sigma behave in a similar manner; 80 to 90% of the ^3H DNA fragment from the core polymerase-TNP-sigma (or sigma)-DNA complex can be competed out by a 10 fold excess of single stranded T7 DNA. About 80% of the ^3H DNA fragment of the initially core polymerase bound DNA can be competed out.

<u>T7 DNA</u>. Experiments similar to those done with T5 DNA were carried out using a HaeIII digest of T7 DNA. As seen in Figure 18 the core polymerase, in presence of sigma, is able to recognize and form a tight binary complex with the promoter-containing fragment, C, and the rest of the fragments are discriminated. This effect of sigma is abolished upon trinitrophenylation. It should be pointed out that fragment C has 3 equal sized fragments, C1, C2 and C3 and only C1 has the major A promoters (Studier et al., 1979). Promoter

recognition studies with progressive trinitrophenylation (Figure 19) indicate that with 1 or 3 lysyl groups of sigma modified the TNP-sigma-core polymerase complex is still able to recognize the promoter-containing fragment although the binding is not as strong as with the holoenzyme. Upon modification of 5 or more lysyl groups the sigma seems to be completely inactive in this respect.

BINDING STUDIES WITH NICKED T7 DNA.

Since the conditions employed to create single strand breaks in T7 DNA genome are those described by Hinkle et al. (1972) the number of nicks introduced per genome is assumed to be the same as described by Hinkle et al. (1972). Figure 20 shows that the binary complex formed using core polymerase with sigma or TNP-sigma and nicked DNA is strong irrespective of the number of nicks in the DNA. The labelled DNA cannot be removed from the binary complex by a 2 fold excess unlabelled intact competitor T7 DNA with 30 minutes of competition. Competition for 90 minutes gives the same results. The affinity of core polymerase for the formation of binary complex with nicked DNA depends on the number of nicks in the DNA. With approximately 5 nicks per DNA molecule (T7 DNA treated with $5x10^{-4}$ ug DNase I) about 70% of the labelled DNA is in the binary complex. With 20 (10^{-3} ug DNase I) and 145 (10^{-2} ug DNase I) nicks, respectively 50 and 20% of the nicked DNA is in the binary complex and with 400 nicks ($5x10^{-1}$ ug DNase I) almost no binary complex can be detected.

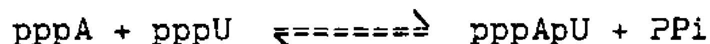
EFFECT ON RNA CHAIN INITIATION.

Experiments on RNA chain initiation were carried out using natural templates such as T7 DNA or synthetic DNAs such as $d(A-T)_n$. Initiation has been defined as the formation of the first phosphodiester bond between the initiation nucleoside triphosphate and elongation nucleotide (Krakow et al., 1976). Recently initiation has been defined as all the steps upto and including the release of sigma which occurs after about 10 nucleotides are polymerized (Hansen and McClure, 1980). Experiments on initiation with T7 DNA were carried out using abortive initiation or single round transcription where sigma is released. By the addition of heparin a few seconds after the RNA chain initiation in the T7 DNA directed reaction the unbound and loosely bound polymerase can be inhibited. Only RNA polymerase in open promoter and ternary complexes is refractory to heparin attack and under such conditions most of the RNA polymerase molecules transcribe the DNA till the termination signal is reached which is about 7 minutes for T7 DNA (Chamberlin et al., 1979) and then are inhibited by heparin. Such an assay involving single round transcription is very sensitive to the presence of sigma and covers the second definition for initiation. As seen in Figure 21 at the end of the chain termination there is about a 4 fold increase in the amount of ^3H UMP incorporated in the presence of sigma (curve A and C) and the trinitrophenylation renders the sigma inactive (curve B).

Another way of studying initiation in natural templates

is by transcribing the DNA in presence of either of the initiation nucleoside triphosphates (ATP or GTP) and UTP or CTP. RNA chain elongation under such conditions is prevented due to the absence of the other two substrate nucleotides. Figure 22 shows the results of such an experiment. A greater than 7 fold difference can be observed between core polymerase and holoenzyme in the synthesis of pppApU and a 2 fold difference in the case of pppGpU synthesis. The modification of sigma results in complete loss of this effect in either of the assay systems.

Several $d(A\text{-}T)_n$ directed initiation reactions are also sensitive to the presence of sigma. The $d(A\text{-}T)_n$ directed RNA chain initiation can be represented by the following reaction:



$$\text{pppA} + \text{pppU} \rightleftharpoons \text{pppApU} + \text{PPi}$$

In the presence of excess added PPi the equilibrium of the above reaction can be shifted towards the left and by employing ^{32}P PPi the label can be exchanged for the PPi of the elongation nucleoside triphosphate, UTP (Krakow and Fronk, 1969). Such an assay system shows a large difference between the activity of core polymerase and holoenzyme; a 2 to 3 fold increase in the amount of PPi exchanged can be observed with holoenzyme compared to core polymerase (Figure 23, curves A and C) and TNP-sigma is unable to support this stimulation. Under the conditions employed the rate of PPi ecchange is approximately 25 pmol per minute for core polymerase. This

rate can be stimulated to approximately 60 pmol per minute by sigma but not by TNP-sigma.

Nucleotides such as AMP and UpA can be used to support initiation but not chain elongation in the $d(A-T)_n$ directed assay (Hansen and McClure, 1979). Figure 24 shows a gradual increase in the amount of UpApU formed with increasing amounts of sigma in the presence of 10 mM Mg^{++} (curve A). A greater than 3 fold increase in the amount of UpApU can be seen upon addition of a full equivalence of sigma to core polymerase. The progressive addition of TNP-sigma has no stimulatory effect on core polymerase (curve B). In presence of 2 mM Mn^{++} although the core polymerase synthesizes about the same amount of UpApU as it does in presence of Mg^{++}, addition of sigma inhibits the synthesis of the trinucleoside diphosphate by core polymerase. At saturating amounts of sigma a greater than 80% inhibition of UpApU synthesis can be observed (curve A) compared to core polymerase. The trinitrophenylation of sigma again fails to support this inhibition of the UpApU synthesis by core polymerase (curve B). The most striking difference between core polymerase and holoenzyme can be seen in their ability to synthesize pApU in presence of AMP and UTP directed by $d(A-T)_n$ (Hansen and McClure, 1979). The presence of sigma enhances the synthesis of pApU by core polymerase by greater than 12 fold (Figure 25, curve A) and TNP-sigma has no stimulatory effect (curve B).

Figure 26 shows the results of nonspecific transcription

of T7 DNA following the introduction of single strand nicks by DNaseI. Transcription of T7 DNA with no DNase treatment is taken as 1 and other values are expressed relative to 1. Peak values for core polymerase, holoenzyme or core polymerase with TNP-sigma are obtained by using T7 DNA treated with 4×10^{-5} ug of DNase. Under this condition the transcription of the nicked T7 DNA is increased by 80% using core polymerase and only 20% using holoenzyme. This inhibition of core polymerase nonspecific transcription by sigma is abolished upon trinitrophenylation. The transcription by core polymerase with TNP-sigma is about 50% greater than the transcription by core polymerase alone. Introduction of more nicks in T7 DNA brought the level of transcription by core polymerase, holoenzyme or core polymerase with TNP-sigma to or below the base level.

EFFECT OF MODIFICATION ON CONFORMATION OF SIGMA.

The protein content of sigma or TNP-sigma digested with proteases for various periods was determined by the TCA precipitation method of Schaffner and Weissmann (1973). Figure 27 shows such a study. Digestion of sigma with SAP results in a loss of 10% of the protein by 5 minutes and 18% by 60 minutes. Digestion of TNP-sigma results in 10 to 20% loss of the protein. The protein content of the tryptic digest of sigma is 25% less by 30 minutes and stays constant till 60 minutes. Digestion of TNP-sigma with trypsin results in about 20 to 60% loss of the protein over

the range of 5 to 60 minutes. The protein content of chymotryptic digest of sigma remains unchanged over 60 minutes of digestion. Digestion of TNP-sigma with chymotrypsin results in 35% loss of protein by 60 minutes. It is interesting to note that the percentage loss of protein is gradually reduced with progressive trinitrophenylation; tryptic digestion of 1 TNP-sigma for 60 minutes results in a loss of over 55% of the protein whereas the digestion of TNP-sigma with 4, 8 and 14 lysyl groups modified results in a loss of 52, 48 and 44% of the protein respectively. Studies with SAP and chymotrypsin show a similar response.

The SAP digest pattern of sigma and modified sigma after SDS polyacrylamide gel electrophoresis (7.5%) is shown in Figure 28. The molecular weights of sigma estimated using SDS polyacrylamide gel electrophoresis do not agree with the molecular weight based on the sequence data. Using the Tris-glycine buffer system molecular weights of 85 to 89000 can be estimated (Burgess, 1976). Sodium phosphate buffer system gives a molecular weight of 82000 (Lowe et al., 1979). The DNA sequence of rpoD gene suggests a molecular weight of 70263 (Burton et al., 1981). Because of this the estimation of molecular weights of the peptide bands generated by protease digestion was difficult. Digestion of sigma with trypsin, chymotrypsin or SAP results in the production of a stable peptide with different molecular weights and several minor peptides. The protease digest pattern of the sigma modified with phenylglyoxal or TNBS is different from the

digest pattern of unmodified sigma. Trypsin is lysine and arginine specific, chymotrypsin is phenylalanine, tyrosine and tryptophan specific and SAP is aspartic acid and glutamic acid specific proteases. The digestion patterns of phenylglyoxal-sigma and TNP-sigma do not seem to be identical suggesting that the conformation of the TNP-sigma is different from the conformation of phenylglyoxal-sigma (Figure 29). It is interesting to note that the modified sigma seems to be more sensitive to tryptic digestion and more resistant to chymotryptic or SAP digestion than sigma. The band pattern is identical between 1, 6 and 11 TNP-sigma digests suggesting that the modification of as low as 1 lysyl group is sufficient to change the conformation.

Sigma subunit was modified using various reagents and experiments similar to the ones described above were carried out using the modified sigma.

Tables 3A and 3B show the results of carbamylation and succinylation of lysyl residues. The results suggest the importance of 1 lysyl group in the function of sigma. Inactivation following succinylation is reversible following dialysis; 90% of the activity of sigma can be recovered following overnight dialysis at $4^{o}C$. The protease digest pattern of the succinylated sigma is indistinguishable from the sigma digest. Succinylation of sigma was carried out in a volume of 10 ul for 30 minutes and the effect of modification on the template binding, promoter recognition

and initiation was carried out in 100 ul volume as described under methods and materials. A control experiment with no sigma had no effect on the core polymerase activity. In general, the effects of succinylation and carbamylation seem to parallel the effects of trinitrophenylation. A comparison of the second order rate constants indicates that the lysyl groups of sigma are more reactive towards TNBS, less towards succinic anhydride and least towards KCNO.

The reaction of arginyl residues with ^{14}C phenylglyoxal does not seem to plateau even after prolonged incubation (Figure 30). However, it is possible to limit the modification to a few arginyl residues by employing proper conditions such as incubation time and phenylglyoxal concentration. For example, approximately 5 arginyl residues can be modified using 4 mM phenylgloxal and incubating for 60 minutes at 37°C. The number of arginine residues modified shows a linear relationship with respect to the concentration of phenylglyoxal upto 10 mM and begins to plateau at 15 mM at which concentration a maximum of approximately 16 arginine residues are modified (Figure 31). Thus 35% of the total arginine residues seem to be accessible to the modification. The data in Figure 32 indicate that complete loss of sigma activity results in following the modification of 3 arginine residues. The inactivation reaction follows pseudo first order kinetics. Table 4 shows the values for n, the reaction order, at different pH. The value for n averages to approximately 2 suggesting that reaction of 2 molecules of phenyl-

glyoxal per sigma, that is, modification of 1 arginine residue per sigma is sufficient to inactivate 1 molecule of sigma.

The reaction with NEM plateaus at about 40 minutes (Figure 33). The sequence analysis of rpoD gene indicates that sigma subunit has 3 sulfhydryl groups (Burton et al., 1981). At 15 mM NEM all the 3 cysteinyl residues can be modified within 40 minutes of incubation (Figure 33). One of the 3 sulfhydryl groups can be modified with no observable loss in the activity of sigma. Complete loss of sigma activity results in when 3 sulfhydryl groups have been modified (Figure 34).

Tables 5A and 5B summarize the effects of modification of arginyl, sulfhydryl and carboxyl groups of sigma using various reagents. Comparison of the results in Tables 3 and 5 indicates that the effects of modification with these reagents, in general, parallel the effects of trinitrophenylation. Sigma modified with CMC or EDC was not only inactive in stimulating the T7 DNA transcription by core polymerase but also unable to form holoenzyme complex with core polymerase. While the approximate pK values of 9 for the critical lysyl group and 8 for the sulfhydryl group were not unusual, the pK value of 8 for the arginyl group was considerably low compared to the pK value of 12 for the guanidino group of free arginine.

DISCUSSION

Several lines of evidence suggest that trinitrophenylation is specific for lysyl groups and not cysteinyl groups. The modification of sulfhydryl groups should also be considered because modification of sigma subunit with NEM, PCMB or DTNB results in complete inactivation of the sigma subunit. Although spectral analysis suggests only trinitrophenylation of lysyl groups any cysteinyl groups modified along with lysyl groups would be difficult to detect. However, at alkaline pH no trinitrophenylation of cysteinyl groups occurs because S-TNP-cysteine is labile at pH 9 and the sulfhydryl groups are regenerated. Also, if the inactivation observed is due to the modification of sulfhydryl groups treatment of the modified sigma with mercaptoethanol should reverse the inactivation and no such effect was observed. Also, at alkaline pH the lysyl residues are unprotonated and only unprotonated lysyl groups can be trinitrophenylated. Finally, paper chromatography of the acid hydrolysate of TNP-sigma suggests that trinitrophenylation of sigma is lysine specific. This procedure should be considered only qualitative because following hydrolysis and extraction of TNP-amino acids less than 50% of the radioactivity could be recovered. However, by using high specific activity TNBS (100 to 150 cpm per pmol) trinitrophenylation of as low as 1 amino acid residue per sigma can be

detected. No modified cysteine or methionine was detected in the hydrolysate.

The data presented in Figure 5 suggest that following modification of 5 lysyl groups 100% inactivation of the subunit occurs. Such a correlation between the activity and degree of modification can also be obtained by using the kinetic analysis of the data (Hollenberg et al., 1971; Marcus et al., 1976 and Marshel and Bodley, 1979). The assumption that the trinitrophenylation reaction is a pseudo first order reaction is valid because even at the lowest concentration of TNBS (0.4 mM) (Figure 6) in the experiment there is more than a 50 fold excess of the ligand and the concentration of TNBS remains virtually unchanged at the end of the reaction. The data suggest that binding of 1.2 or approximately 1 molecule of TNP per sigma subunit would lead to complete inactivation (Figure 7). The kinetic data do not agree with the results of Figure 5. It is possible that there are at least 5 lysyl groups in sigma with equal reactivity towards TNBS and one of them is critical for the activity of sigma. Such a finding is not uncommon; MarSchel and Bodley (1979) report that elongation factor Ts of E.coli has 2 equally reactive arginine groups out of a total of 10 residues. Only one of the two seems to be involved in the interaction between elongation factors Ts and Tu. It has been shown that lysyl groups of sigma can be modified with Celite adsorbed dansyl chloride

without affecting the activity of sigma (Wu et al., 1976). A similar experiment was performed with 1000 fold molar excess of CDC in 20 mM KP buffer (pH 9) and the unreacted CDC was removed by dialysis. The CDC-sigma was fully active. An experiment corresponding to Figure 5 was repeated with CDC-sigma (Figure A6). The results suggest that 2 of the 5 lysyl groups available for trinitrophenylation can be protected by dansylation.

Not all the lysyl groups of sigma are of equal reactivity towards trinitrophenylation. The obvious biphasic profile of the modification reaction (Figure 2) could be due to the presence of 3 sets of lysyl groups with approximately 10 lysyl groups in each set. The first set containing the critical residue has lysyl groups readily accessible to trinitrophenylation. The second set contains less readily accessible lysyl groups and the third set contains readily accessible lysyl residues which can be modified at TNBS concentrations above 5 mM.

The various assays available for sigma make use of either synthetic DNAs such as $d(A-T)_n$ or natural DNAs such as T phage DNAs. The higher k'' values in the T5 DNA assay system compared to the $d(A-T)_n$ assay system could be due to the difference in the two assay systems; in the pApU system the sigma subunit is not cycled. Since the pK value of the sulfonate group of TNBS is about 5.7 (Figure A7) the observed pK value of about 9 seems to

be associated with the critical lysyl group of sigma.

The possibility of inactivation due to an aggregation of sigma (following modification) has to be ruled out because recently it was reported that sigma from a temperature sensitive mutant of E.coli forms hexameric aggregates at temperatures above 37°C affecting the binding to the core polymerase and resulting in the inhibition of sigma (Lowe et al., 1981). No aggregates of TNP-sigma (10 TNP per sigma) could be detected using ultracentrifugation or gel filtration. Thus the inactivation of sigma seems to be due to the trinitrophenylation of the critical lysyl group.

The effect of sigma subunit on transcription involves the following steps: the binding of sigma to core polymerase, the binding of core polymerase-sigma complex to the promoter (or the binding of sigma to the core polymerase-DNA complex) and initiation of RNA. The modification of sigma could affect any of these steps. The ultracentrifugation results (Figure 9) suggest that TNP-sigma (8 TNP per sigma) still binds to the core polymerase. Progressive modification of sigma has no effect on the binding to the core polymerase. However, TNP-sigma with 20 or more TNP bound per sigma was found to form pentameric or larger aggregates as judged by sedimentation velocity of TNP-sigma. For this reason the reconstitution experiments on such a TNP-sigma preparation could not be

performed. Binding of sigma to core polymerase is strong. The binding constant for sigma-core polymerase interaction has been estimated to be 10^{10} M^{-1} (Wu et al., 1976a). Thus inhibition could also result from any change in the binding constant due to the modification. For example, very loose or tight binding of the modified sigma (compared to the unmodified sigma) to the core polymerase might result in inactivation. Experiments involving competition between the modified and unmodified sigmas for binding to the core polymerase (Figure 10) suggest that the binding affinity of TNP-sigma (8 TNP per sigma) to the core polymerase is comparable to that of sigma. The presence of a high glycerol concentration (50%) is known to strengthen the core polymerase-sigma interaction (Gonzalez et al., 1977). Therefore it was necessary to examine the reconstitution of modified sigma with core polymerase in the absence of a high glycerol concentration. This was accomplished by DNA cellulose chromatography. The modified sigma and a stoichiometric amount of core polymerase were incubated in 100 ul of TGED with 0.1 M NaCl at 37°C for 5 minutes, loaded on a 1 cm x 1 cm DNA cellulose column, washed and eluted. Fractions (0.5 ml) were collected and analyzed for sigma. The results suggested that sigma modified with NEM, phenylglyoxal or TNBS reconstitutes with core polymerase and the afinity of binding is comparable to that of sigma in 5% glycerol.

Attempts were made to determine if the critical lysyl

group is in the core polymerase binding domain of sigma. One approach is to modify holoenzyme, separate the modified holoenzyme into the core polymerase and sigma components and analyze the sigma subunit. Holoenzyme was modified with various concentrations of TNBS (0.05 mM to 5 mM) and dialyzed. The TNP-holoenzyme when passed through a 1 cm x 1 cm Bio-Rex 70 column was not separated into core polymerase and sigma; the core polymerase was not retained by the Bio-Rex 70 column, perhaps due to the modification. Also the modification of holoenzyme with TNBS at 1 mM or greater concentrations results in the dissociation of sigma from core polymerase (still intact) as judged by ultracentrifugation (Figure A8) and the core polymerase binding domain of sigma is no longer protected. These results suggest the importance of the lysyl groups of the core polymerase and not of the sigma subunit in the core polymerase-sigma interaction. A synthetic copolymer comprised of glutamic acid and tyrosine (Glu_9,Tyr_1) inhibits RNA polymerase by interacting with polymerase (Krakow, 1974). The interaction of sigma with core polymerase could be similar to the interaction of the polypeptide. This was confirmed by the modification of sigma with carboxyl group specific reagents, CMC and EDC. Kinetic analysis indicated the presence of 1 critical carboxyl group required for the activity of sigma. The modified sigma was not able to form a holoenzyme complex with core polymerase. While the critical

sulfhydryl, arginyl or lysyl groups of sigma do not seem to be involved in the core polymerase-sigma interaction, the acidic groups of sigma seem to be important in binding to core polymerase. Interaction of the acidic amino acids of sigma with basic amino acids of core polymerase, perhaps with lysyl groups, may be involved. Of all the subunits of the RNA polymerase sigma subunit is the most acidic subunit with an estimated pI of 4.4 (Burton et al., 1981). Acidic amino acids form 20% of the total amino acids in sigma and 50% of the acidic amino acids are clustered in the N-terminal third of the peptide (Burton et al., 1981). It is possible that the N-terminal area of sigma is involved in core polymerase-sigma interaction. Since the modified sigma also binds to core polymerase bound to DNA (Figure 11) and core polymerase-TNP-sigma complex binds to DNA it seems likely that the inhibition could be at the level of promoter recognition and/or initiation.

TEMPLATE BINDING STUDIES.

Synthetic DNAs such as $d(A-T)_n$ have no promoters and RNA polymerase does not go through the process of site selection. Both core polymerase and holoenzyme form an open complex with $d(A-T)_n$ (Hansen and McClure, 1979 and 1980). However, there is difference in the behavior of core polymerase or holoenzyme towards $d(A-T)_n$ binding just as with the promoter where a

difference of 3 to 5 orders of magnitude in binding constant is observed between core polymerase and holoenzyme binary complex. The binding constant for holoenzyme $d(A\text{-}T)_n$ open complex is 3 orders of magnitude greater than the core polymerase $d(A\text{-}T)_n$ open complex; k for core polymerase binary complex is 10^6 M^{-1} and for holoenzyme binary complex is 10^9 M^{-1} (Hansen and McClure, 1980). While the data presented in Figure 12 do not reflect the binding constants for the formation of polymerase $d(A\text{-}T)_n$ binary complexes they do suggest that the open binary complex with $d(A\text{-}T)_n$ or $d(I\text{-}C)_n$ is more stable with holoenzyme than with core polymerase. This stimulatory effect of sigma on the core polymerase DNA interaction is inhibited by trinitrophenylation.

With natural promoters the core polymerase, unlike the holoenzyme, is unable to form a stable and open complex (Hinkle and Chamberlin, 1972). The filter binding studies (Figure 13) suggest that the holoenzyme containing unmodified sigma forms a stable open complex with T7 DNA which is refractory to the competitor DNA whereas the core polymerase-TNP-sigma complex is unable to form a stable complex with T7 DNA. In order to minimize the effects of general DNA sites the BclI 'B' fragment containing the major T7 promoters (Rosenberg et al., 1979) was used. These results also suggest the inability of the TNP-sigma-core polymerase complex to form the open promoter binary complex (Figure 14). Filter binding studies and agarose

gel electrophoresis using restriction endonuclease fragments of T7 or T5 DNA give a better understanding of the polymerase interaction with the promoter and non-promoter DNA sites. The data shown in Figures 15 and 16 (for T5 HindIII fragments) and 18 and 19 (for T7 HaeIII fragments) confirm the previous findings that the holoenzyme discriminates between promoters and nonpromoters while the core polymerase shows no affinity for promoters. The holoenzyme is able to form tight binary complex with only promoter containing fragments whereas core polymerase forms a loose complex with promoter or nonpromoter fragments. The results also suggest that while TNP-sigma-core polymerase complex is unable to form an open complex with the promoters it is still able to discriminate between promoter and nonpromoter fragments; the T5 DNA fragments with no promoter are still discriminated. This aspect of similarity between sigma and TNP-sigma was further confirmed by filter binding studies using HaeIII 'A' fragment of T7 DNA containing no major promoters (Studier et al., 1979). Core polymerase-sigma or core polymerase-TNP-sigma forms complexes with the fragment which are dissociated by competitor DNA (Figure 17). These results suggest that core polymerase can form stronger complexes with general DNA sites compared to the holoenzyme or core polymerase-TNP-sigma. The tight binding of core polymerase to general DNA sites is well documented. The binding constant for

the core polymerase-general DNA complex is 3 to 4 orders of magnitude greater than the holoenzyme-general DNA complex (Hinkle and Chamberlin, 1972). This effect of sigma remains unaltered by the modification of lysyl groups. The tight binding of holoenzyme to the single strand nicks in T7 DNA was demonstrated by Hinkle et al. (1972). Core polymerase with sigma or TNP-sigma forms a tight binary complex with nicked T7 DNA whereas the stability of the core polymerase T7 DNA complex decreases with the number of nicks in the DNA. In this respect also sigma is resistant to lysine modification.

There is overwhelming evidence suggesting the importance of lysyl groups of various proteins in nucleotide or DNA binding function. Modification of 3 lysyl groups of dihydrofolate reductase with 2,4-pentanedione has been shown to prevent the binary complex formation of the enzyme with NADPH (Otwell et al., 1979). Using the lysyl specific reagent, 2,4-pentanedione, lysyl groups have been implicated in the activity of the pig heart diphosphopyridine nucleotide dependent isocitrate dehydrogenase (Hayman and Colman, 1977). Hollenberg et al. (1971) have shown using TNBS that a critical lysyl group in the active site of rabbit muscle pyruvate kinase is involved in the interaction of the enzyme with ADP. Extensive chemical modification studies of the gene 5 protein of bacteriophage fd, a DNA binding protein, have revealed that acetylation of 6 lysyl groups

results in the inhibition of DNA binding ability (Coleman and Oakley, 1980). Modification of lysyl groups of the cAMP receptor protein using TNBS is known to inhibit the DNA binding function of the protein (Pampeno, 1979). Similar studies have shown the importance of lysyl groups in RNA polymerase also. Modification of lysyl groups of core polymerase in the presence of DNA using methyl acetimidate indicates that about 12 lysine residues are protected by DNA from modification (Makoff and Malcolm, 1980). Similar studies using TNBS have suggested that $d(A-T)_n$ protects about 10 lysyl groups from trinitrophenylation. These lysyl groups could be located in the DNA binding domain of the core polymerase and be involved in electrostatic interactions with nucleotides in template binding (Krakow et al., 1976). Although a direct binding of sigma to promoters has not been demonstrated, many studies have implicated the interaction of free sigma or sigma in the holoenzyme with DNA. Hillel and Wu (1978) demonstrated that UV irradiation of the T7 DNA promoter-holoenzyme binary complex results in the crosslinking of DNA to the sigma or beta subunits. Similar studies with bromouracil labelled lac UV 5 promoter-holoenzyme complex suggest that the sigma and beta subunits can be cross-linked with DNA by UV irradiation (Simpson, 1979). These studies indicate the specific interaction of sigma with promoters. The results presented here implicate a sigma lysyl group critical for the interaction with the promoter.

A possible electrostatic interaction between the ϵ -amino group of the critical lysyl group of sigma and the promoter is feasible. Hillel and Wu (1978) have shown that the sigma subunit can also crosslink with general DNA sites of T7 DNA. Recently a direct interaction of sigma with DNA has been demonstrated. Using antibodies against sigma and a micro complement fixation assay free sigma (or sigma in the holoenzyme) was shown to interact with synthetic DNA as well as natural DNAs such as T7 and lambda DNAs (Stender, 1980). Kudo and Doi (1981) have shown using gel electrophoresis under native conditions that sigma binds to supercoiled plasmid DNA pBRH 4. Since the effect of sigma on the core polymerase-general DNA interaction or on the core polymerase-nicked T7 DNA interaction is unaffected by the modification, the lysyl group critical for promoter recognition may not be involved in the sigma-general DNA interaction.

INITIATION STUDIES.

The process of initiation has been studied extensively using synthetic DNAs such as $d(A-T)_n$ (Krakow and Fronk, 1969 and Hansen and McClure, 1979) and natural DNAs (Johnston and McClure, 1976 and Chamberlin et al., 1979). However, it has been difficult to properly define the process of initiation. Earlier, it was defined as the formation of the first phosphodiester bond in RNA synthesis (Chamberlin, 1976; Krakow et al., 1976 and

Johnston and McClure, 1976). Recently it has been proposed by Hansen and McClure (1980) that initiation involves all the steps up to and including the release of sigma and the formation of a stable ternary complex which occurs following the synthesis of first 9 ± 2 ribonucleotides (Krakow and von der Helm, 1971 and Hansen and McClure, 1980). It became necessary to test the effect of the modification of sigma on initiation and related steps using several assays. These include heparin challenge, abortive initiation in the T7 DNA directed reaction, single step synthesis of pApU or UpApU and PPi exchange in the $d(A-T)_n$ directed reaction. In the heparin challenge assay RNA polymerase goes through a complete cycle of RNA chain synthesis and core polymerase or sigma is not cycled. In the single step synthesis of pApU sigma is not released. In all the other assays sigma is cycled. In all these assays and in the nonspecific initiation at the single strand T7 DNA nicks the core polymerase-TNP-sigma complex behaved in a manner similar to the core polymerase alone. In one system, the $d(A-T)_n$ directed synthesis of UpApU, in the presence of Mn^{++} the holoenzyme was less active than the core polymerase. In the presence of Mg^{++} the holoenzyme synthesized UpApU at a more rapid rate than the core polymerase. With either Mn^{++} or Mg^{++} as the divalent cation the TNP-sigma holoenzyme behaved like the core polymerase. The effect of sigma on initiation could be related to the 60 fold

reduction observed in the Km value for the elongation nucleoside triphosphate due to the presence of sigma (Hansen and McClure, 1979, 1980). One lysyl group of sigma may be critical in these functions.

The effects of modification on sigma could be a consequence of either blocking the ϵ -amino group of lysine or the introduction of the bulky trinitrophenyl group. The lysyl groups of sigma were also modified using succinic anhydride or KCNO where the functional groups are less bulkier than the trinitrophenyl group. The results presented in Tables 3A and 3B indicate that there are no major differences in the properties between carbamylated, succinylated and trinitrophenylated sigma.

CONFORMATION OF SIGMA.

Of the several methods available the difference in the protease digest pattern was used as a criterion to study any possible conformational change. Data shown in Figures 28 and 29 suggest that there is a change in the conformation of sigma following the modification. It is crucial to prove that following the modification all the sigma molecules in the population are modified. The ligand, TNBS, in trinitrophenylation reactions is present in excess amount compared to sigma and hence all the sigma molecules are expected to be modified. For example, in a typical experiment with 100 ul reaction mix containing 100 or 10 ug sigma and 0.1 mM TNBS (at which

concentration 1 lysine group per sigma is expected to be modified) the molar ratio of TNBS to sigma is respectively 7 and 70 and the ratio is 0.2 and 2 if the number of moles of lysyl groups in sigma is considered. The SAP digest pattern between the two modified sigmas was indistinguishable. Also the band pattern is indistinguishable between 1 TNP-sigma and 3 or 6 TNP-sigma and since most of the activity of sigma is still retained with 1 TNP-sigma the inactivation observed with 6 TNP-sigma could not be due to the change in the conformation of sigma. The results also suggest that the modification of the first lysyl group results in the change in conformation and the critical lysyl group is modified subsequently. The protein loss (Figure 27) in the digestion of TNP-sigma (using various proteases) is maximal with 1 TNP-sigma, minimal with 14 TNP-sigma and intermediate with 4 or 8 TNP-sigma. If 1 TNP-sigma has a mixed population of sigma (for example, 50% of sigma and 50% of 2 TNP-sigma) the loss of protein observed would be in the reverse order; that is the loss would increase with progressive modification. It is possible that the loss of protein in the protease digest of the modified sigma is related to the conformational change and the results reflect a conformational change with as low as 1 lysyl group modified per sigma.

MODIFICATION OF ARGINYL GROUPS.

The results indicate that sigma is completely inactivated following the modification of 3 arginine residues. Just as with the modification of lysyl groups the critical arginine residue reacts more rapidly with phenylglyoxal than the remainder of the 46 arginine groups. The apparant pK value of about 8 for the critical arginine, which is substantially lower than the pK of 12 for the free arginine, reflects a possible effect of the environment on the critical arginine residue. Such a finding is not uncommon. The critical arginyl residue of elongation factor G of E.coli has a pK value of 8.8 (Rohrbach and Bodley, 1977). Optimal inactivation of elongation factor Ts of E.coli by butanedione occurs at around pH 9 (MarSchel and Bodley, 1979). The second order rate constant of inactivation of aspartate transcarbamylase by phenylglyoxal increases by 40 fold from pH 7 to pH 10 (Kantrowitz and Lipscomb, 1976). Inactivation of porcine phospholipase A2 (Vensel and Kantrowitz, 1980) or creatine kinase (Borders and Riordan, 1975) by phenylglyoxal also shows a similar pH behavior. Arginyl residues of several nucleotide binding proteins are known to be involved in nucleotide binding function of proteins. Arginyl residues of ovine brain glutamine synthetase and E.coli carbamyl phosphate synthetase seem to be involved in ATP binding function (Powers

and Riordan, 1975). Modification of cardiac myosin S-1 by phenylglyoxal decreases the Mg ADP binding ability markedly (Morkin et al., 1979). Nucleotides such as AMP, ADP and Mg-ATP protect adenylate kinase from inactivation by phenylglyoxal and arginyl residues may be involved in the interaction with these nucleotides (Berghauser, 1975). Arginyl residues of several template-requiring polymerases are known to be important in template binding; avian myeloblastosis virus and Rauscher murine leukemia virus reverse transcriptases, calf thymus DNA polymerase β , E.coli DNA polymerase I and E.coli RNA polymerase are inhibited by phenylglyoxal whereas the calf thymus terminal deoxynucleotidyl transferase which does not require a template is resistant to inhibition by phenylglyoxal (Srivastava and Modak, 1980). Chemical modification of nucleosome cores using butanedione suggests that arginyl residues of histones play an important role in the formation of nucleosome structure. Electrostatic interactions between guanidinium ions of arginyl residues and the phosphate groups of DNA have been proposed in the nucleosome cores (Ichimura et al., 1982). Therefore it is possible that arginyl residue(s) of sigma is(are) interacting directly with promoter nucleotides, in an electrostatic manner, in the promoter holoenzyme complex. The binding of sigma is known to induce

conformational change in holoenzyme which may result in increased affinity for promoter sites (Wu et al., 1976). Although PG-sigma binds to core polymerase with an affinity comparable to that of sigma, the binding of PG-sigma to core polymerase may not be able to induce a specific conformational change. MarSchel and Bodley (1979) have shown the importance of arginyl residue in protein-protein interaction; modification of one arginine residue of elongation factor Ts prevents its interaction with elongation factor Tu.

MODIFICATION OF SULFHYDRYL GROUPS.

Of the 3 sulfhydryl groups in the sigma subunit one can be modified at 5 mM NEM with relatively no loss in the activity of sigma subunit. Following the modification of the other two sulfhydryl groups the activity of sigma is completely lost. Just as with the modification of lysyl and arginyl residues, the modification of sulfhydryl groups does not affect either the binding or the binding affinity of NEM-sigma to core polymerase. Sulfhydryl groups of RNA polymerase are known to be important in the maintainance of quaternary structure of the enzyme. Modification of *Azotobacter vinelandii* RNA polymerase with phenylmercurisulfonate above pH 9 results in the dissociation of the protomer (Krakow, 1975). Extensive modification of *E.coli* RNA polymerase with PCMB results in the release of α subunits

(Ishihama, 1972). Sulfhydryl groups of β' may be involved in subunit-subunit interaction (Ito and Ishihama, 1973). While the sulfhydryl groups of the core polymerase subunits may be involved in the subunit-subunit interaction, those of the sigma subunit do not seem to be crucial in the sigma-core polymerase interaction. However, sulfhydryl groups of sigma may be crucial in bringing a conformational change in holoenzyme which is thought to be essential for promoter recognition. Alternatively, sulfhydryl groups of sigma in the holoenzyme may interact directly with promoter nucleotides. Sulfhydryl groups of RNA polymerase are known to be involved in the template binding function. Modification of E.coli RNA polymerase holoenzyme with 0.01 M sodium tetrathionate results in complete loss of $d(A-T)_n$ binding activity (Yarbrough and Wu, 1974). E.coli RNA polymerase holoenzyme modified with 1 uM phenylmercurisulfonate shows a complete loss of $d(A-T)_n$ binding activity (Krakow, 1975).

MODIFICATION OF CARBOXYLIC ACIDS.

Water soluble carbodiimides have a broad specificity; depending on the reaction conditions WSC can react with carboxylic acids, tyrosine, cysteine, unprotonated amines, threonine and serine (Means and Feeney, 1971). Sigma subunit was modified with WSC at room temperature

and neutral pH. Under these conditions threonine and serine are not reactive (Ramachandran and Colman, 1977). Reaction of WSC with tyrosine can be reversed by treating the modified protein with 0.5 M hydroxylamine (Carraway and Koshland, 1972). Following incubation of the (^{3}H) glycine-sigma (5 glycine per sigma) in 0.5 M hydroxylamine (pH 7) at 25°C for up to 5 hours over 90% of the (^{3}H) glycine incorporated is stable. Sigma activity is also not recovered after this treatment (Table 6). This rules out the possibility that tyrosine modification could be responsible for the loss of sigma activity. After modification of sigma with EDC plus glycine (5 glycine per sigma) the three cysteine residues were still available for reaction with (^{3}H) NEM. The results suggest that the reaction of WSC with sigma is specific for carboxylic amino acids. The incorporation of glycine and loss of sigma activity are linearly related. Complete loss of sigma activity occurs when 1.3 mol glycine are incorporated per mol sigma (Figure 35). The data presented in Figure 36 indicate that following reaction of sigma with EDC in the presence of (^{3}H) glycine the (^{3}H) glycine-sigma is unable to form a stable holoenzyme complex. The carboxylic amino acids of sigma, clustered within the first 215 residues from the N-terminus may be involved in the core-sigma interaction. A possible electro-

static interaction between carboxylic acids of sigma and the positively charged amino acids in the sigma binding domain of core polymerase can be proposed. Since the binding of sigma to core polymerase is not sensitive to ionic strength other interactions must exist in the binding of sigma to core polymerase.

PARTIAL PEPTIDE MAPPING OF THE CRUCIAL AMINO ACIDS.

Chemical cleavage of sigma subunit at methionine residues using CNBr was used as a probe to partially locate the critical amino acids. Sigma subunit has 25 methionine residues (Burton et al., 1981). Table 7 shows the expected fragments (and amino acids) produced by complete cleavage of sigma subunit with CNBr. SDS polyacrylamide gel electrophoresis (15%) of CNBr fragments of sigma (Figure 37) shows a sharp band with mobility comparable to that of CRP (Mr, 22500) and 3 diffused bands migrating between lysozyme and insulin. These 4 major bands are assigned to 8 fragments as indicated in the figure. Figure 38 is an autoradiogram of the CNBr fragments of (^{3}H) TNP-sigma. The lysyl groups in fragment A do not seem to be trinitrophenylated. Assuming that the 2 lysyl groups in the fragment Glu_2-Met_{47} are not trinitrophenylated, it can be concluded that the critical lysyl group is not in the first 288 amino acids from the N terminus. Similar studies were carried out with (^{3}H) NEM-sigma with 1, 2 or 3 sulfhydryl

groups modified (Figure 39). The results indicate that fragment A containing 1 sulfhydryl group is labelled in sigma subunit with 1, 2 or 3 sulfhydryl groups modified. It can be concluded that the SH group that is not essential for sigma function is cys_{132}. Peptide mapping was also done on sigma modified with (^{14}C) phenylglyoxal. The autoradiograms indicated that both the large and the small fragments were labelled. The interpretation of the data was difficult. Autoradiogram of the (^{3}H) glycine-sigma fragments is shown in Figure 40. Only fragment A is labelled in sigma modified with 1, 2, 3 or 5 glycines. These results suggest that the N terminal half of sigma may be the core polymerase binding domain and the C terminal half of sigma containing the critical lysyl, cysteinyl and possibly arginyl residues may be involved in the promoter recognition and the initiation effects of sigma.

CONCLUSIONS.

1. Lysyl groups of sigma can be divided into 3 sets with respect to the reactivity towards TNBS. Two sets of readily accessible and one set of not readily accessible lysyl groups.

2. A critical lysyl group with a pK value of about 9 and readily accessible is involved in sigma function.

3. At least 4 more lysyl groups with the same reactivity as the critical lysyl group can be modified

with TNBS.

4. TNP-sigma forms a holoenzyme complex with core polymerase with a binding affinity comparable to that of sigma.

5. TNP-sigma bound to the core polymerase can bind to DNA.

6. Holoenzyme formed with TNP-sigma is unable to form a tight binary complex with $d(A-T)_n$ or $d(I-C)_n$.

7. While the core polymerase-TNP-sigma complex is unable to form a tight binary complex with T5 or T7 promoters, TNP-sigma still retains the ability to lower the affinity of core polymerase for DNA general sites.

8. TNP-sigma-core polymerase complex was similar to holoenzyme with respect to forming a tight binary complex with T7 DNA containing single stranded nicks.

9. TNP-sigma-core polymerase complex behaves in a manner similar to core polymerase in the initiation assays using natural or synthetic DNA or natural DNA with single stranded nicks.

10. A change in conformation of sigma can be observed when the first available lysyl group is modified with TNBS.

11. The inactivation of sigma following trinitrophenylation does not seem to be due to the change in conformation.

12. Modification of lysyl groups with KCNO or succinic anhydride also inhibits the promoter recognition and initiation properties of sigma.

13. Approximately 35% of the total arginines of sigma

can be modified using phenylglyoxal.

14. One arginyl residue with a low pK value of 8 seems to be critical for sigma function. At least 2 more arginyl residues are modified along with the critical arginyl residue.

15. One of the 3 sulfhydryl groups of sigma can be modified with NEM with no loss of activity.

16. Complete loss of sigma activity occurs after modification of 3 sulfhydryl groups. One sulfhydryl group with a pK of about 8 is critical for sigma function.

17. Modification of sulfhydryl groups with PCMB or DTNB also inhibits the promoter recognition and initiation effects.

18. The critical arginyl and sulfhydryl groups of sigma are involved in the promoter recognition and initiation effects of sigma.

19. Modifying arginyl or sulfhydryl groups results in a change in the conformation of sigma without affecting the ability of sigma to form a holoenzyme complex with core polymerase. The binding affinity of modified sigma was comparable to that of sigma.

20. Carboxylic amino acids of sigma can be modified specifically using EDC or CMC and labelled with (^{3}H) glycine in the presence of EDC or CMC.

21. Modification of 1 carboxylic amino acid group results in the loss of sigma function due to the inability of the modified sigma to form a stable holo-

enzyme complex with core polymerase.

22. Critical carboxyl group is in the N terminal half of sigma. Sulfhydryl group that is not essential for sigma function is identified as cys_{132}.

23. The critical lysyl, sulfhydryl and possibly arginyl groups are in the C terminal half of sigma.

24. The N terminal half of sigma may be involved in binding of sigma to core polymerase. The C terminal half may be involved in the promoter recognition and initiation functions.

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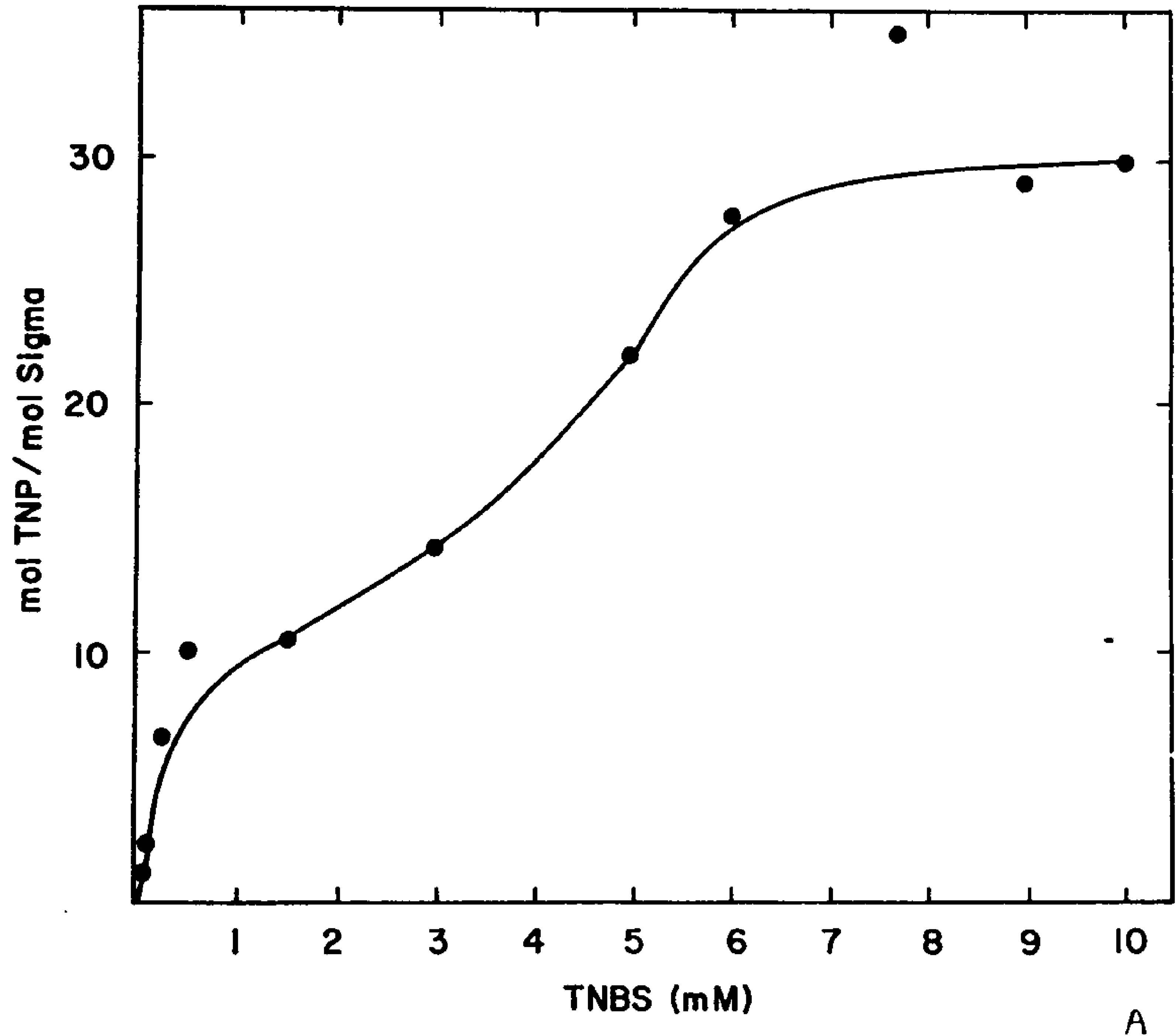
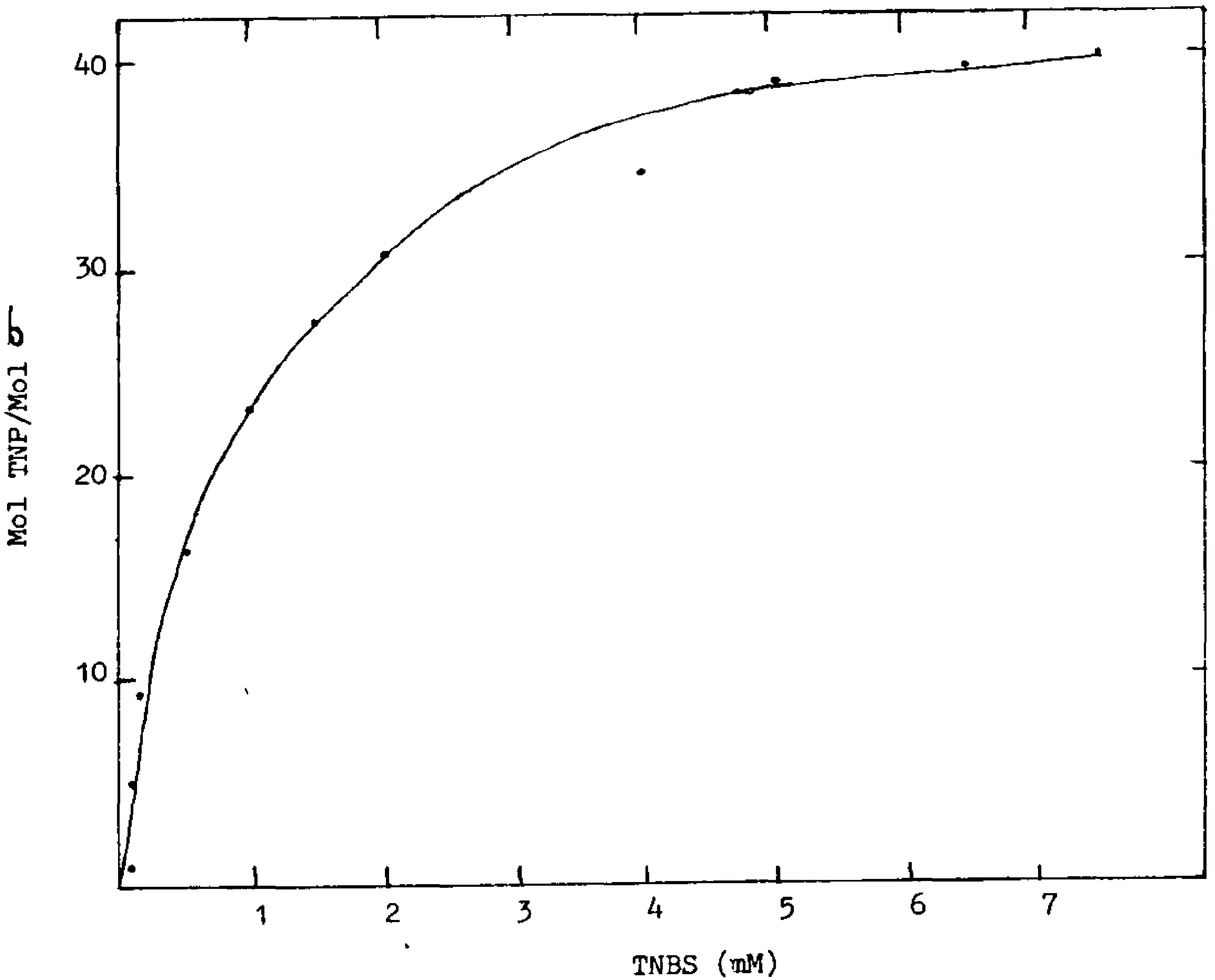
FIGURE 1: SDS polyacrylamide gel electrophoresis of sigma fractions. Aliquots of fractions (20 ul) were diluted with SDS sample buffer, boiled for 2 minutes and electrophoresed on 7.5% polyacrylamide gels as described under methods. Top. Fractions from DEAE column. Holoenzyme standard is on extreme right. Bottom. Fractions from Ultrogel ACA-34 gel filtration column. Holoenzyme standard is on extreme left.

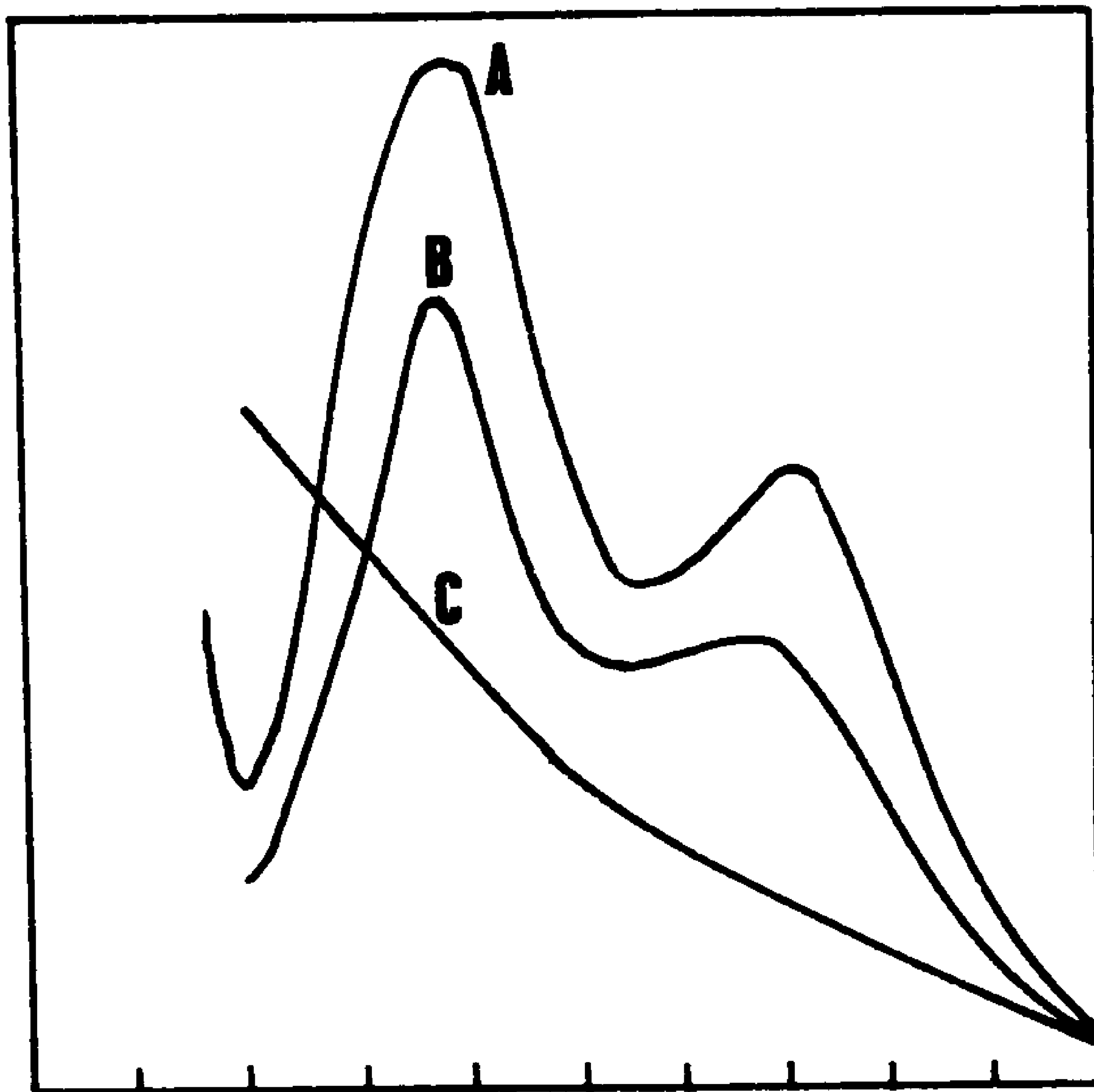
FIGURE 2: Concentration dependent trinitrophenylation of sigma. The reaction mix (50 ul) containing 25 ug sigma, 40 mM BTP buffer (pH 9) and varying concentration of (^{3}H) TNBS was incubated at 37°C. At the end of 30 minutes 10 ul aliquot was TCA precipitated, filtered on GF/C glass fiber filters and counted. The remaining was dialyzed overnight against 20 mM Tris-HCl (pH 8), 1 mM DTT and the protein content determined. An aliquot (5 ug) was TCA precipitated, filtered and counted. Mol TNP incorporated per mol sigma was calculated using a molecular weight of 70263 daltons for sigma. A, with dialysis; B, without dialysis.


mol TNP / mol Sigma
10
20
30
1
2
3
4
5
6
7
8
9
10
TNBS (mM)
A



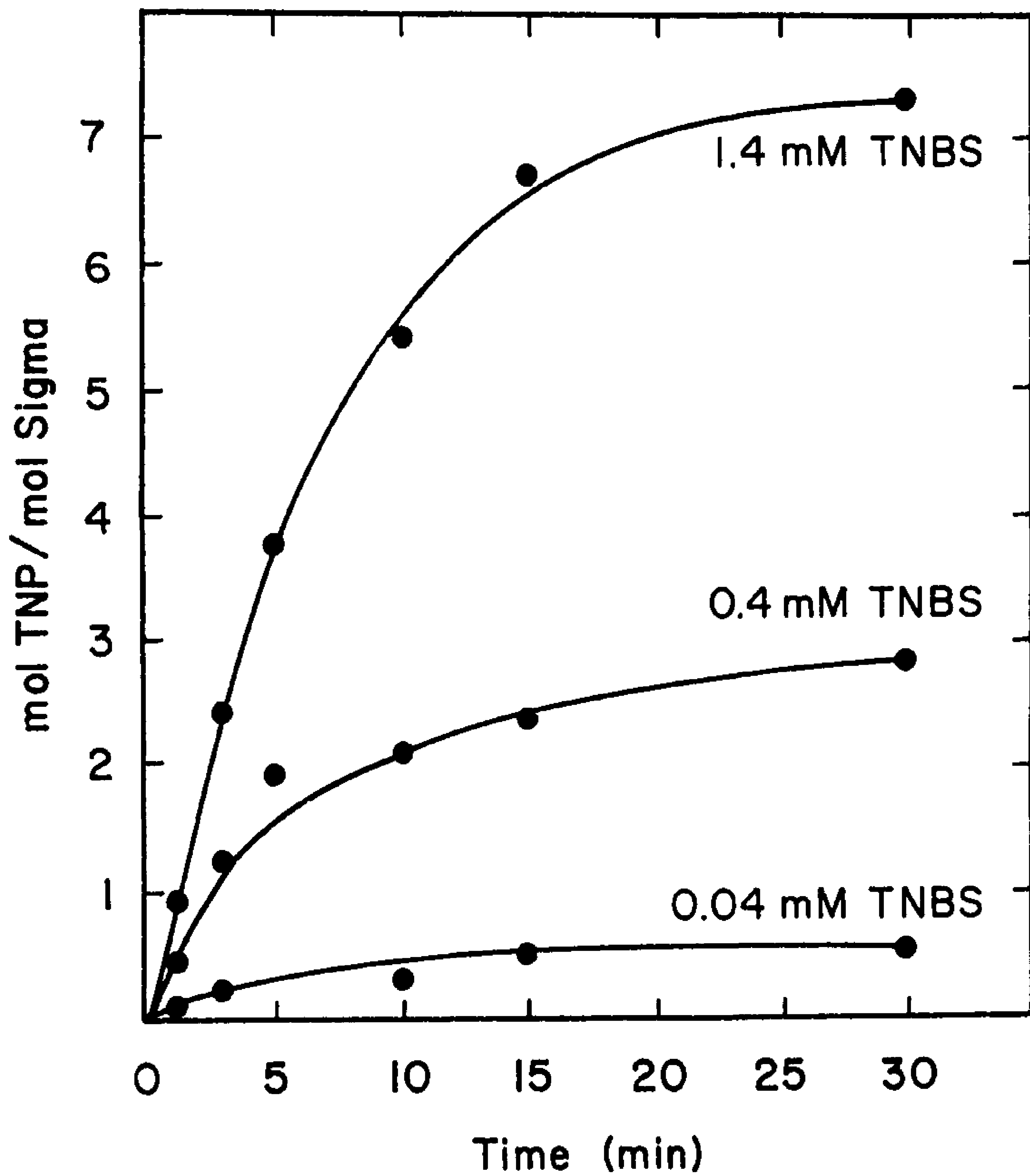
40
30
20
10
Mol TNP/Mol b
1
2
3
4
5
6
7
TNBS (mM)
B

FIGURE 3: Absorbance spectra of TNP-sigma and the standards. The spectra of authentic TNP-lysine (curve B) and S-TNP-cysteine (curve C) were obtained by scanning the uthentic materials prepared according to the procedure described by Hollenberg et al. (1971). TNP-sigma (7 TNP per sigma) was prepared in a 300 ul mix containing 300 ug of sigma, 1.4 mM TNBS and BTP buffer. Following the dialysis the protein content and the mol TNP incorporated per mol sigma were determined and TNP-sigma was scanned (curve A).



A
B
C
Absorbance
300
400
500
Wavelength (nm)

FIGURE 4: Time dependent trinitrophenylation of sigma. The reaction mix (70 ul) containing 70 ug sigma, BTP buffer (pH 9) and varying concentrations of (^{3}H) TNBS was incubated and 10 ul aliquots were added to 500 ul of 1 M L-lysine in 20 mM BTP buffer (pH 9) in a dialysis bag. Following overnight dialysis the mol TNP incorporated per mol sigma was calculated. TNBS concentration: A, 1.4 mM; B, 0.4 mM and C, 0.04 mM.



1.4 mM TNBS
0.4 mM TNBS
0.04 mM TNBS
mol TNP/mol Sigma
Time (min)
0
5
10
15
20
25
30
1
2
3
4
5
6
7

FIGURE 5: Relationship of lysine modification to loss of sigma activity. Reactions were run with 10 ug sigma, BTP buffer (pH 9) and varying concentrations of (^{3}H) or unlabelled TNBS. Following overnight dialysis aliquots containing 2.5 ug of sigma from the unlabelled TNBS reaction mix was taken for the determination of activity using T7 DNA as template and a stoichiometric amount of core polymerase (10 ug). Aliquots from the (^{3}H) TNBS reaction mix were taken to determine the mol TNP incorporated per mol sigma.

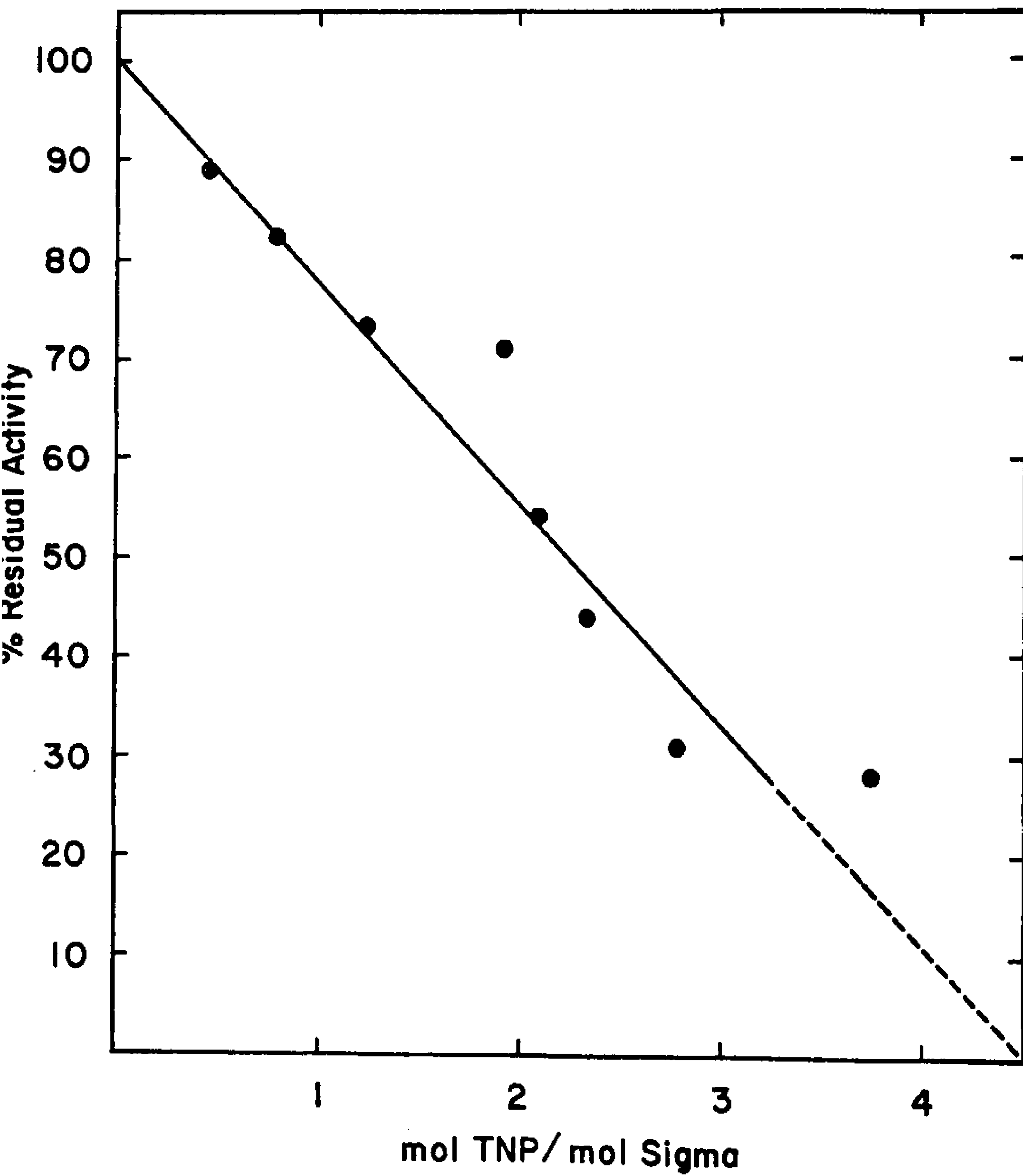
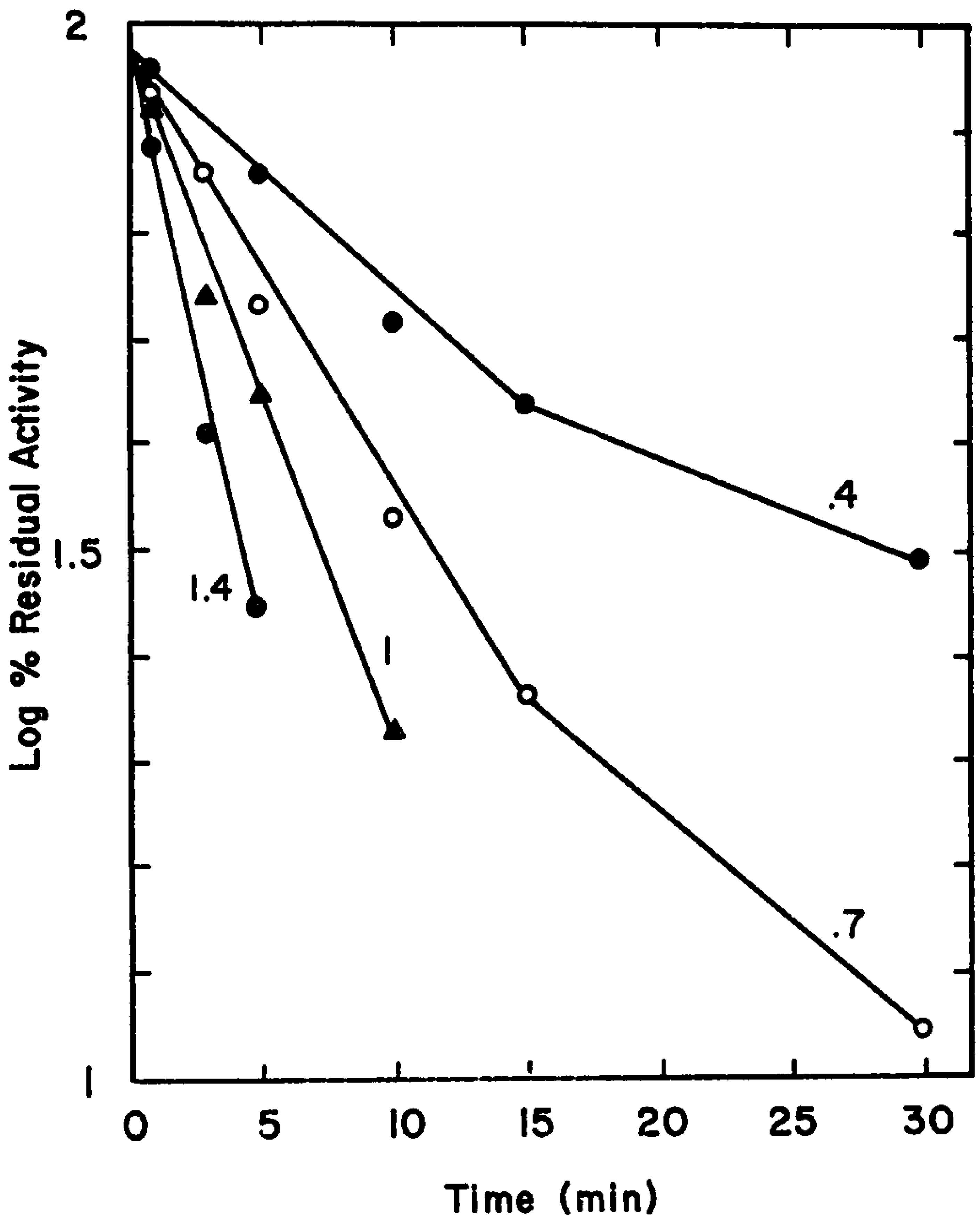
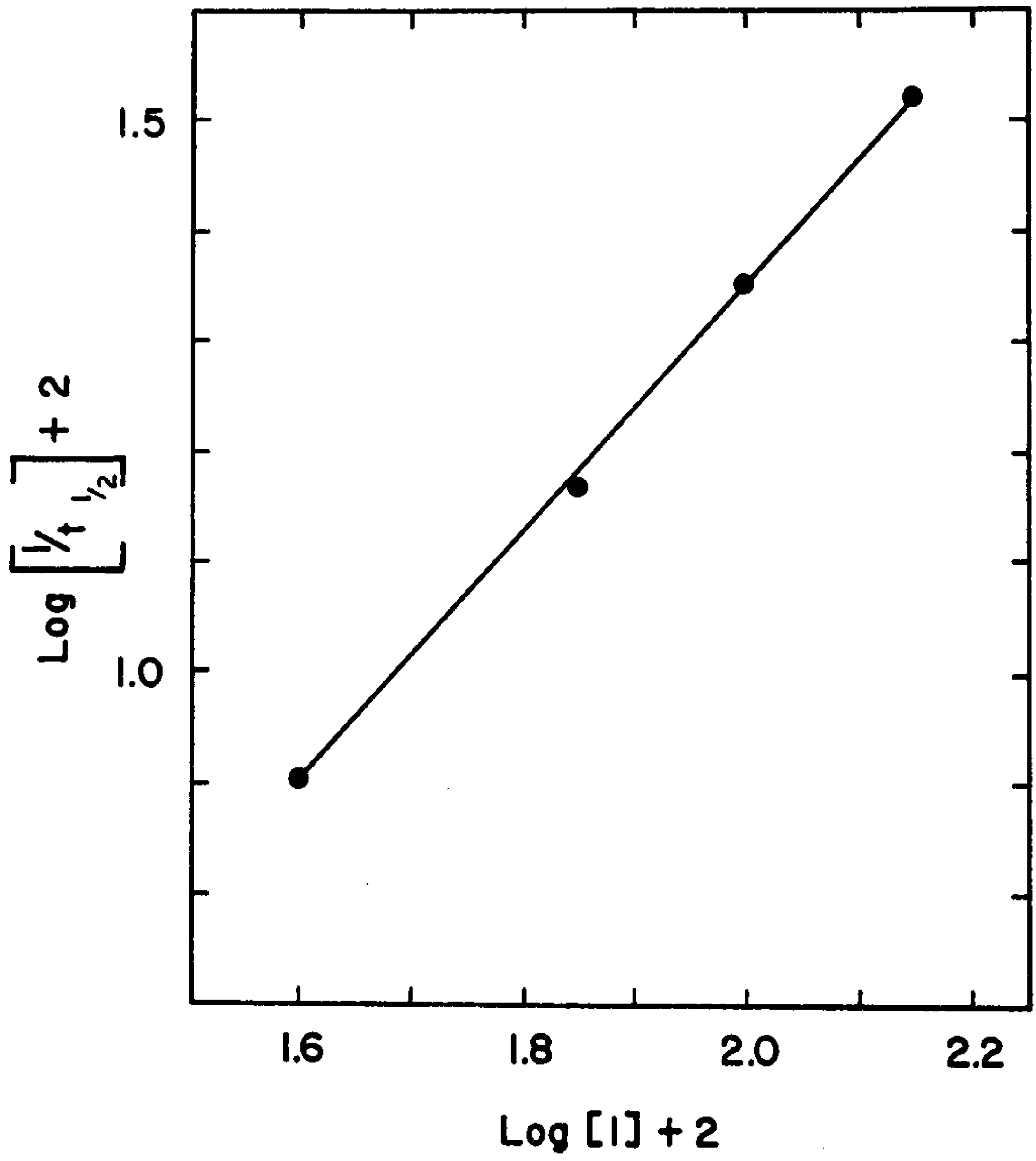

% Residual Activity
100
90
80
70
60
50
40
30
20
10
1
2
3
4
mol TNP/ mol Sigma

FIGURE 6: Inactivation of sigma by TNBS. In a 35 ul reaction mix, 17.5 ug sigma with BTP buffer and varying concentrations of TNBS were incubated. At various time intervals 5 ul aliquots were removed and added to the RNA polymerase assay system containing 10 ug core polymerase and 20 ug T5 DNA. Appropriate controls with no TNBS added and a blank with TNBS (1.5 mM) but not sigma added were also run.



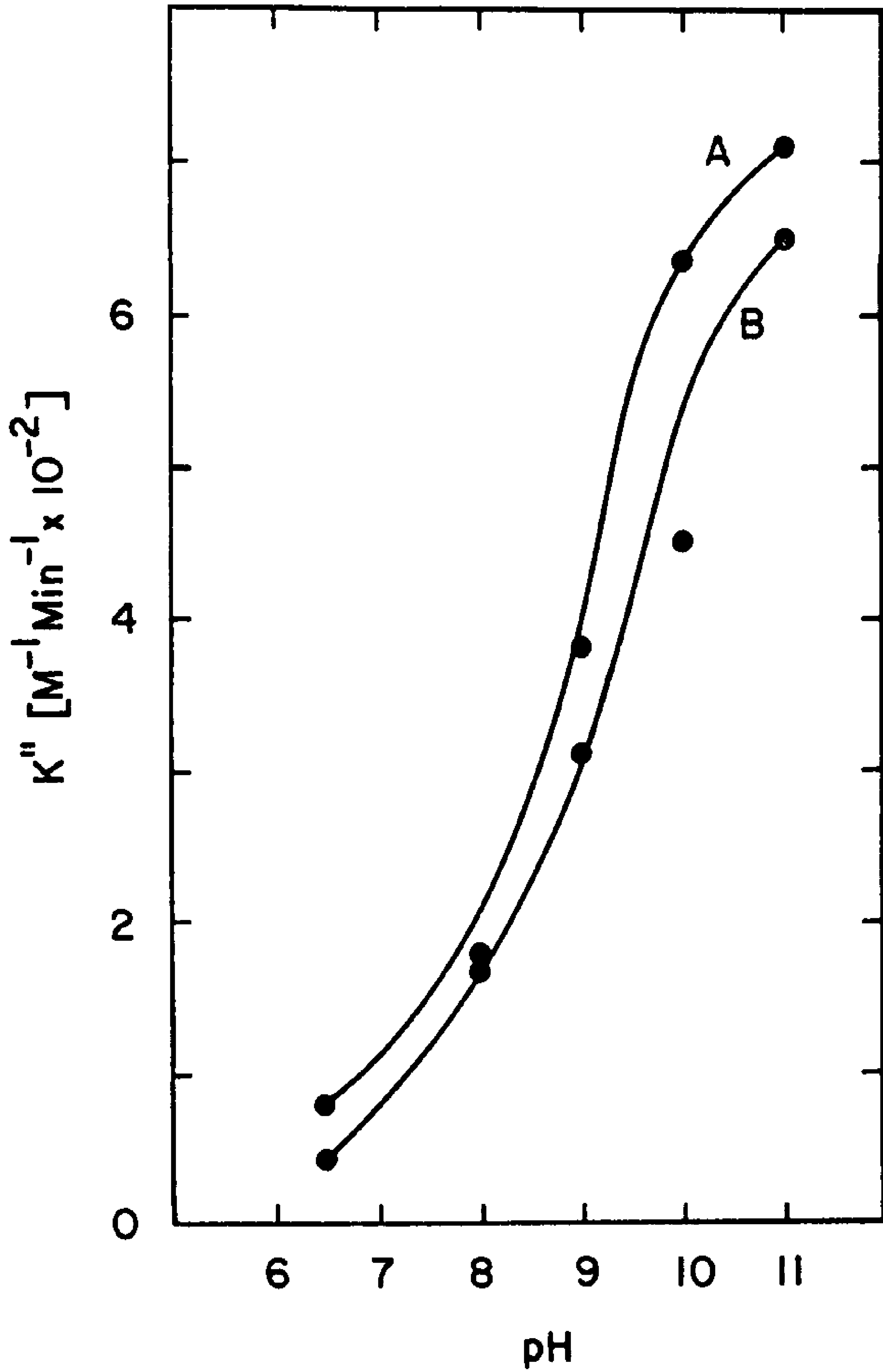
Log % Residual Activity
2
1.5
1
0
5
10
15
20
25
30
Time (min)
1.4
1
.7
.4

FIGURE 7: Reaction order of sigma inactivation with respect to TNBS concentration. Logarithm values of $1/t_{\frac{1}{2}}$ (Log k') were plotted against the respective Log TNBS concentrations in mM (Log I). Both values were obtained from Figure 6.



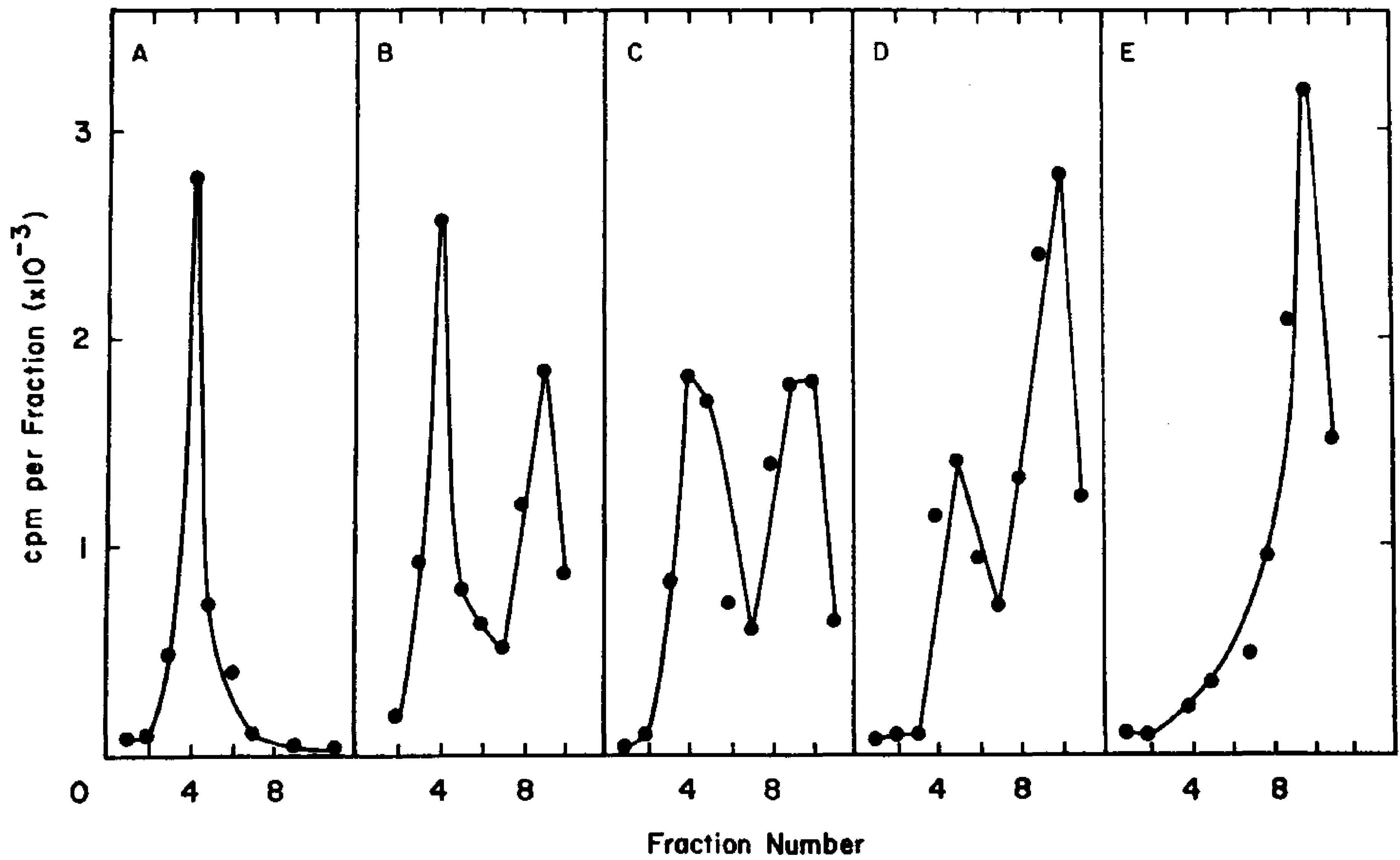
1.5
1.0
Log [1/t 1/2] + 2
1.6
1.8
2.0
2.2
Log [I] + 2

FIGURE 8: Inactivation of sigma by TNBS as a function of pH. Reactions were carried out as described in Figure 6 with the pH of the BTP buffer varying from 6.5 to 11. The second order rate constant k'' for each of the pH values was calculated from the equation $k'' = k'/(I)^n$. A, pApU assay system; B, T5 DNA assay system.



K'' [M⁻¹Min⁻¹ x 10⁻²]
0
2
4
6
A
B
6
7
8
9
10
11
pH

FIGURE 9: Reconstitution of TNP-sigma with core polymerase. In 50 ul TMS buffer containing 0.5 M NaCl, 100 ug core polymerase was mixed with varying amounts of (^{3}H) TNP-sigma (6 TNP per sigma) and incubated at 37°C for 5 minutes and layered on the gradient and centrifuged as described in methods. Fractions of 0.4 ml were collected and aliquots were used for determination of protein, radioactivity and analyses by SDS polyacrylamide gel electrophoresis. The core polymerase to (^{3}H) TNP-sigma ratios are A, 0:1; B, 0.25:1; C, 0.5:1; D, 0.75:1 and E, 1:1.



A
B
C
D
E
cpm per Fraction (x10^{-3})
3
2
1
0
4
8
4
8
4
8
4
8
4
8
Fraction Number

FIGURE 10: Binding affinity of TNP-sigma to core polymerase. In 50 ul TMS buffer containing 0.5 M NaCl, 25 ug (^{3}H) TNP-sigma (6 TNP per sigma) was mixed with 12.5, 25 or 50 ug of sigma (curves A, B or C respectively) and incubated with 100 ug of core polymerase at 37°C for 5 minutes. Following the gradient centrifugation the collected fractions were analyzed as described in Figure 9. The molar ratios of core polymerase to (^{3}H) TNP-sigma to sigma are A, 1:1:0.5; B, 1:1:1 and C, 1:1:2.

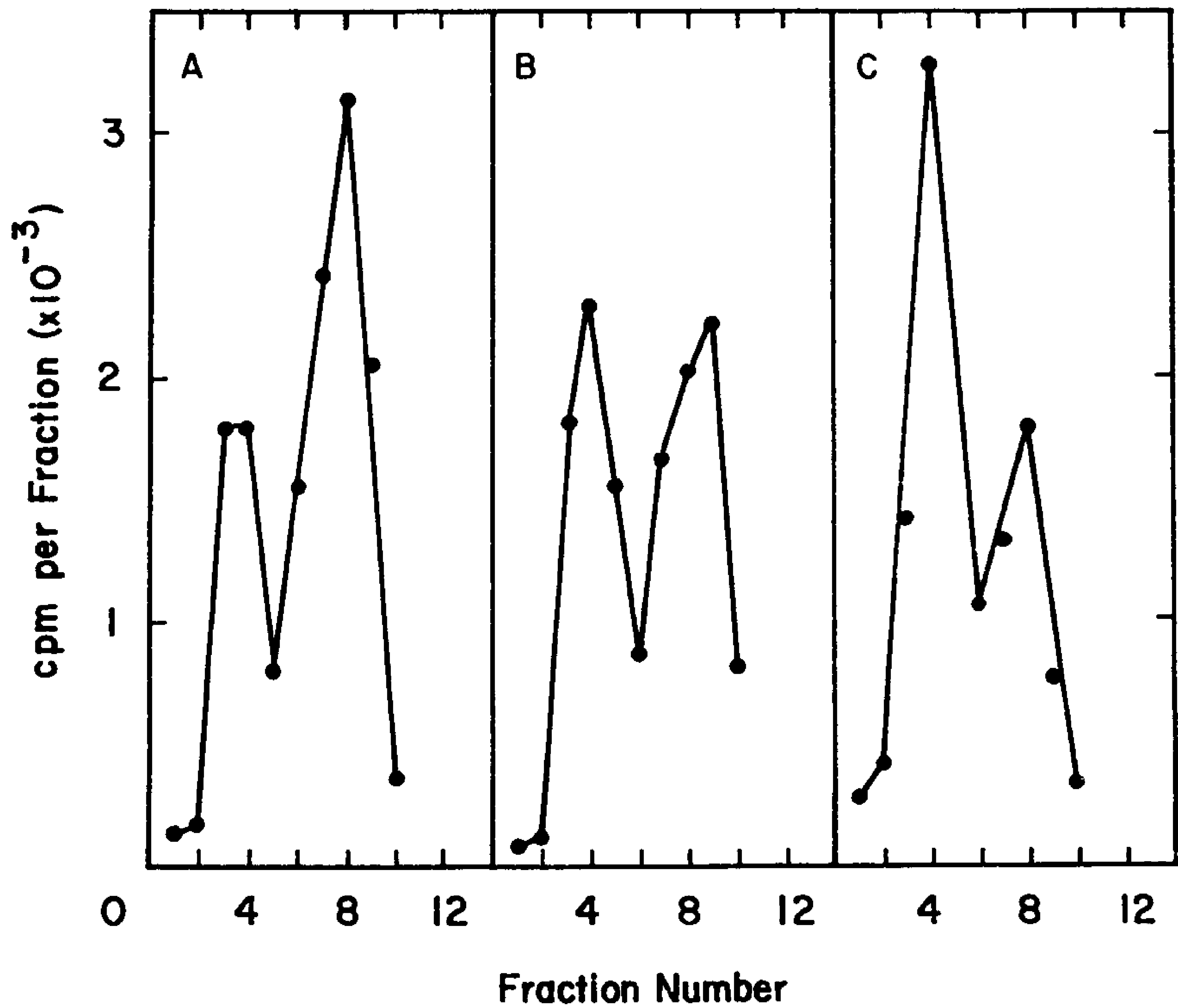
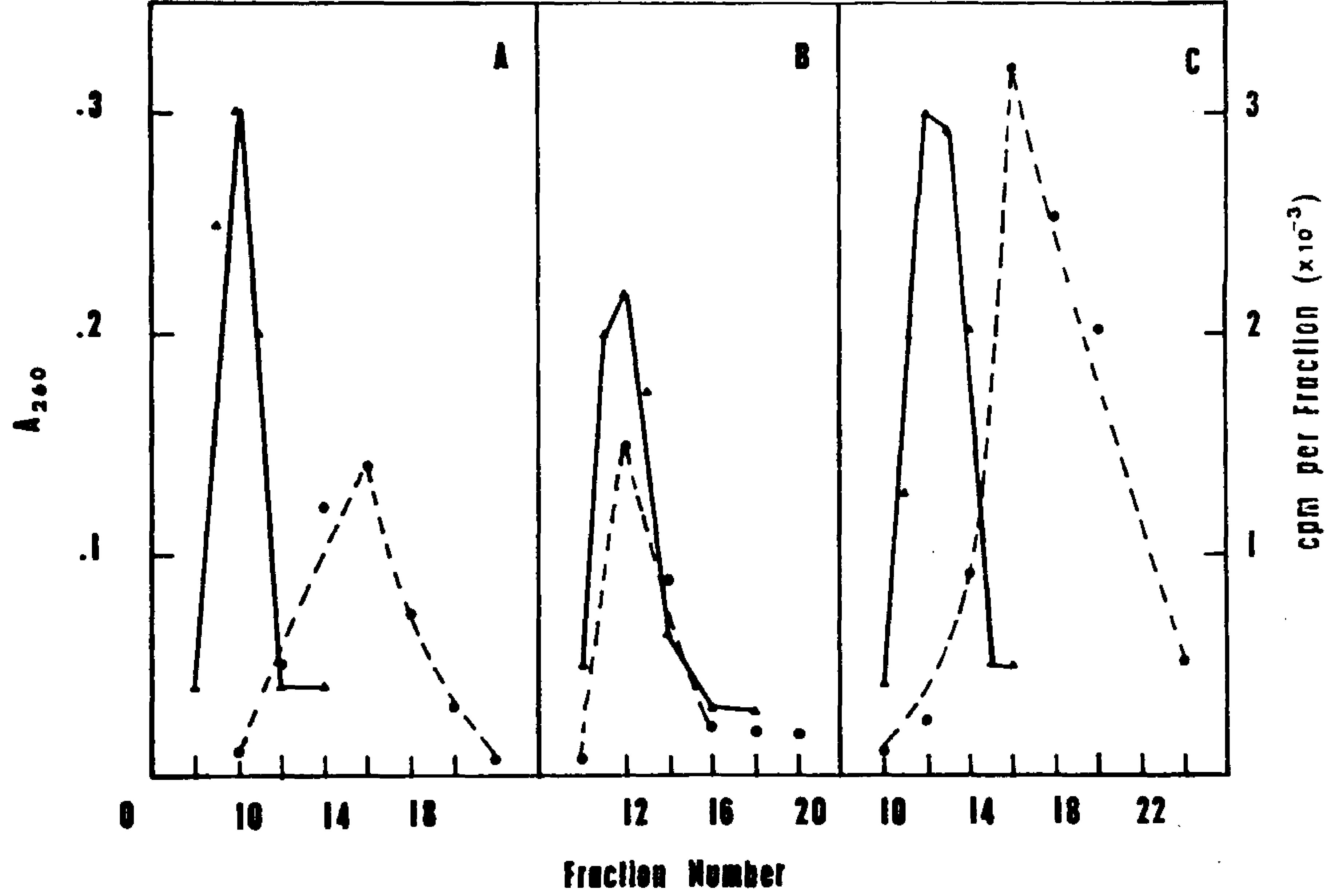
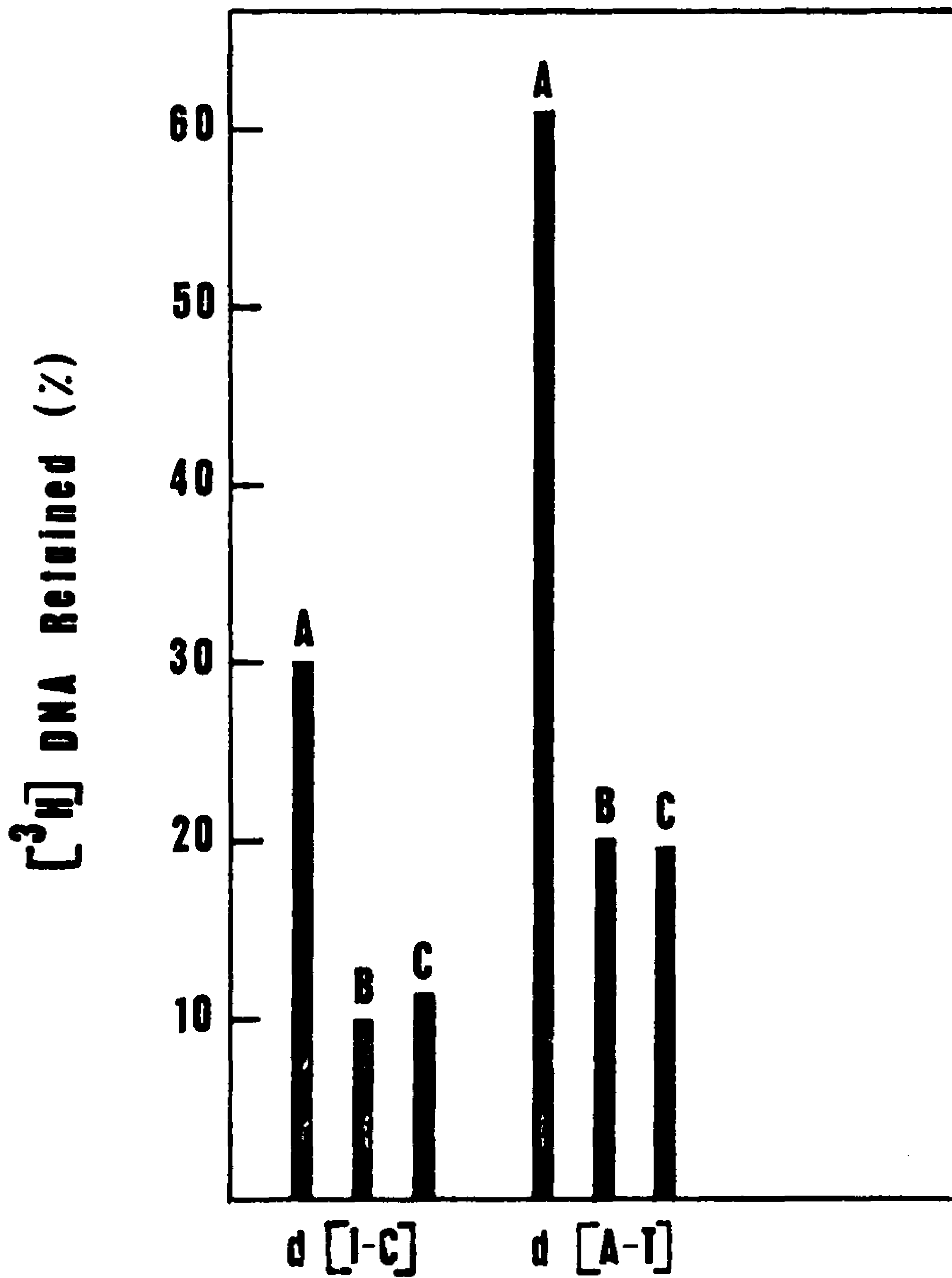

A
B
C
cpm per Fraction (x10^-3)
3
2
1
0
4
8
12
4
8
12
4
8
12
Fraction Number

FIGURE 11: Reconstitution of TNP-sigma with core polymerase bound to DNA. A) In a 50 ul TMS buffer 25 ug of (^{3}H) TNP-sigma (10 TNP per sigma) was mixed with 75 ug T7 DNA or $d(A-T)_n$, incubated at 37°C for 5 minutes and filtered through Ultrogel ACA-34 column with TMS buffer containing no NaCl. B) Core polymerase and DNA (100 ug each) were incubated in 50 ul TMS buffer for 5 minutes at 37°C. Later 25 ug (^{3}H) TNP-sigma was added and incubation continued for 5 minutes. The mixture was filtered through the column with TMS buffer containing no NaCl. C) The experiment was similar to B except that the gel filtration was in the presence of 0.5 M NaCl. Dotted line, radioactivity and solid line, A_{260}.



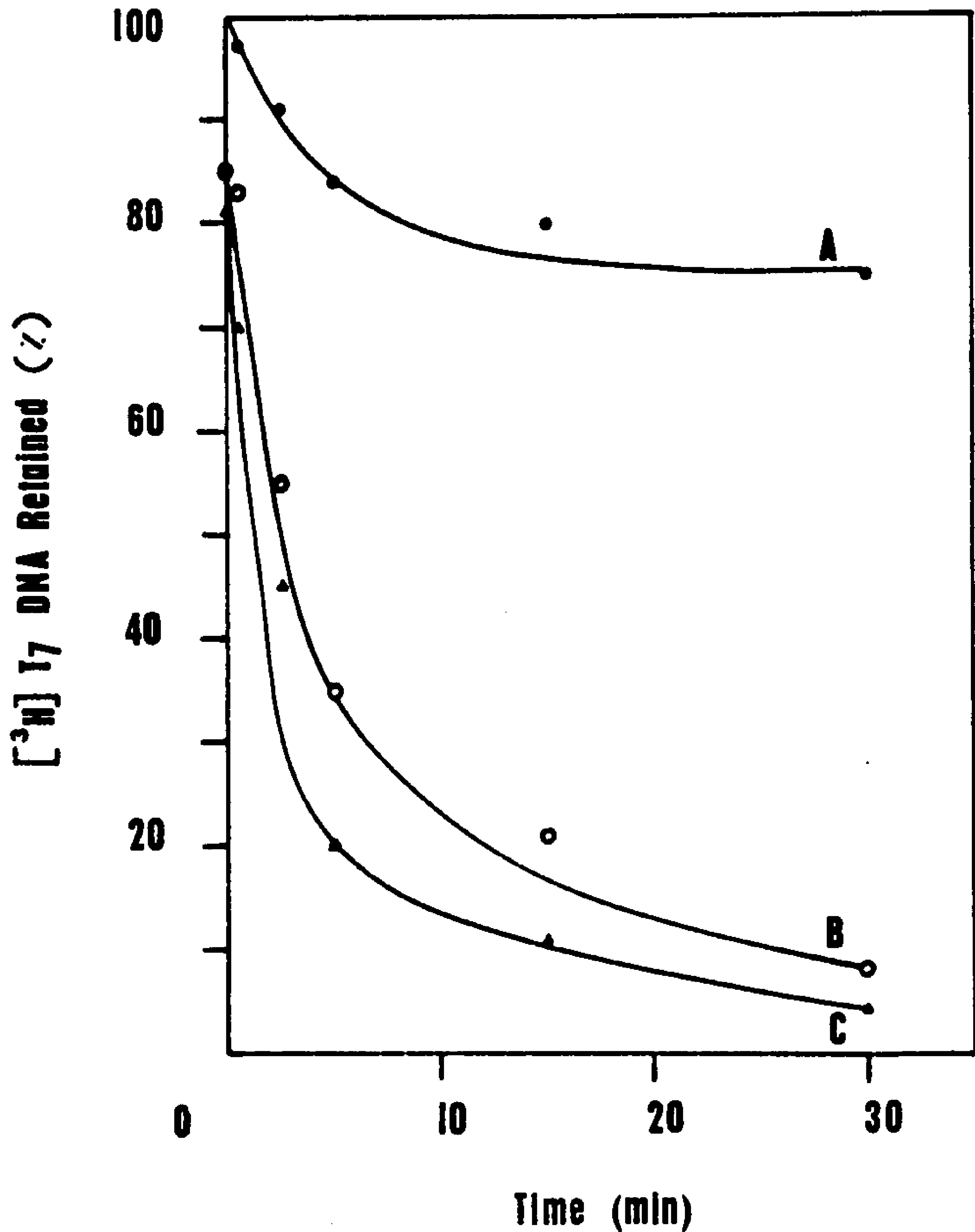
A
B
C
A260
.1
.2
.3
cpm per Fraction (x10^-3)
1
2
3
0
10
14
18
12
16
20
22
Fraction Number

FIGURE 12: Formation of a heparin stable template complex. The reaction mix contained, in 100 ul of binding buffer A, 5 nmol (^3H) $d(A\text{-}T)_n$ or $d(I\text{-}C)_n$ (approximate specific activity of 2000 cpm per nmol) and 5 ug core polymerase + sigma (A), core polymerase (B) or core polymerase + TNP-sigma (7 TNP per sigma) (C). The mix was incubated at 37°C for 10 minutes. A solution of 0.25 ug heparin (in water) was added and incubation continued for 10 minutes. The mix was filtered on a nitrocellulose filter, washed with 1 ml of binding buffer A and the radioactivity determined.



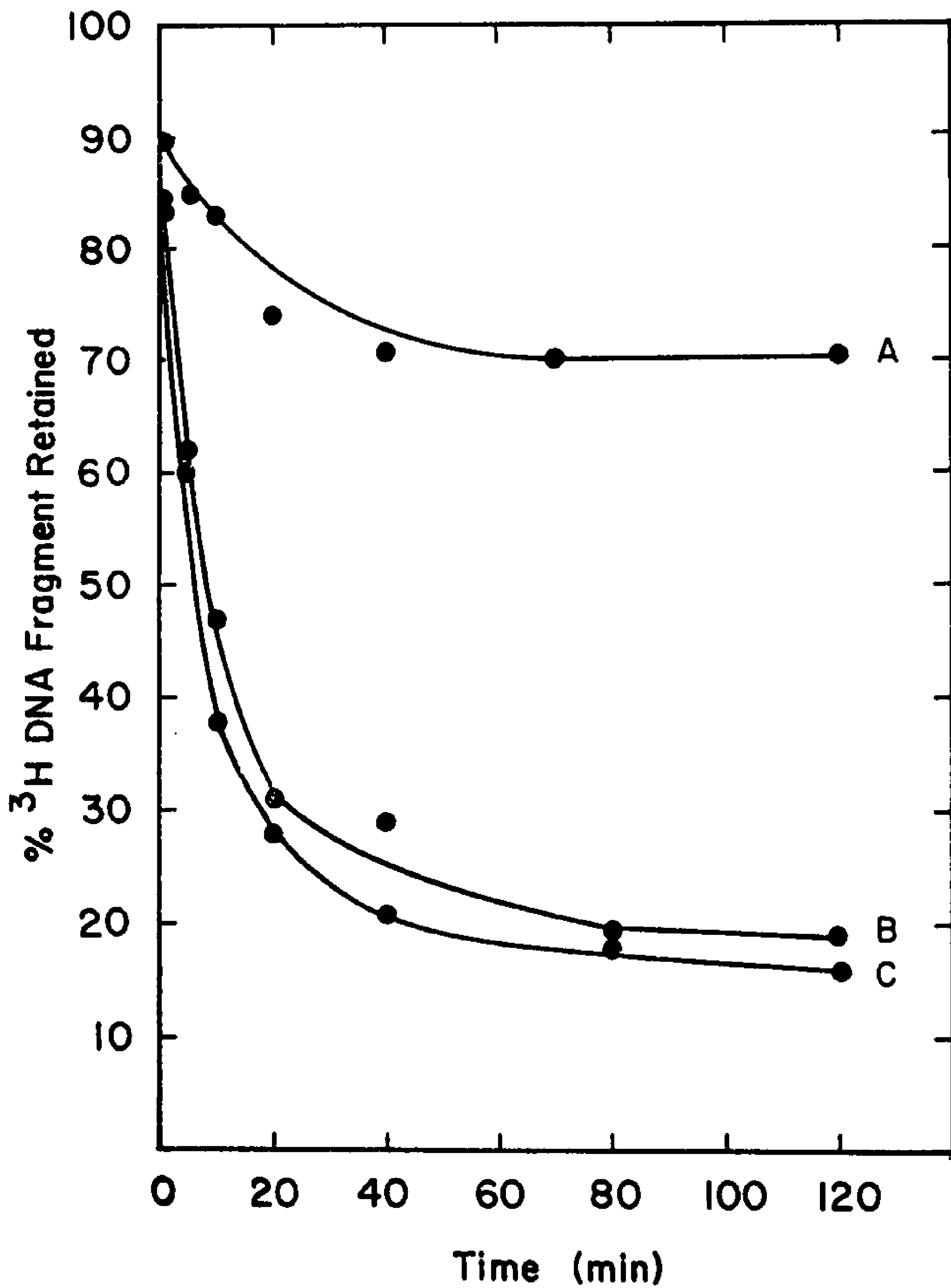
[³H] DNA Retained (%)
60
50
40
30
20
10
A
B
C
A
B
C
d [I-C]
d [A-T]

FIGURE 13: Formation of a stable T7 DNA-polymerase complex. In 800 ul binding buffer A, 25 nmol (^3H) T7 DNA (7500 cpm per nmol) was incubated at 37°C with 2.4 ug core polymerase (curve C), 1.8 ug core polymerase + 0.6 ug sigma (curve A) or 1.8 ug core polymerase + 0.6 ug TNP-sigma (6 TNP per sigma) (curve B). At the end of 5 minutes (0 time) a 100 ul aliquot was diluted to 1 ml with binding buffer A and filtered on a nitrocellulose filter. To the remiaining mix 250 nmol of unlabelled T7 DNA was added, the incubation continued and 100 ul aliquots were removed at intervals, diluted with 1 ml binding buffer A and filtered.



100
80
60
40
20
A
B
C
0
10
20
30
Time (min)
[³H] T7 DNA Retained (%)

FIGURE 14: Formation of a stable promoter-polymerase complex. These studies were carried out as described for Figure 13 except that 25 nmol of BclI 'B' fragment of (^{3}H) T7 DNA were used.



100
90
80
70
60
50
40
30
20
10
% ³H DNA Fragment Retained
A
B
C
0
20
40
60
80
100
120
Time (min)

FIGURE 15: Formation of T5 DNA promoter-polymerase complex. T5 DNA (25 nmol) restricted with HindIII and 1 ug core polymerase (B,C,D), 1 ug core polymerase + 0.25 ug sigma (E, F, G, H) or 1 ug core polymerase + 0.25 ug TNP-sigma (6 TNP per sigma) (I,J,K,L) were preincubated at 37°C for 2 minutes in 50 ul binding buffer B. Denatured calf thymus DNA (15 ug) was added and incubation continued for 10 minutes, 1, 3 or 6 hours, filtered and electrophoresed as described in methods. A, 9 nmol of T5 DNA fragments; B,C,D, core polymerase-DNA fragments competed by calf thymus DNA for 10 minutes, 1 and 3 hours; E,F,G,H, core polymerase + sigma-DNA fragments competed for 10 minutes, 1, 3 and 6 hours; I,J,K,L, core polymerase + TNP-sigma-DNA fragments competed for 10 minutes, 1, 3 and 6 hours.

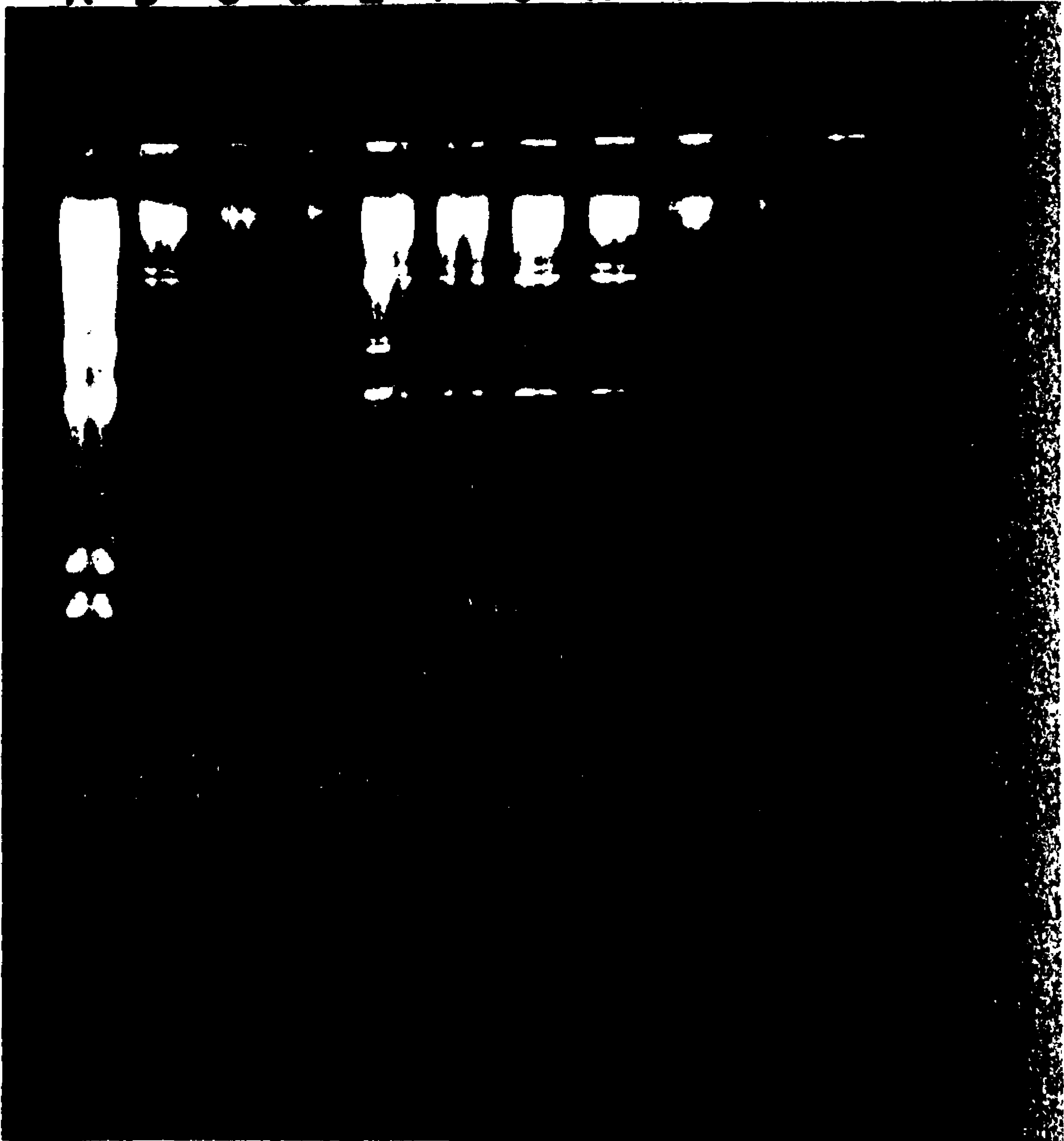

A B C D E F G H I J K L

FIGURE 16: Progressive trinitrophenylation of sigma and promoter recognition. The study was done as described in Figure 15 using core polymerase and TNP-sigma modified to various degrees. The competition was for 10 minutes, 1 or 5 hours. A,B,C, 1 TNP per sigma; D,E,F, 3 TNP per sigma; G,H,I, 4 TNP per sigma and J,K,L, 8 TNP per sigma.

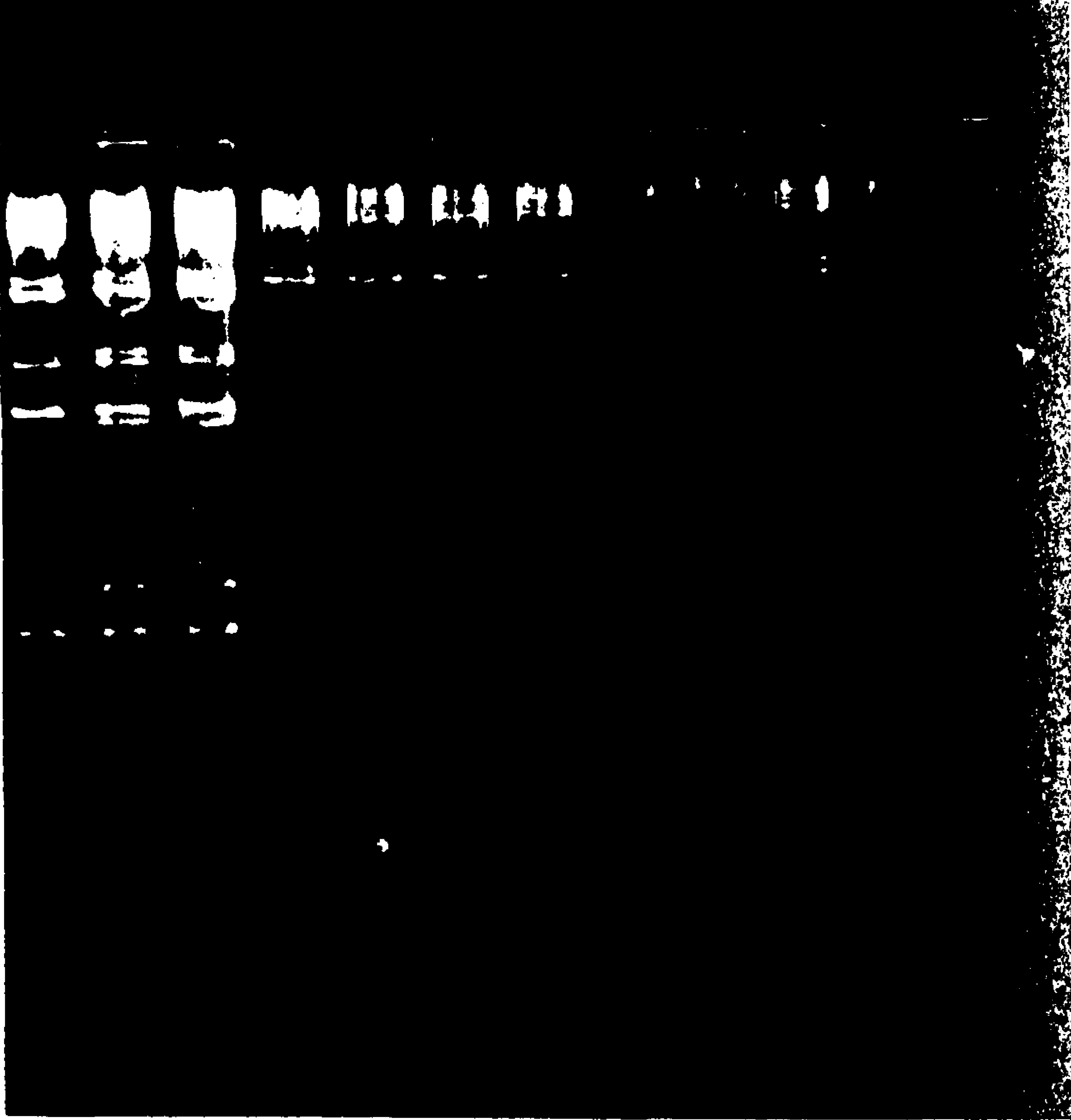
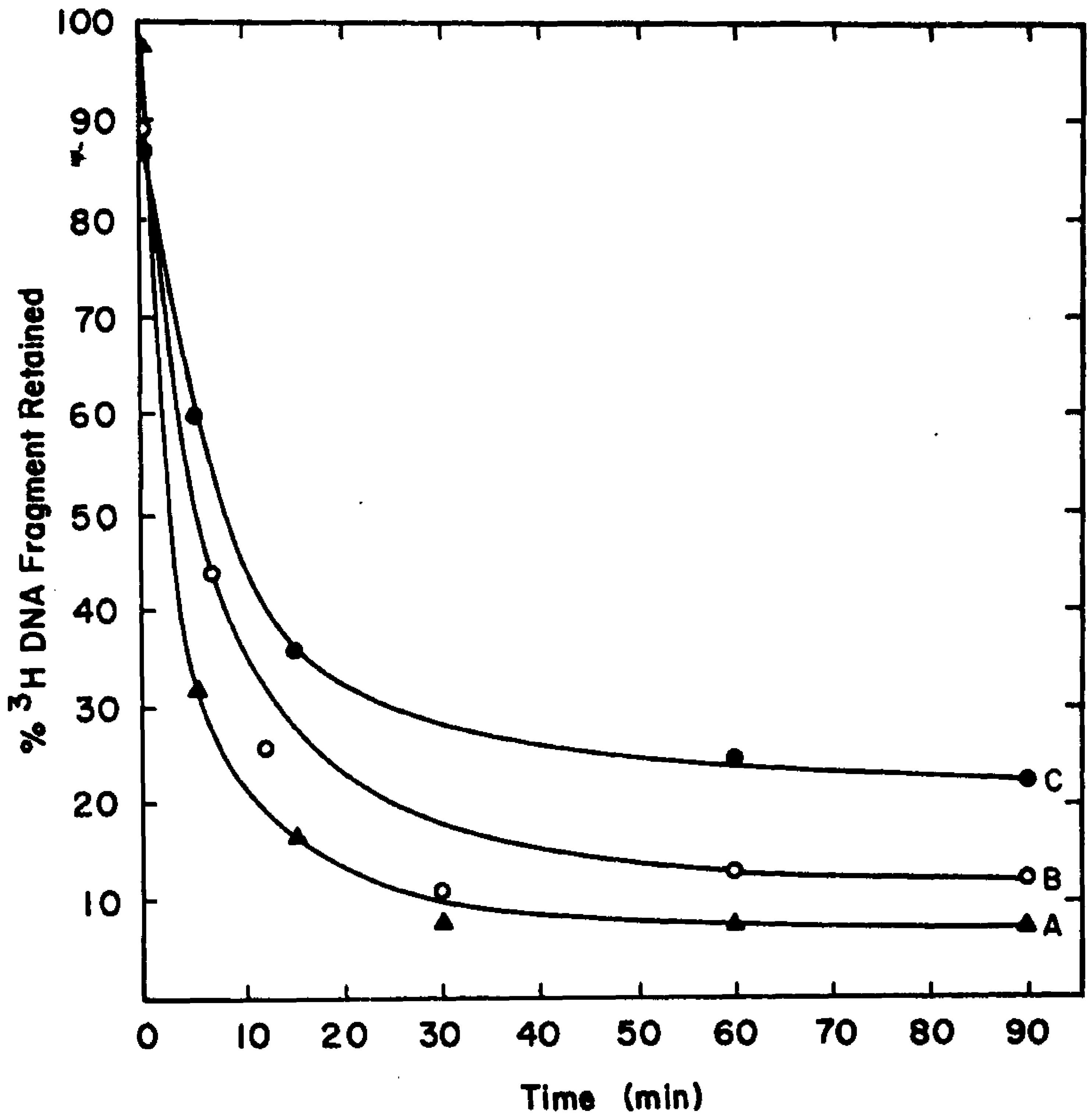

A B C D E F G H I J K L

FIGURE 17: Formation of general DNA-polymerase complex. (^{3}H) T7 HaeIII fragment 'A' (Studier et al., 1979) (15 nmol) in 60 ul binding buffer A was incubated at 37°C with 1 ug core polymerase or 1 ug core polymerase with sigma or TNP-sigma (7 TNP per sigma). At the end of 10 minutes (0 time) 10 ul was diluted into 1 ml of binding buffer A and filtered. To the remainder was added 75 nmol of unlabelled denatured T7 DNA and 10 ul aliquots removed at 10, 30, 60 or 90 minutes, diluted with 1 ml of binding buffer A and filtered. A, core polymerase + sigma; B, core polymerase + TNP-sigma and C, core polymerase.



100
90
80
70
60
50
40
30
20
10
% ³H DNA Fragment Retained
0
10
20
30
40
50
60
70
80
90
Time (min)
C
B
A

FIGURE 18: Formation of T7 DNA promoter-polymerase complex. The studies were done as described in Figure 15 using T7 DNA HaeIII fragments. A,B,C, core polymerase + TNP-sigma (6 TNP per sigma); D,E,F, core polymerase + sigma. The competition in both cases were for 10 minutes, 3 and 6 hours. G,H,I,J,K, core polymerase. The competition was for 10, 30 minutes, 1, 3 or 6 hours. L, HaeIII T7 DNA fragments.

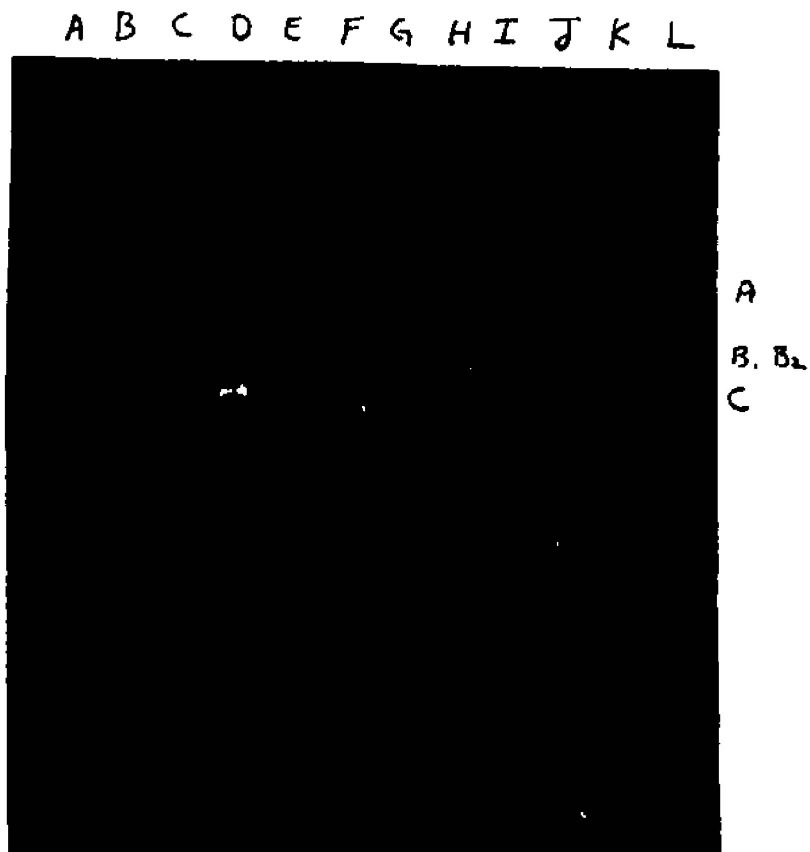


FIGURE 19: Progressive trinitrophenylation of sigma and promoter recognition. The study was done as described in Figure 15 using core polymerase and TNP-sigma modified to various degrees and T7 DNA HaeIII fragments. A,B,C, 2 TNP per sigma; D,E,F, 3 TNP per sigma; G,H,I, 6 TNP per sigma; J,K, 8 TNP per sigma and L, HaeIII T7 DNA fragments.

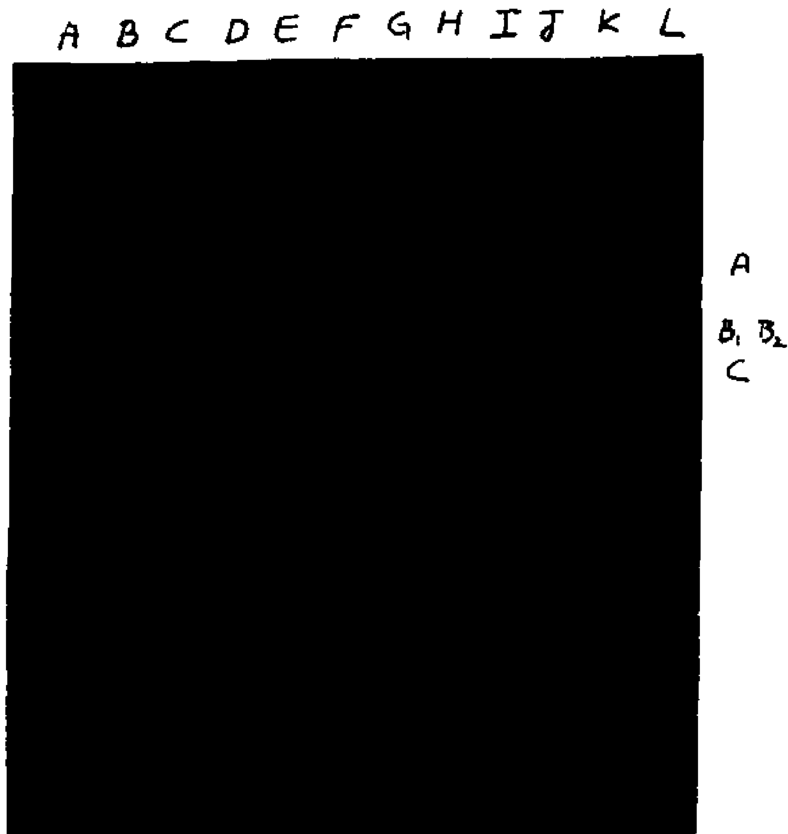


FIGURE 20: Binding studies with nicked T7 DNA. The binary complex between nicked T7 DNA and polymerase was formed by incubating 10 nmol of (^{3}H) T7 DNA with various number of nicks (Hinkle et al., 1972) and 1 ug core polymerase (curve C) or core polymerase with sigma (curve A) or TNP-sigma (6 TNP per sigma) (curve B) in 100 ul binding buffer A at 37°C. At the end of 30 minutes, a 2 fold excess of unlabelled intact competitor T7 DNA was added and incubation continued for 30 minutes. The mix was diluted with 1 ml of binding buffer A, filtered on a nitrocellulose filter and counted.

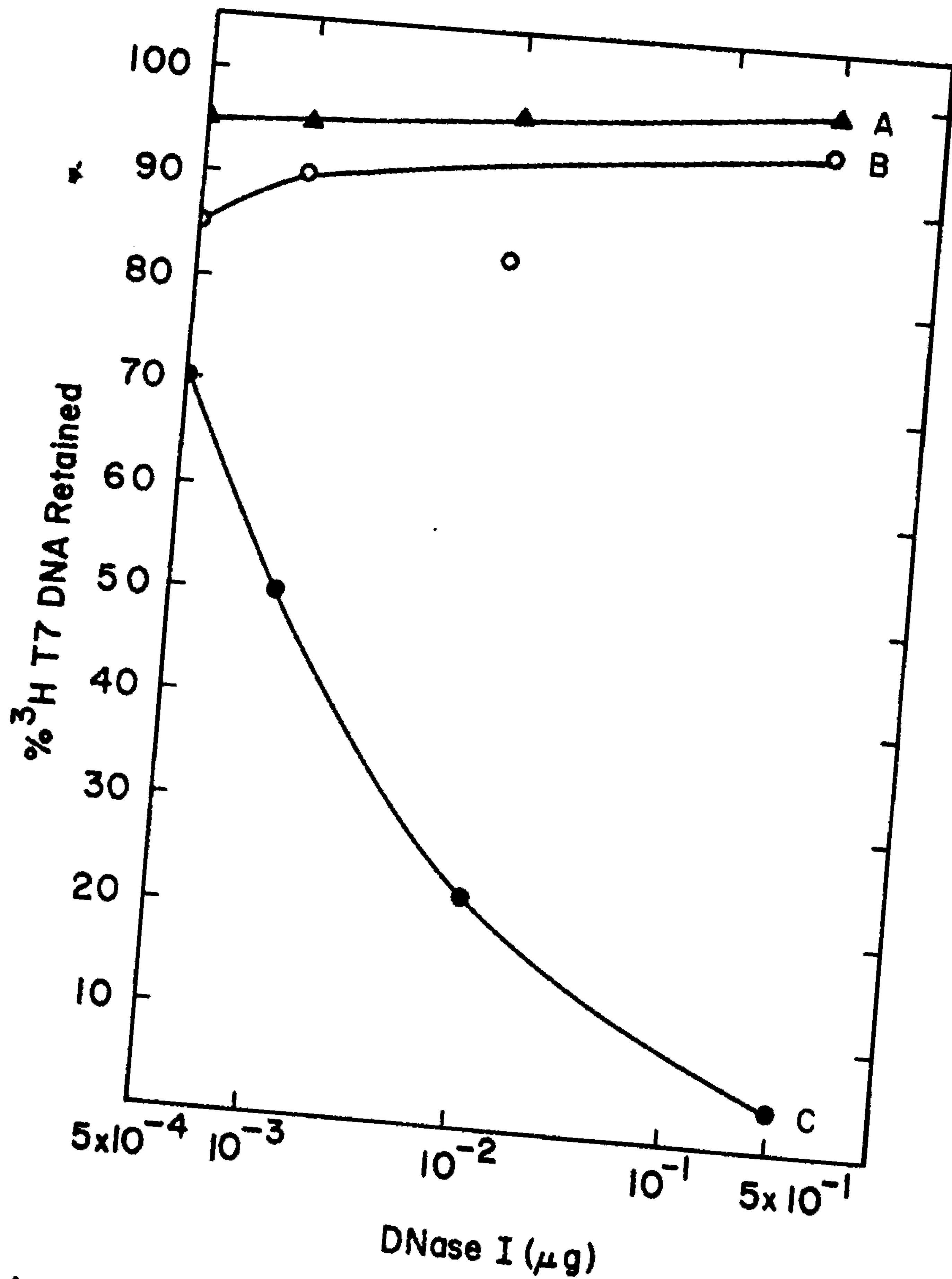
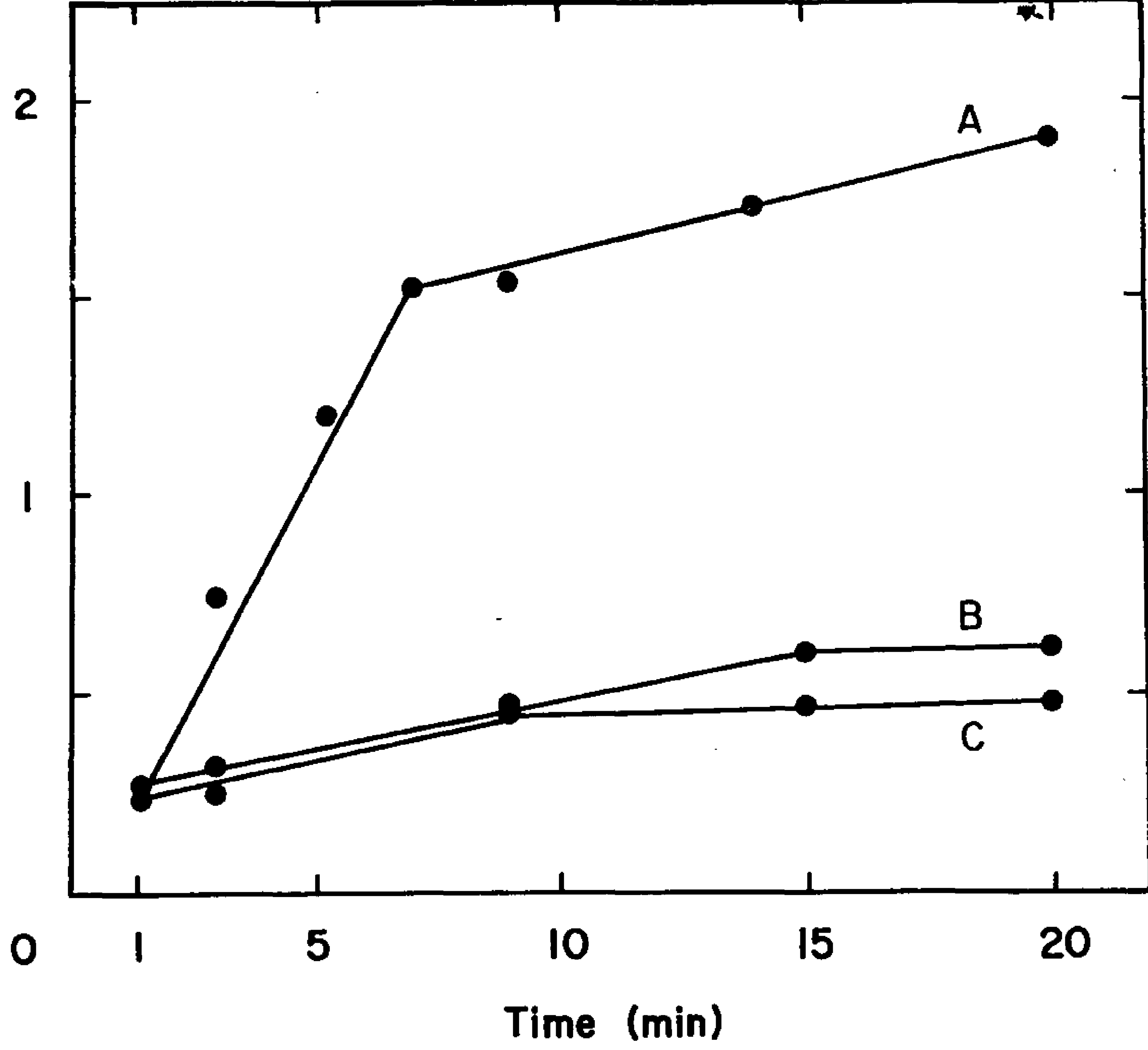

100
90
80
70
60
50
40
30
20
10
% ^{3}H T7 DNA Retained
A
B
C
5x10^{-4}
10^{-3}
10^{-2}
10^{-1}
5x10^{-1}
DNase I (μg)

FIGURE 21: Single round transcription of T7 DNA in presence of heparin. The reaction mix (1 ml) containing 1 mM each of ATP, GTP, CTP and (^{3}H) UTP (specific activity, 15 to 20000 cpm per nmol), 80 mM Tris-HCl (pH 7.8), 40 mM mercaptoethylamine, 20 mM $MgCl_2$, 0.5 mg BSA per ml, 100 pmol core polymerase or core polymerase with 100 pmol sigma or TNP-sigma (5 TNP per sigma) and 1.2 umol of T7 DNA was incubated at 37°C. At the end of 1 minute a 100 ul aliquot was removed for the determination of (^{3}H) UMP incorporated into acid precipitable material. To the remainder was added 15 ul of 20 ug per ul heparin. At intervals 100 ul aliquots were removed for analysis. A, core polymerase + sigma; B, core polymerase + TNP-sigma and C, core polymerase.



(^{3}H) UMP Incorporated (nmol)
2
1
0
1
5
10
15
20
Time (min)
A
B
C

FIGURE 22: Abortive initiation reaction directed by T7 DNA. The reaction mix contained 20 pmol of core polymerase or core polymerase with 20 pmol of sigma or TNP-sigma (6 TNP per sigma), 10 mM $MgCl_2$, 1 mM DTT, 50 mM Tris-HCl (pH 8) and 200 nmol of T7 DNA. Following a preincubation of 2 minutes at 37°C the reaction mix was brought to 1 mM ATP or GTP and (^3H) UTP (5 to 8000 cpm per nmol). The final volume was 200 ul and 50 ul aliquots were removed at intervals, the reaction terminated with 10 ul of 0.5 M EDTA (pH 7.6) and chromatographed using the WASP system. A, core polymerase + sigma; B, core polymerase + TNP-sigma and C, core polymerase.

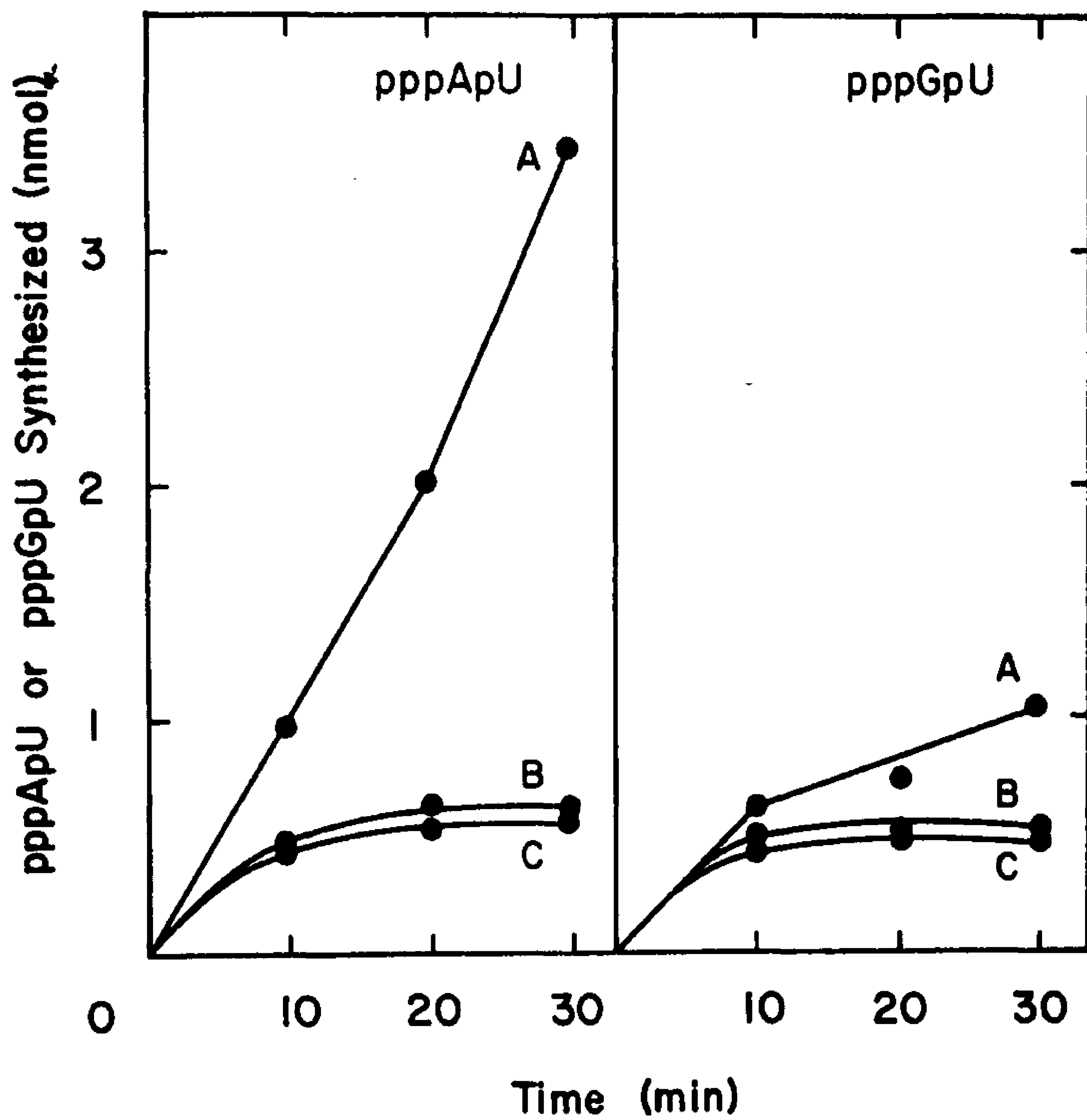
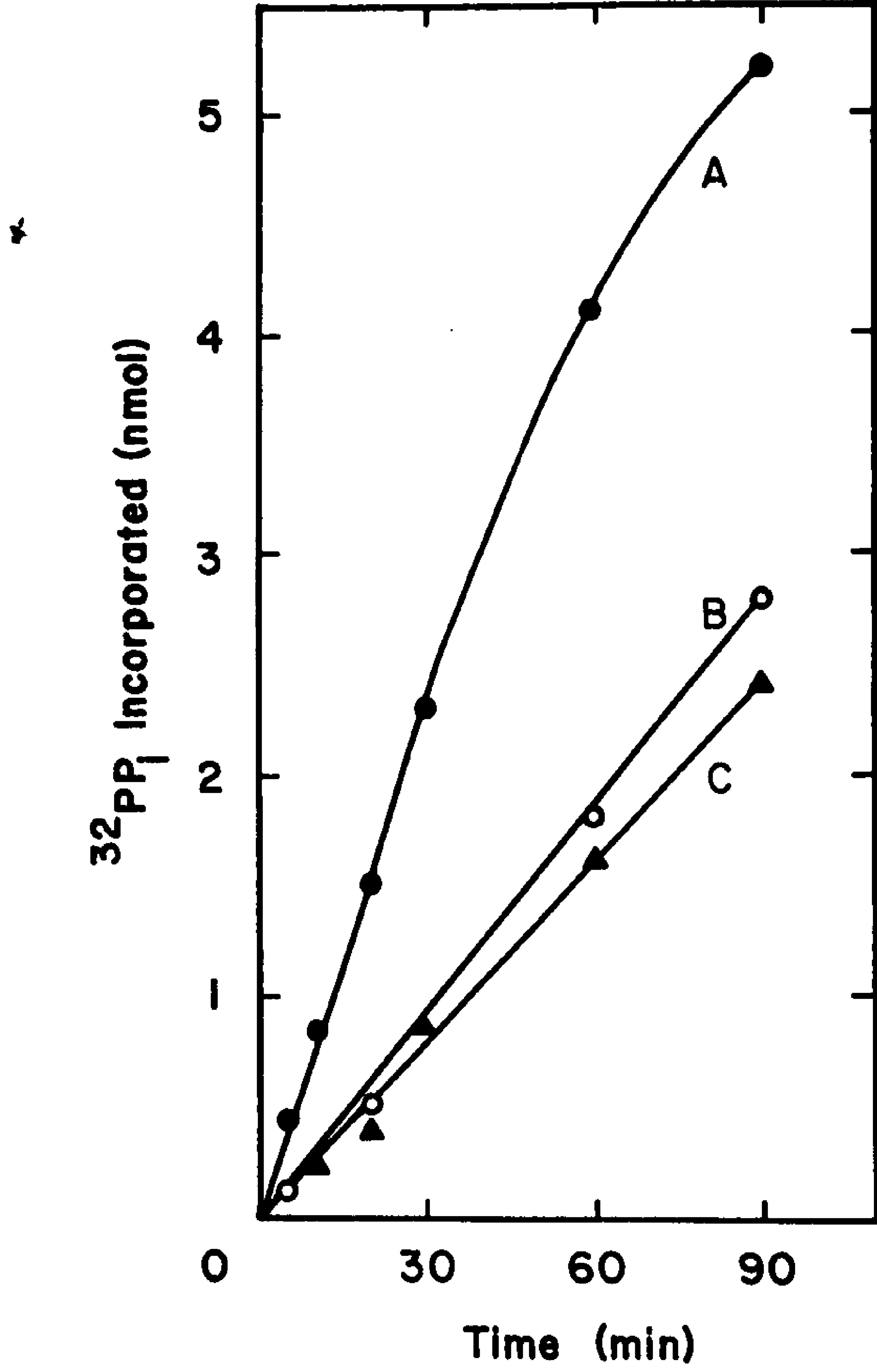

pppApU
pppGpU
A
B
C
A
B
C
pppApU or pppGpU Synthesized (nmol)
1
2
3
0
10
20
30
10
20
30
Time (min)

FIGURE 23: Pyrophosphate exchange assay directed by $d(A\text{-}T)_n$. The reaction mix containing 1.5 nmol of $d(A\text{-}T)_n$, 10 pmol of core polymerase or core polymerase with sigma or TNP-sigma (6 TNP per sigma), 80 mM Tris-HCl (pH 8), 40 mM mercaptoethylamine, 4 mM $MgCl_2$ was incubated for 10 minutes at 37°C. The mix was brought to 1 mM (^{32}P) PPi (5 to 8000 cpm per nmol), 4 uM ATP and 400 uM UTP (the final volume of the mix was 800 ul) and 250 ul aliquots were removed at intervals, the reaction was terminated with unlabelled pyrophosphate and the amount of (^{32}P) PPi incorporated into the charcoal adsorbable form was determined as described by Krakow and Fronk (1969). A, core polymerase + sigma; B, core polymerase + TNP-sigma and C, core polymerase.



$^{32}PP_i$ Incorporated (nmol)
1
2
3
4
5
0
30
60
90
Time (min)
A
B
C

FIGURE 24: Abortive initiation reaction directed by $d(A-T)_n$ using UpA as initiation dinucleoside monophosphate. Core polymerase (10 pmol) with varying amounts of sigma or TNP-sigma (5 TNP per sigma) and 10 nmol of $d(A-T)_n$ were incubated at 37°C for 10 minutes in a reaction mix containing 50 mM Tris-HCl (pH 8), 1 mM DTT, 2 mM Mn^{++} or 10 mM Mg^{++}. The mix was brought to 1 mM (^3H) UTP (5000 cpm per nmol) and 1 mM UpA (50 ul final volume of the mix) and further incubated for 10 minutes. The reaction was terminated and chromatographed. A, core polymerase + sigma; B, core polymerase + TNP-sigma and ▲ , core polymerase.

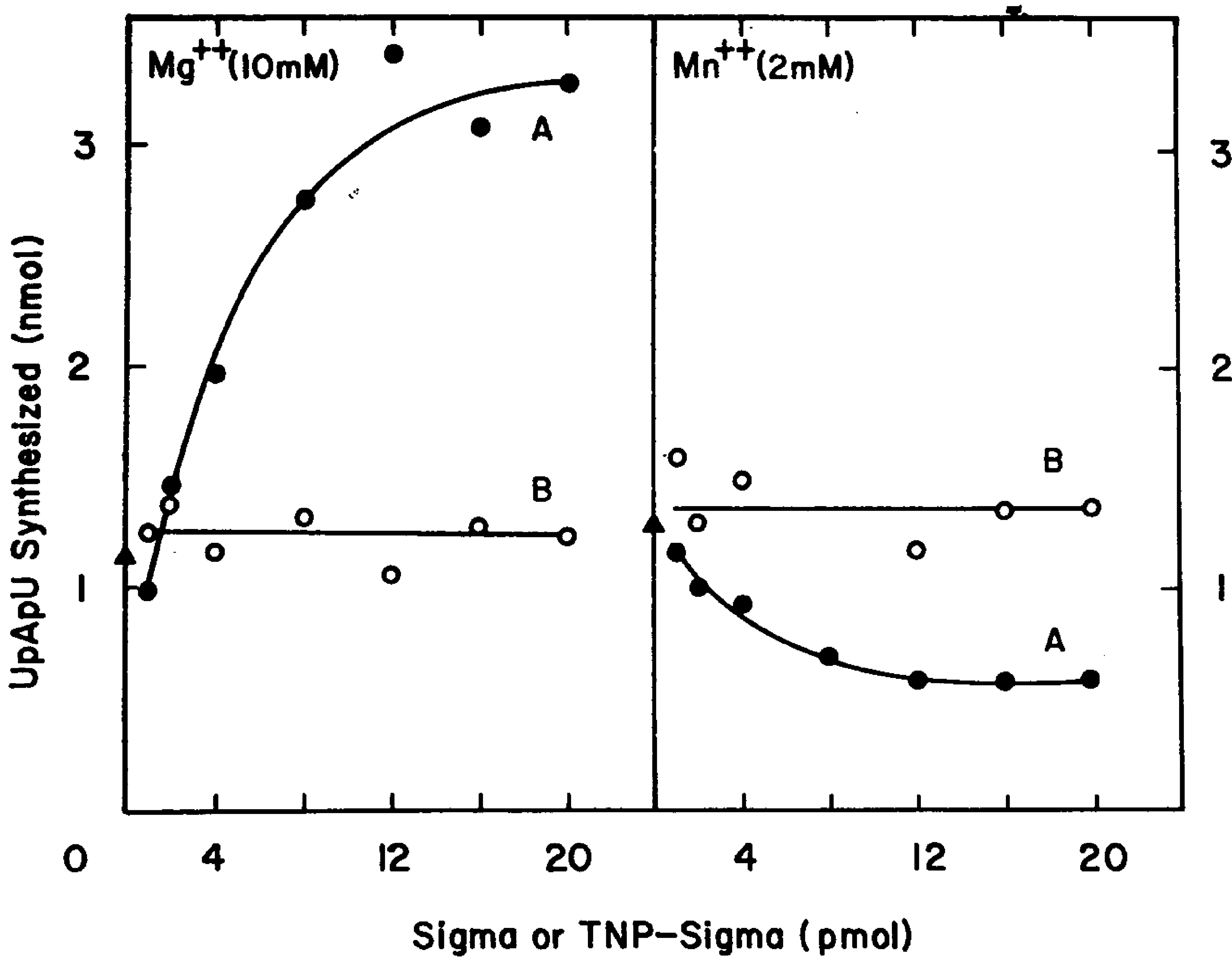

Mg++(10mM)
Mn++(2mM)
A
B
A
B
UpApU Synthesized (nmol)
Sigma or TNP-Sigma (pmol)
0
4
12
20
4
12
20
1
2
3
1
2
3

FIGURE 25: $d(A-T)_n$ directed abortive initiation reaction using AMP as initiation nucleoside monophosphate. Core polymerase (10 pmol) and $d(A-T)_n$ (10 nmol) with varying amounts of sigma or TNP-sigma (5 TNP per sigma) were incubated with 40 mM Tris-HCl (pH 8), 80 mM KCl, 1 mM DTT, 10 mM $MgCl_2$ for 10 minutes at 37°C. The mix (final volume, 50 ul) was brought to 2 mM AMP and 200 uM (^3H) UTP (150 cpm per pmol) and incubation continued for 10 minutes. The reaction was stopped and chromatographed. A, core polymerase + sigma; B, core polymerase + TNP-sigma and ▲, core polymerase.

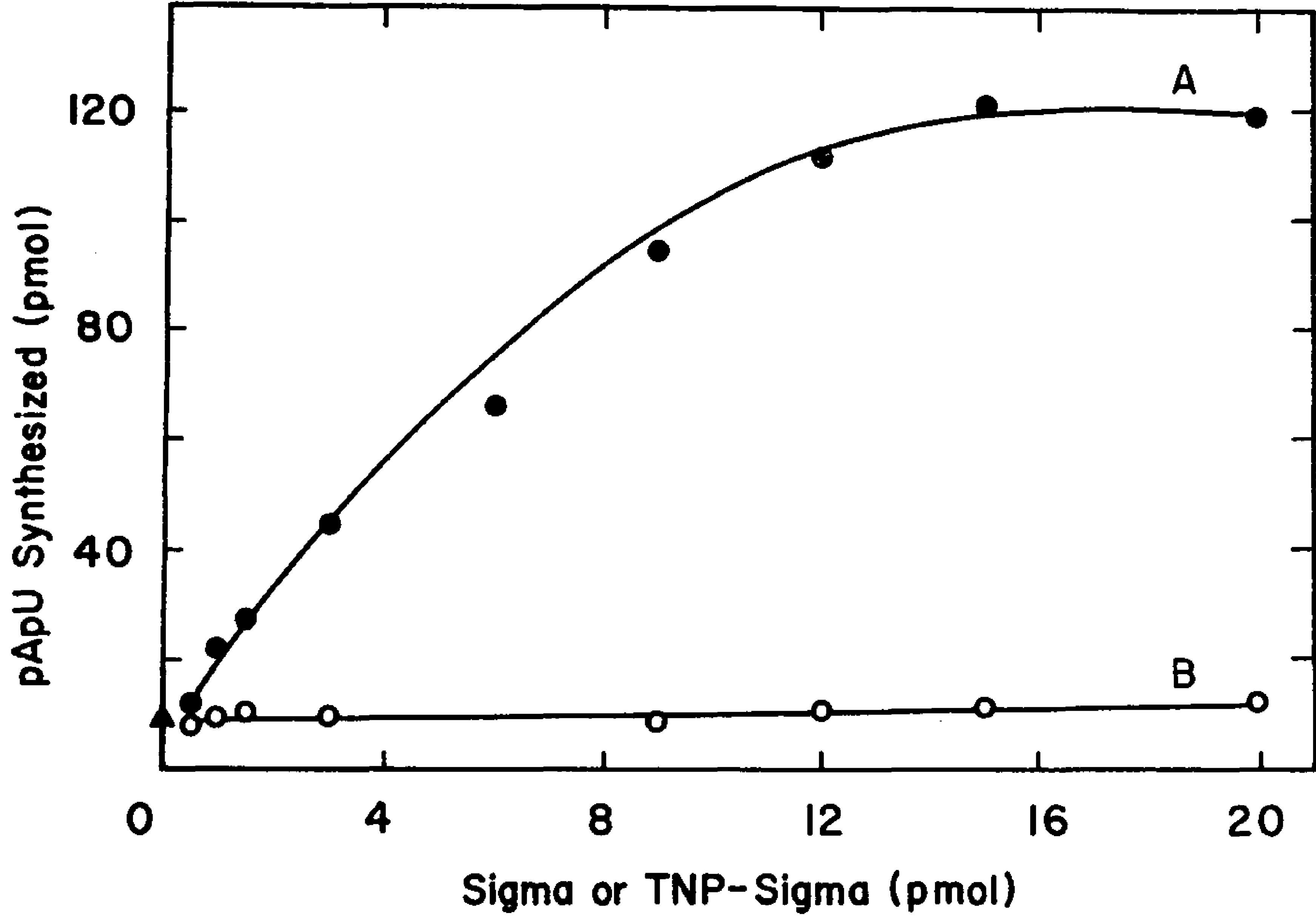

pApU Synthesized (pmol)
120
80
40
0
4
8
12
16
20
Sigma or TNP-Sigma (pmol)
A
B

FIGURE 26: Nonspecific transcription at single stranded nicks on T7 DNA. The reaction mix (250 ul) containing 1 mM each of ATP, GTP, CTP and (^3H) UTP (25000 cpm per nmol), 40 mM mercaptoethylamine, 80 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 0.5 mg BSA per ml, 10 pmol core polymerase or core polymerase with 10 pmol sigma or TNP-sigma (7 TNP per sigma) and 120 nmol T7 DNA (treated with the indicated amounts of DNase I) was incubated at 37°C. At the end of 1 minute, 5 ul of 15 ug per ul heparin solution in water was added, incubation continued for 10 minutes and analyzed for (^3H) UMP incorporated into acid precipitable material. A, core polymerase + sigma; B, core polymerase + TNP-sigma and C, core polymerase.

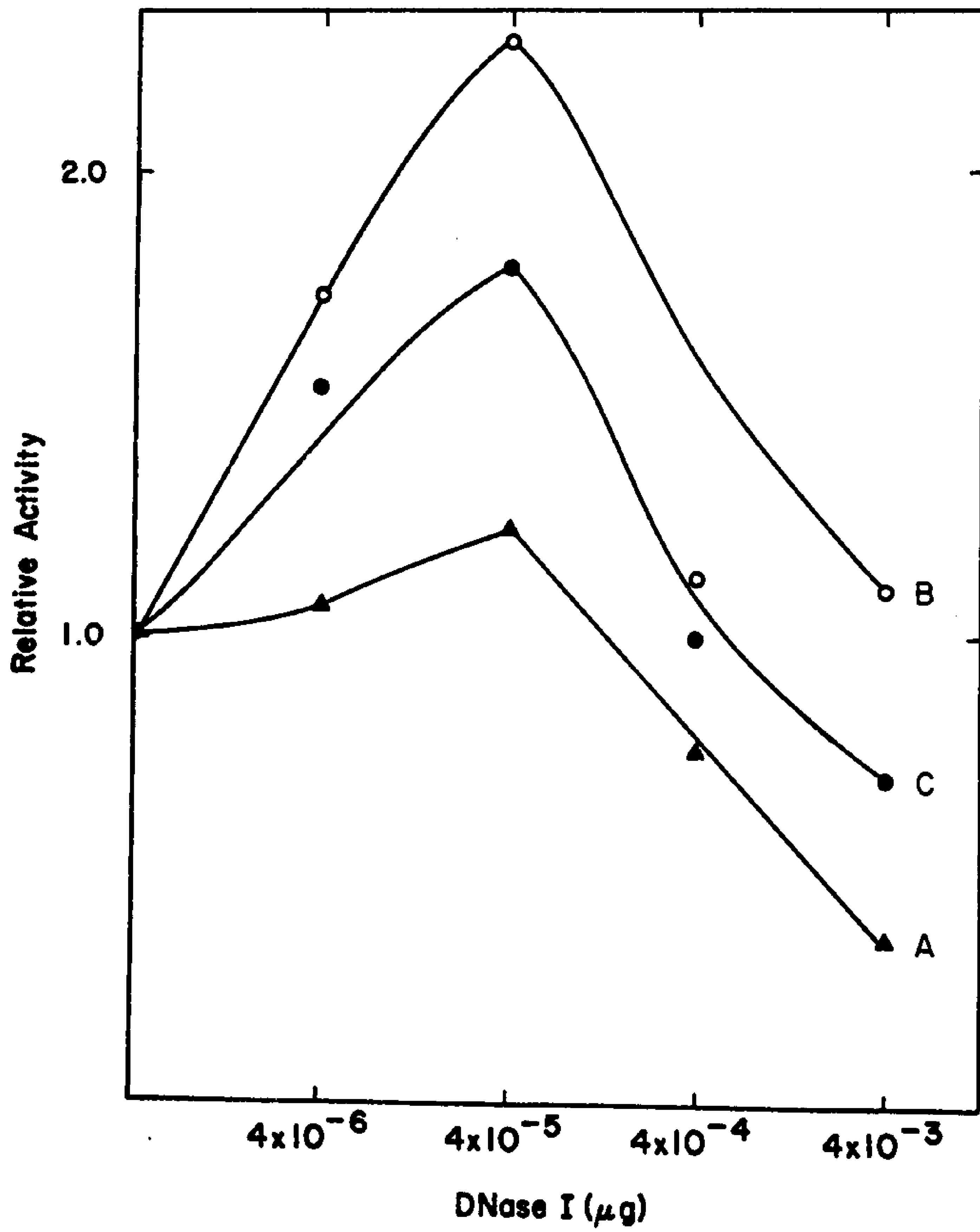

2.0
1.0
Relative Activity
4x10^{-6}
4x10^{-5}
4x10^{-4}
4x10^{-3}
DNase I (μg)
B
C
A

FIGURE 27: Protein determination of the protease digests of TNP-sigma. TNP-sigma (10 ug) modified to various degrees in 20 mM Tris-HCl (pH 8), 0.1 mM DTT was digested with 0.4% (0.04 ug) SAP, 0.25% chymotrypsin or 0.25% trypsin at 37°C for various time. Protein content of the digest was determined according to the procedure described by Schaffner and Weissmann (1973). Degree of modification: 1, 1 TNP per sigma; 2, 4 TNP per sigma; 3, 8 TNP per sigma and 4, 14 TNP per sigma. A, chymotrypsin digest; B, trypsin digest and C, SAP digest.

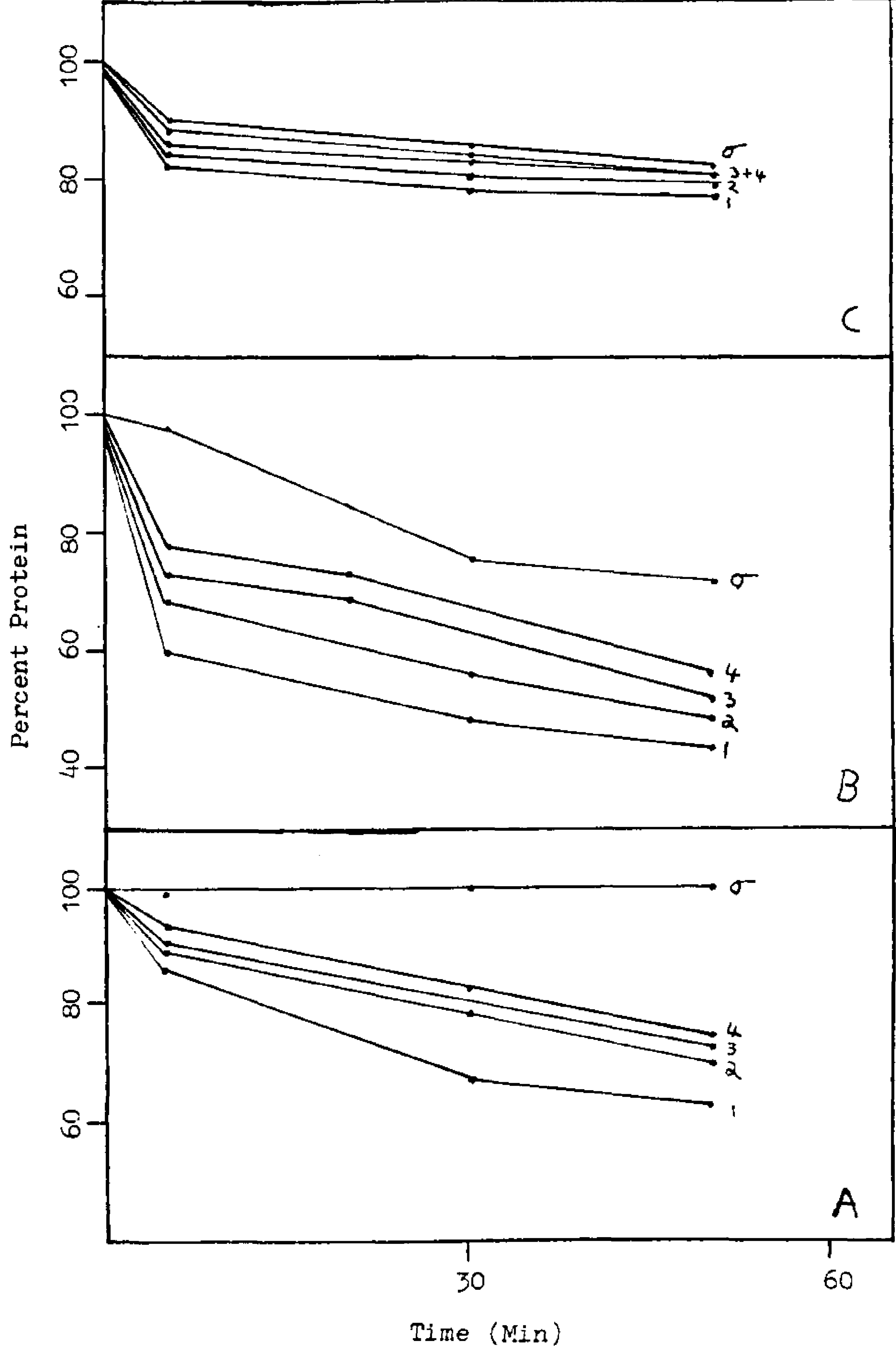
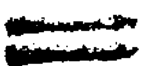
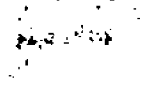
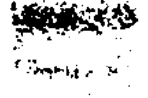
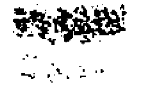

Percent Protein
100
80
60
100
80
60
40
100
80
60
C
B
A
σ
3+4
2
1
σ
4
3
2
1
σ
4
3
2
1
30
60
Time (Min)

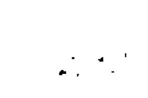
FIGURE 28: A possible conformational change following modification. In a reaction volume of 110 ul, 66 ug of sigma or TNP-sigma (6 TNP per sigma) in 20 mM Tris-HCl (pH 8), 1 mM DTT was digested with 2.6 ug Staphylococcus aureus V8 protease at 37°C. Aliquots of 25 ul were taken at intervals of time and electrophoresed on SDS polyacrylamide gel (7.5%). A-D, TNP-sigma digested for 5, 30, 60 and 90 minutes; E-H, sigma digested for 5, 30, 60 and 90 minutes; I, 10 ug sigma digested for 0 minutes (TNP-sigma shows an identical pattern) and J, 10 ug holoenzyme and 5 ug cAMP receptor protein (mol. wt. 22500).











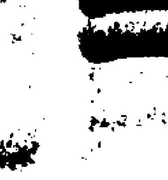

A B C D E F G H I J

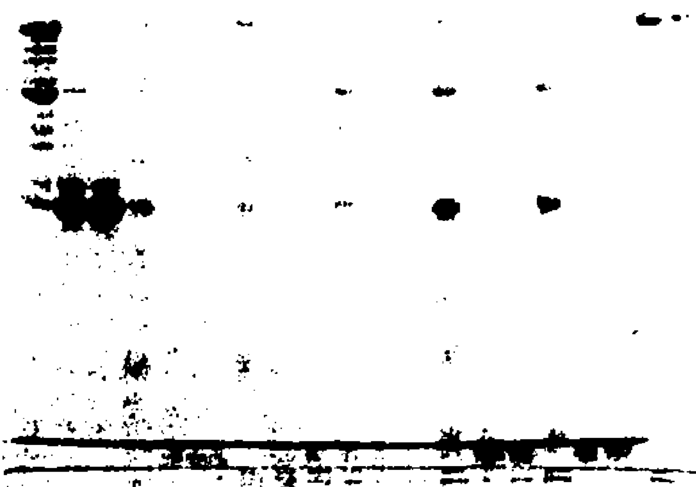
FIGURE 29: A possible conformational change in sigma following modification. In a reaction volume of 40 ul, 10 ug of sigma, TNP-sigma or PG-sigma was digested with 0.4 ug SAP, 0.025 ug trypsin or 0.025 ug chymotrypsin at 37°C for various time intervals in 20 mM Tris-HCl, pH 8, 1 mM DTT. The digests were electrophoresed on SDS polyacrylamide gel (7.5%).

A. Trypsin digest: The digestion was for 5, 10 and 30 minutes. 1,2,3, sigma; 4,5,6, 1 TNP-sigma; 7,8,9, 3 TNP-sigma; 10,11,12, 6 TNP-sigma; 13,14, 15, 2 PG-sigma; 16,17,18, 5 PG-sigma; 19, 5 ug 2 PG-sigma and 20, 5 ug 5 PG-sigma.

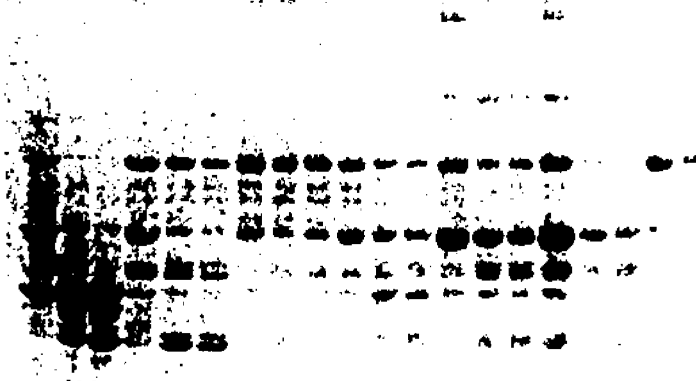
B. SAP digest: The digestion was for 5, 30 and 60 minutes. The legend is same as for A.

C. Chymotrypsin digest: The digestion was for 1, 5 and 15 minutes. The legend is same as for A.

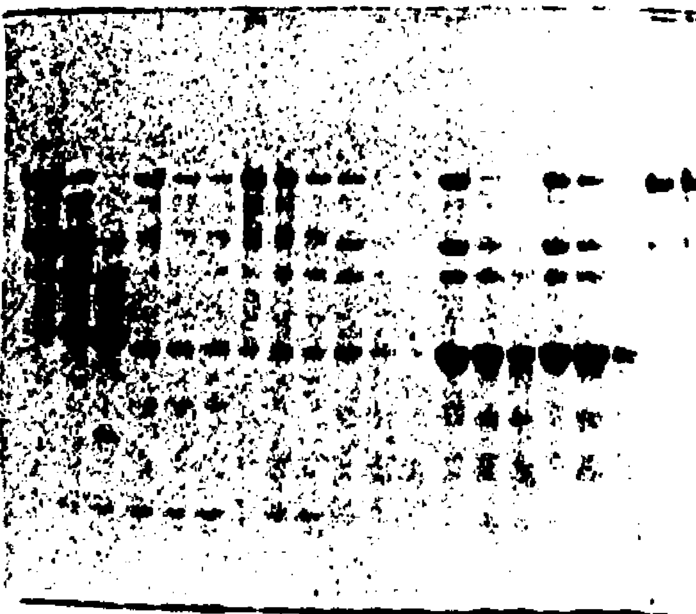
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



TRYPSIN



SAP



CHYMOTRYPSIN

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

FIGURE 30: Time dependent modification of arginyl residues of sigma with phenylglyoxal. In a 20 ul volume 10 ug sigma in 50 mM KP buffer pH 8 was incubated at 37°C with various concentrations of (^{14}C) phenylglyoxal (5000 cpm per nmol). At the end of the incubation period 50 ul of 0.5 M arginine in water was added and incubation continued for 5 minutes. The mix was dialyzed overnight against 40 mM KP buffer pH 7 and number of arginyl residues modified was determined. Concentrations of (^{14}C) phenylglyoxal, A, 1 mM; B, 2.5 mM and C, 5 mM.

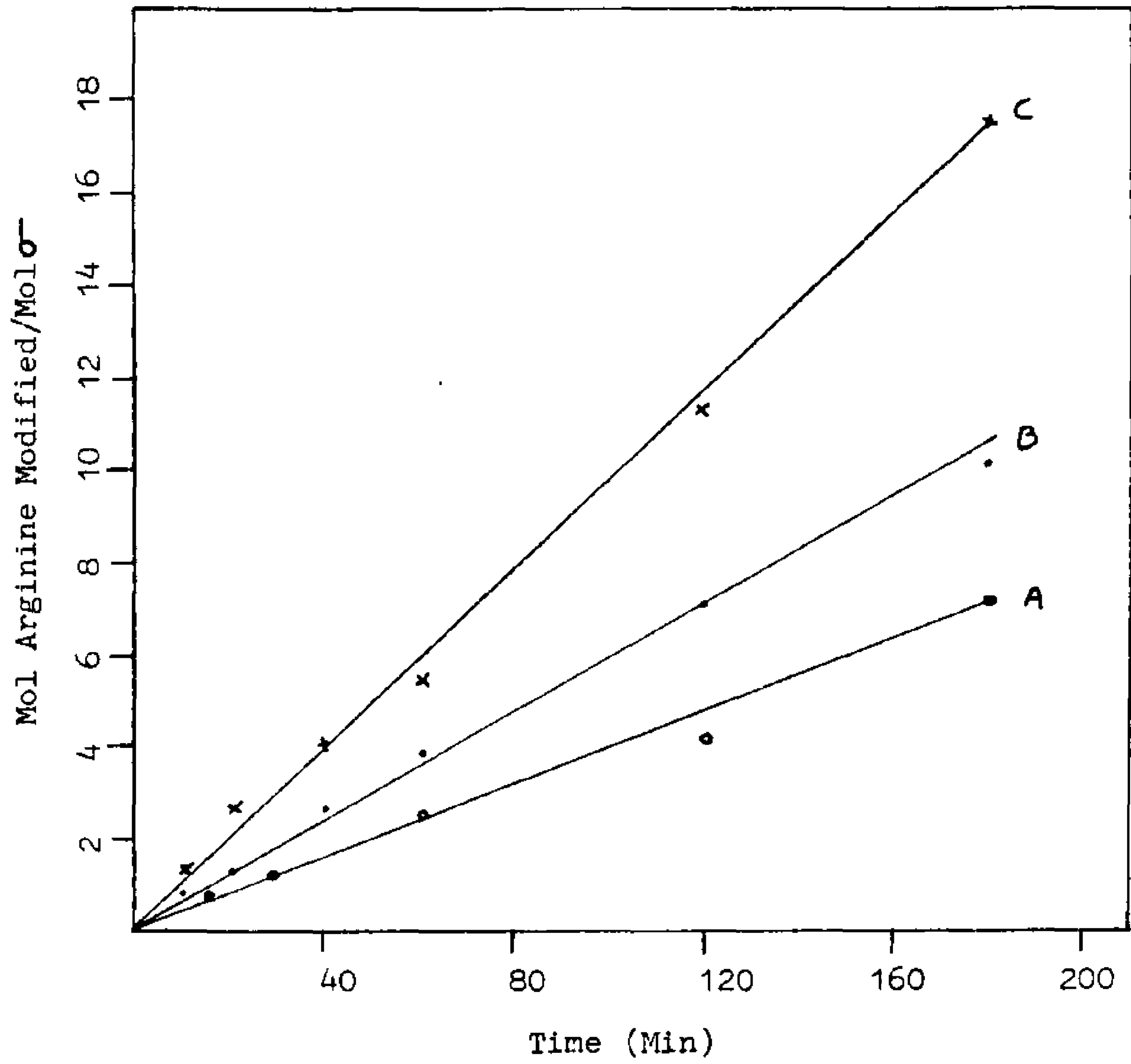

Mol Arginine Modified/Mol σ
2
4
6
8
10
12
14
16
18
40
80
120
160
200
Time (Min)
C
B
A

FIGURE 31: Concentration dependent modification of sigma with (^{14}C) phenylglyoxal. The reaction mix (20 ul) contained 10 ug sigma, KP buffer and various concentrations of (^{14}C) pehnylglyoxal. The mix was incubated at 37°C for 60 minutes and processed and analyzed as described in Figure 30.

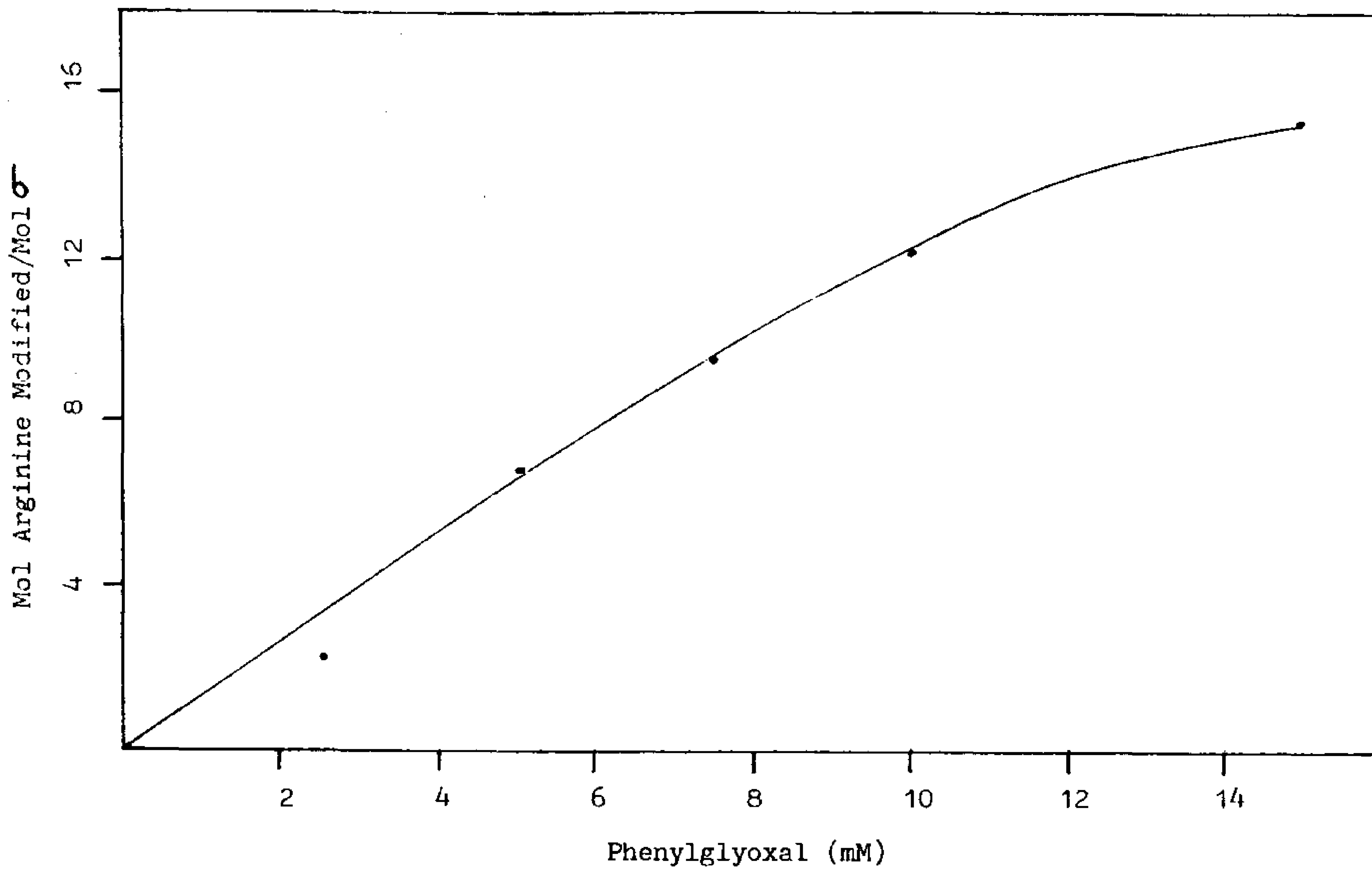
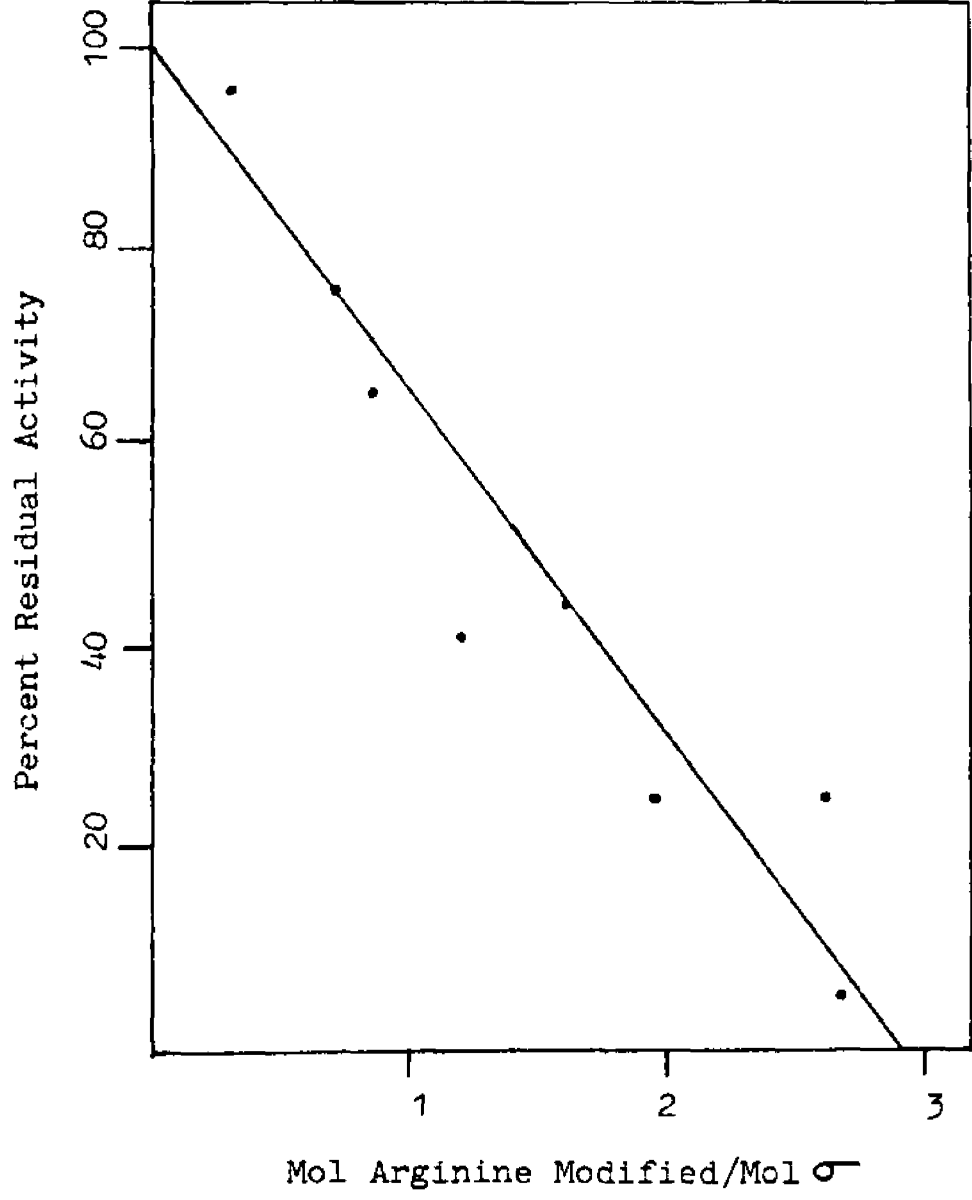
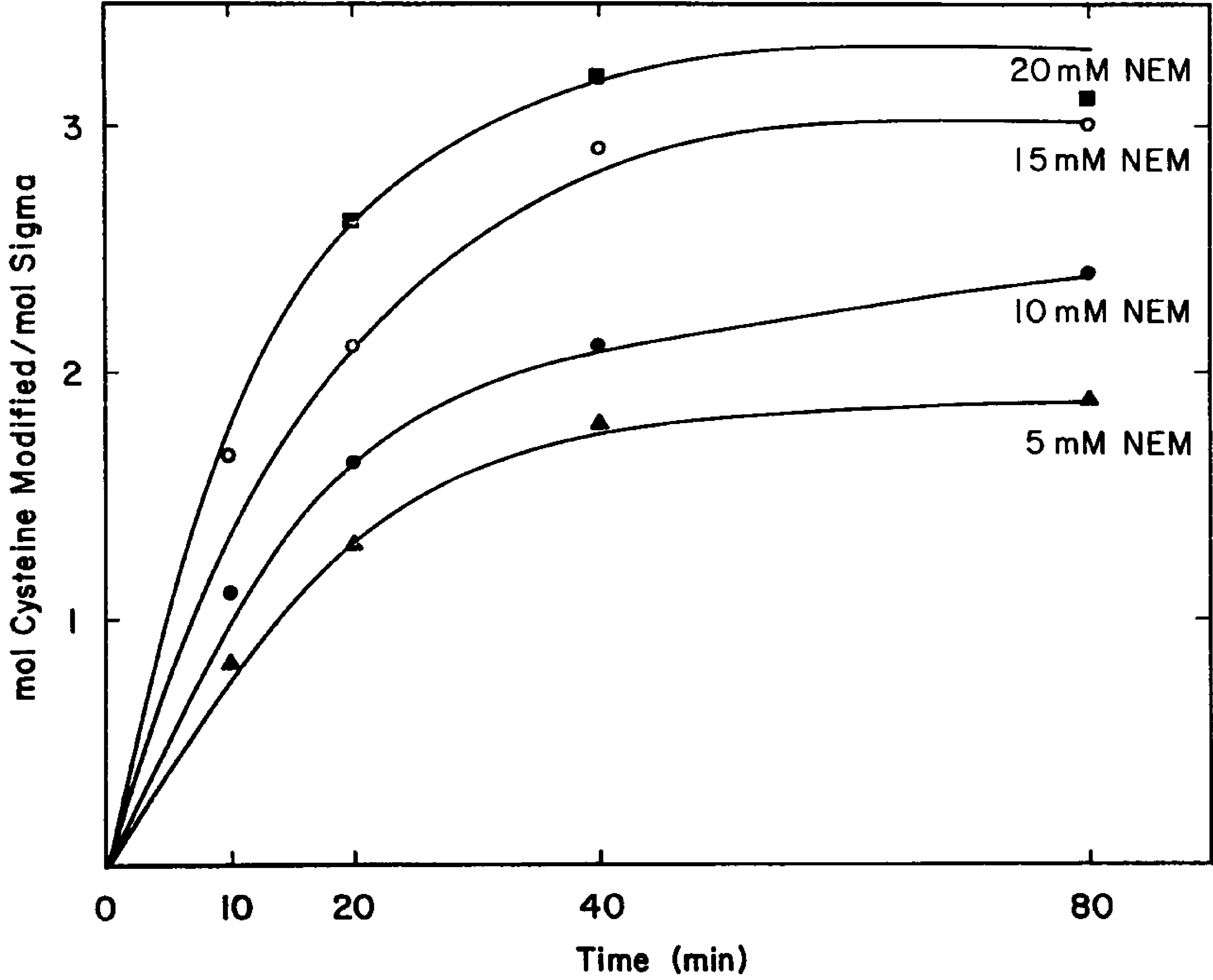

Mol Arginine Modified/Mol σ
4
8
12
15
2
4
6
8
10
12
14
Phenylglyoxal (mM)

FIGURE 32: Relationship of arginine modification to loss of sigma activity. Reactions were carried out as described in Figure 30 using unlabelled or (^{14}C) phenylglyoxal. Aliquots containing 2.5 ug of sigma from the unlabelled phenylglyoxal reaction mix was taken for the determination of sigma activity. Aliquots from the (^{14}C) phenylglyoxal reaction mix were taken to determine the mol arginines modified per mol sigma.



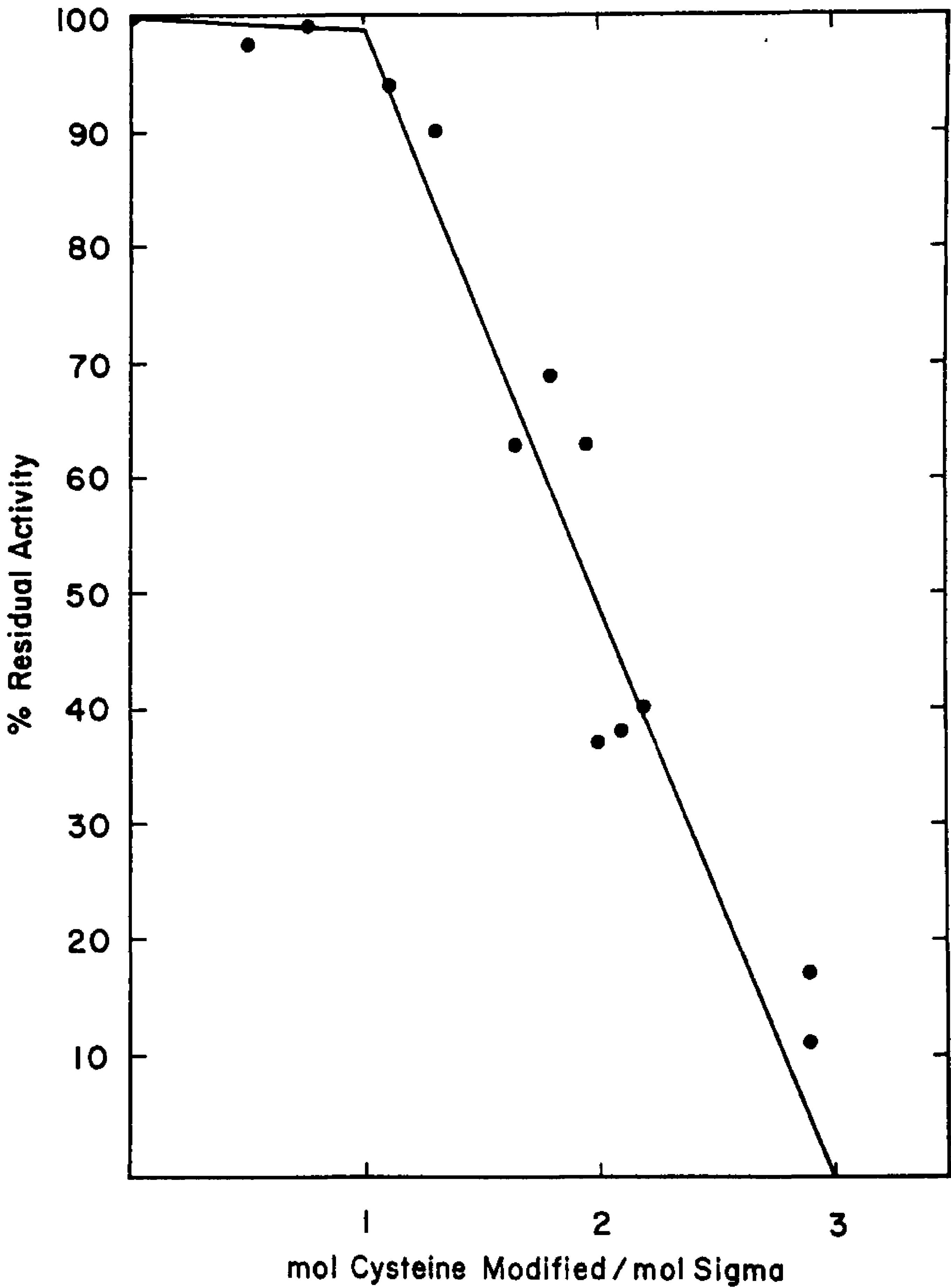
Percent Residual Activity
20
40
60
80
100
1
2
3
Mol Arginine Modified/Mol σ

FIGURE 33: Time and concentration dependent modification of sulfhydryl groups of sigma with NEM. In a 25 ul volume 10 ug sigma was incubated with the indicated concentrations of (^3H) NEM (approximate specific activity, 9600 cpm per nmol) and 100 mM TEA-H_2SO_4 buffer (pH 7) at 37°C for various time intervals. At the end of the incubation period, 5 ul of 0.1 M ME was added and incubation continued for 5 minutes. Sigma was TCA precipitated, filtered and counted. NEM concentration: A, 5 mM; B, 10 mM; C, 15 mM and D, 20 mM.



mol Cysteine Modified/mol Sigma
1
2
3
0
10
20
40
80
Time (min)
20 mM NEM
15 mM NEM
10 mM NEM
5 mM NEM

FIGURE 34: Relationship of sulfhydryl modification to loss of sigma activity. Reactions similar to the ones described in Figure 33 were carried out in 10 ul volume containing 2.5 ug sigma, various concentrations of unlabelled NEM and incubated for various time intervals. At the end of the incubation period the sigma activity was determined. The data from Figure 33 were used to plot the percent residual activity versus the mol sulfhydryl groups modified per mol sigma.



100
90
80
70
60
50
40
30
20
10
% Residual Activity
1
2
3
mol Cysteine Modified / mol Sigma

FIGURE 35: Relationship of carboxyl group modification to loss of sigma activity. The reaction mix (40 ul) containing 20 ug sigma, 100 mM TEA-H_2SO_4 (pH 7), 100 mM (^3H) glycine or 100 mM glycine and varying concentrations of EDC was incubated at 25^oC for 60 minutes. The reaction was stopped by bringing the reaction mix to 0.5 M sodium acetate and then dialyzing overnight Tris-HCl (pH 7). For each concentration of EDC the mol (^3H) glycine incorporated per mol sigma was determined and the residual sigma activity (using the preparation modified in the presence of the unlabelled glycine) was determined using the T5 DNA-directed assay.

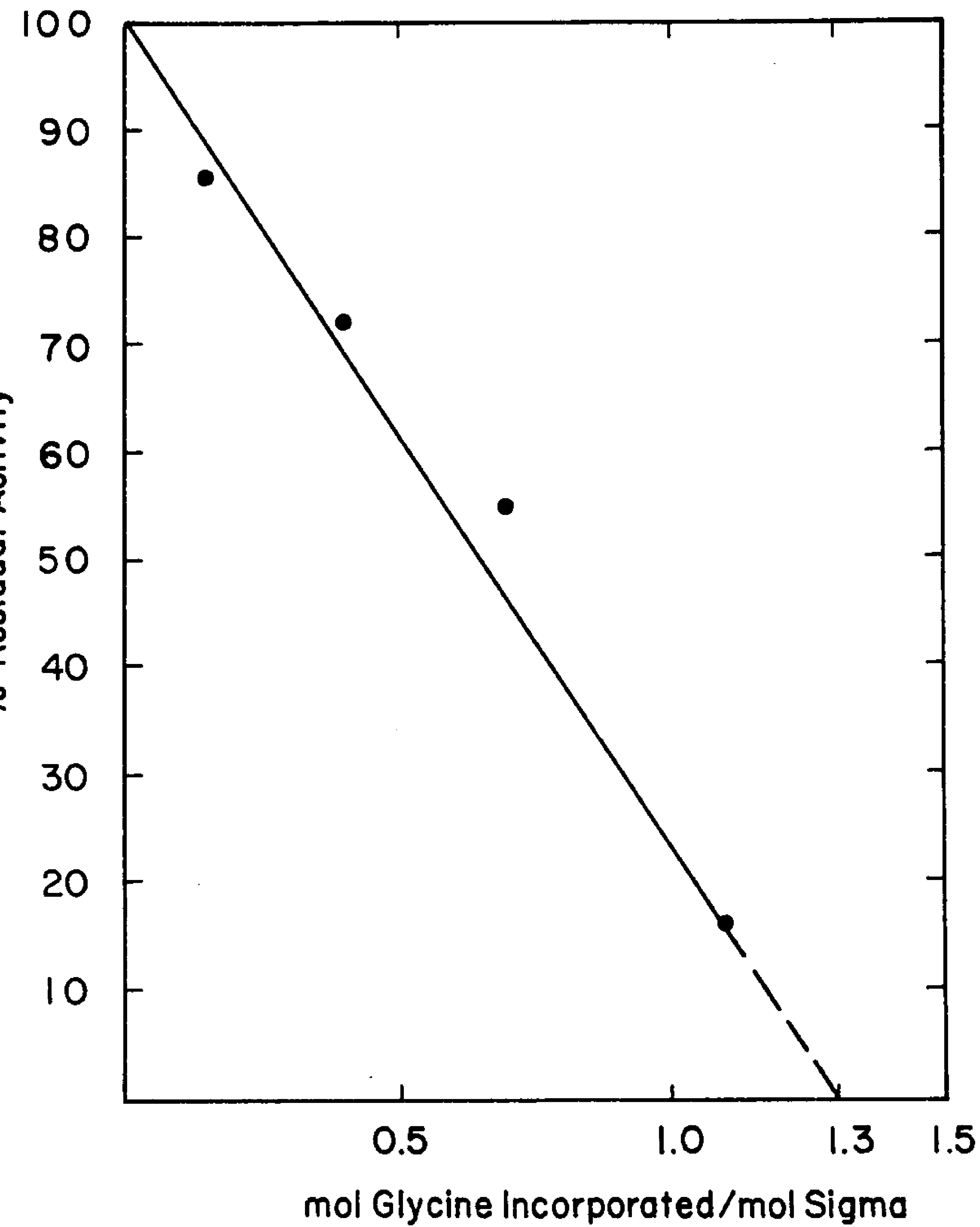

100
90
80
70
60
50
40
30
20
10
0.5
1.0
1.3
1.5
mol Glycine Incorporated/mol Sigma

FIGURE 36: Reconstitution of (^3H) glycine-sigma with core polymerase. (^3H) gly-sigma was prepared as follows: the reaction mix (40 ul) contained 100 ug sigma, 5, 10, 20 or 40 mM EDC, 100 mM (^3H) glycine (20000 cpm/nmol) and 100 mM TEA-H_2SO_4 (pH 7). After incubation at 25°C for 60 minutes the reaction was stopped and the mix was dialyzed as described in Figure 35. At the indicated concentrations of EDC approximately 1, 2, 3 and 5 mol (^3H) glycine were incorporated per mol sigma.

Reconstitution was assayed as follows: in 100 ul TMS buffer, 30 ug (^3H) gly-sigma and 250 ug core polymerase were incubated at 37°C for 5 minutes, layered on the gradient and centrifuged as described under Methods. Fractions of 0.4 ml were collected and analyzed for radioactivity. The mol (^3H) glycine per mol sigma are A, 1; B, 2; C, 3; D, 5. The arrow indicates the position of core polymerase.

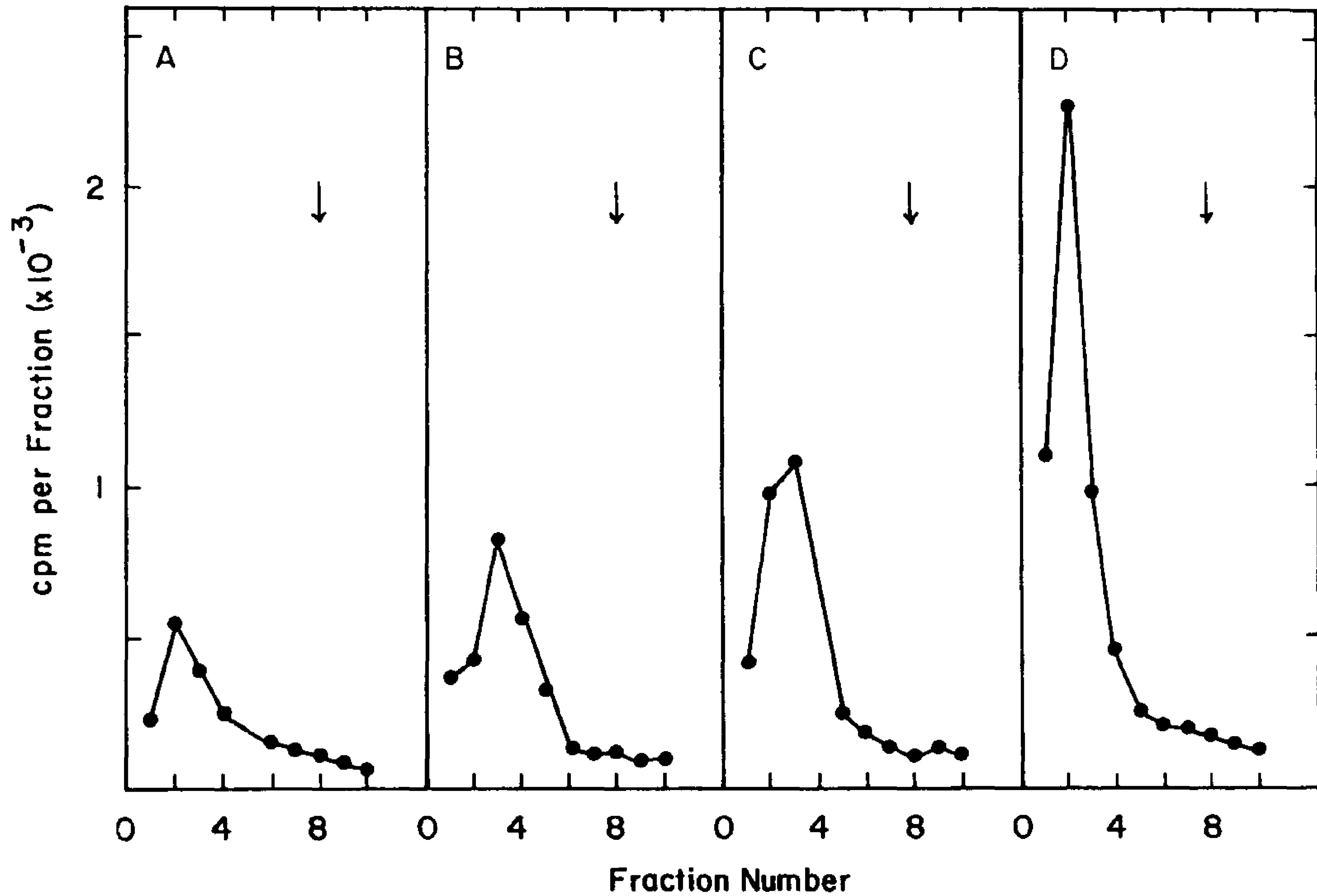

A
B
C
D
cpm per Fraction (x10^{-3})
2
1
0
4
8
Fraction Number

FIGURE 37: Electrophoresis of CNBr fragments of sigma. 25 ug (^{3}H) TNP-sigma (5 TNP/sigma) was treated with CNBr in 70% formic acid overnight in dark at room temperature. The digest was lyophilized and 10 ug digest was electrophoresed on 15% SDS polyacrylamide gel. 1,2,3, 5 ug of respectively insulin, lysozyme and CRP; 4, 5 ug TNP-sigma; 5,6, 10 ug TNP-sigma treated with 20000 fold molar excess of CNBr; 7,8, 10 ug TNP-sigma treated with 50000 fold molar excess of CNBr. The CNBr fragments of sigma showed a band pattern identical to that of TNP-sigma.

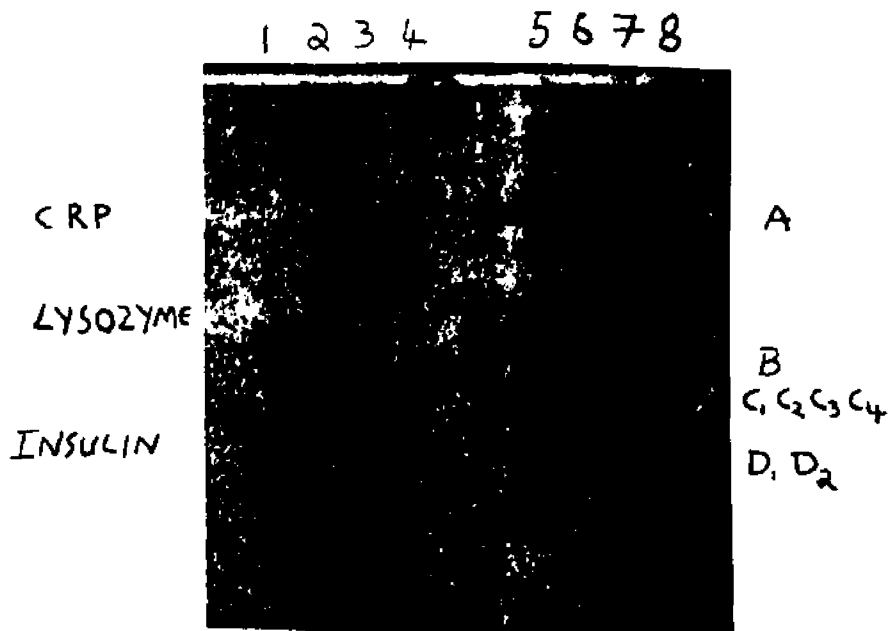
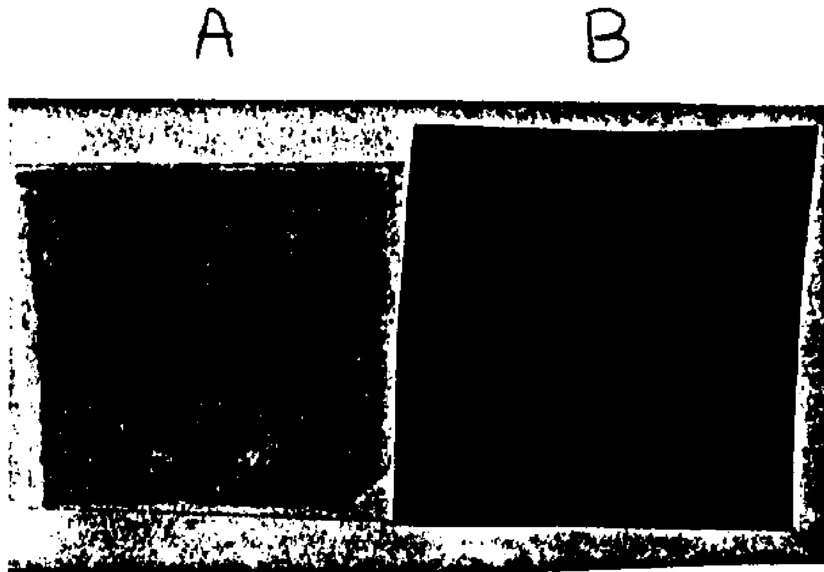


FIGURE 38: Autoradiography of the CNBr fragments of (^{3}H) TNP-sigma (5 TNP/sigma). The gel shown in Figure 37 was dried and exposed to X-ray film for 15 days at -70°C. The film was developed. A, the gel; B, autoradiograms.



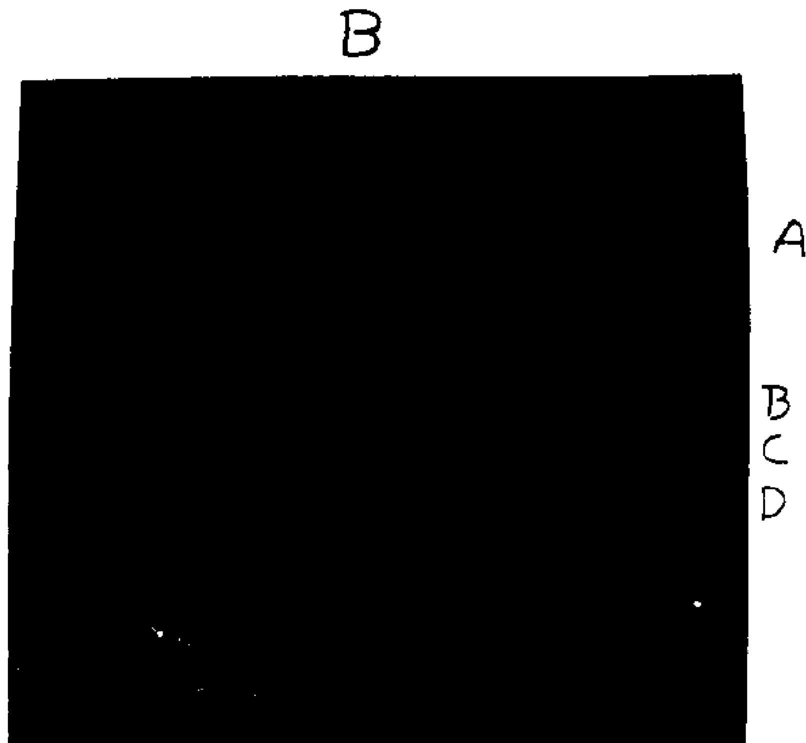
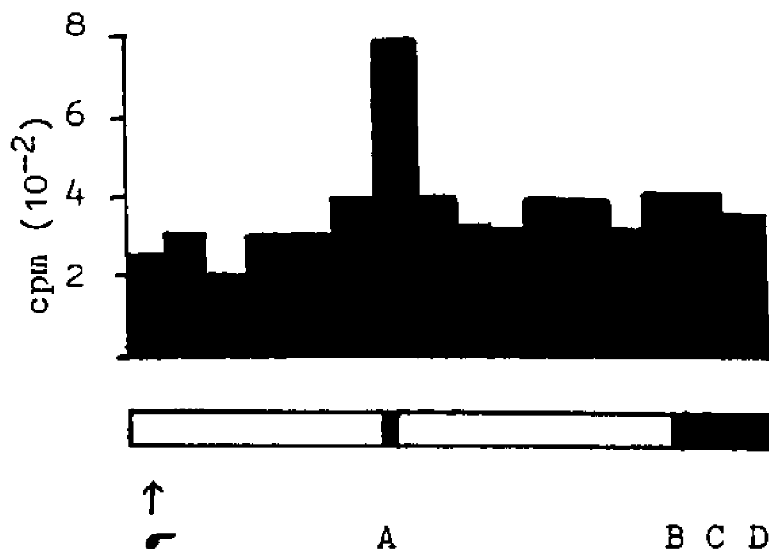


FIGURE 39: Radioactivity associated with the CNBr fragments of SH-sigma.



15 ug of 1 SH-sigma modified with (^3H) NEM was treated with CNBr. The fragments generated were electrophoresed on 15% SDS polyacrylamide gel. The gels were sliced, digested in H_2O_2 overnight at 50°C and counted in 10 ml Aquasol (New England Nuclear). Experiments with 2 or 3 SH-sigma showed a similar pattern of radioactivity distribution. 2100 cpm was associated with 15 ug of 3 SH-sigma.

FIGURE 40: Autoradiography of the CNBr fragments of (^{3}H) glycine-sigma (1, 2, 3 or 5 glycines/sigma). (^{3}H) glycine-sigma preparations were made as described in Figure 36 and treated with 0.5 M CNBr. Following lyophilization the fragments were electrophoresed. The gel was dried and autoradiographed. A, the gel; B, autoradiogram. 1,2,3,4, CNBr fragments of respectively 1, 2, 3, 5 glycines/sigma (10 ug); 5,6, 5 ug of respectively 2 and 5 glycines/sigma.

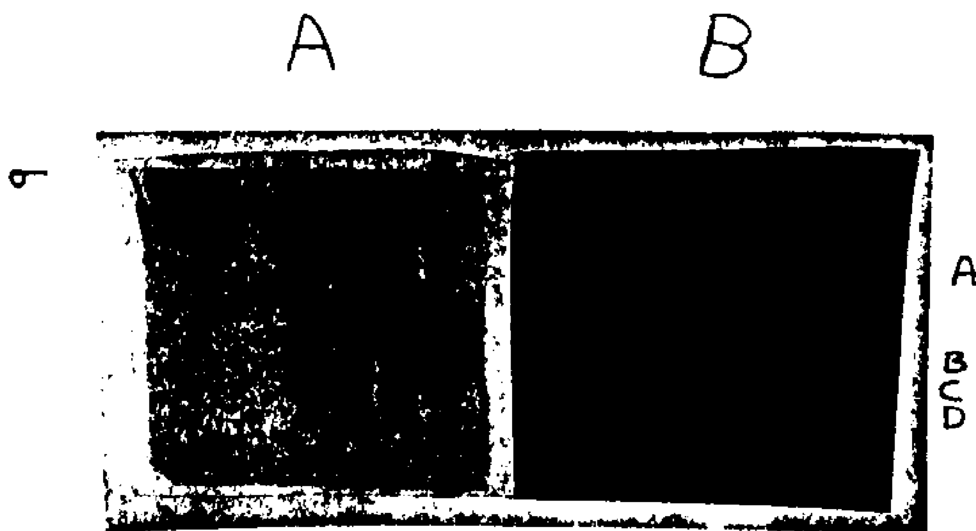


Table 1: Summary of purification of RNA polymerase.

Stage of purification	Total protein (mg)	Total volume (ml)	Total activity (U*)	Specific activity (U/mg)	Percent yield **
0.5 M NaCl extract	13800	1000	24230	2	-
1 M NaCl extract	12250	1000	145230	12	100
DEAE load	12000	1800	125130	10	86
DEAE peak pool	1230	450	60225	49	41
DNA cellulose load	1145	1600	59540	52	41
DNA cellulose peak pool	95	220	39140	412	27
Ultrogel load	93	5	40296	433	28
Ultrogel peak pool	65	85	41775	642	29

* One unit is defined as 1 nmol of (^3H) UMP incorporated by 10 ul of enzyme fraction at 37°C for 10 minutes. The reaction mix contained 40 ug calf thymus DNA, 1 mM each of ATP, CTP, GTP and (^3H) UTP, 80 mM Tris-HCl (pH 7.6), 20 mM $MgCl_2$, 40 mM mercaptoethylamine.

** Percent yield is based on total activity.

Table 2: Summary of paper chromatography of acid hydrolysates of TNP-sigma.

Sample submitted to chromatography	TNBS	ε-TNP-lys	S-TNP-cys	TNP-met
Authentic ε-TNP-lys	-	+ 0.65	-	-
Authentic S-TNP-cys	-	-	+ 0.17	-
Authentic TNP-met	-	-	-	+ 0.5
^3H TNP-amino acids from 10 TNP/σ	-	+ 0.65	-	-
^3H TNP-amino acids from 28 TNP/σ	-	+ 0.65	-	-
^3H TNBS	+ 0.53	-	-	-

The numbers refer to R_f values.

Table 3a: Summary of modification of lysyl groups of sigma using various reagents.

Reaction	Reagent	Buffer pH 9	Incubation time at $37^{o}C$	Order of the reaction	Rate constant (k")	pK of the critical lysine
Trinitro-phenyl ation	TNBS	40 mM BTP	30 min	1.2	320	9.1
Carbamyl-ation	KCNO	200 mM borate	90 to 120 min	0.8	0.017	8.8
Succinyl-ation	Succinic anhydride	50 mM NaP	30 min	1.2	5.4	9

Table 3b: Properties of lysyl groups modified sigma.

Reaction	T7 DNA binding	Promoter recognition	Initiation	Change in conformation	Reconstitution with core
Trinitro-phenyl ation	Inhibitory	Inhibitory	Inhibitory	Yes	Unaffected
Carbamyl-ation	"	"	"	"	"
Succinyl-ation	"	"	"	-	"

Table 4: The values for the reaction order, n, for inactivation of sigma by phenylglyoxal at different pH.

pH	Buffer	n
7	50 mM KP	2.0
8	50 mM KP	1.0
9	40 mM BTP	1.6
10	100 mM Glycine-NaOH	2.5

Amino acid modified	Reagent	Buffer and pH	Incubation time and temperature	No. of residues modified at 100% inactivation	Order of the reaction	Rate constant (k")	pK of the critical group
Arginine	Phenyl-glyoxal	50 mM KP pH 8	60 min. 37°C	3	1.7	20	8
Cysteine	NEM	50 mM TEA pH 7	"	3	0.85	9	8
	PCMB	"	"	-	0.76	70	-
	DTNB	"	"	-	0.69	28	-
Carbaxylic groups	EDC	"	60 min. 25°C	1	1.0	10	-
	CMC	"	"	1	0.83	11	-

Table 5a: Summary of modification of arginyl, cysteinyl and carboxyl groups of sigma.

Amino acid modified	Reagent	Reconstitution with core	Affinity of binding to core	Promoter recognition	Initiation	Change in conformation
Arginine	Phenyl-glyoxal	Unaffected	Comparable to sigma	Inhibitory	Inhibitory	Yes
Cysteine	NEM	"	"	"	"	"
	PCMB	"	–	"	"	–
	DTNB	"	–	"	"	–
Carbaxylic groups	EDC	Inhibitory	–	"	"	–
	CMC	"	–	"	"	–

Table 5b: Summary of properties of arginyl, cysteinyl and carboxyl groups modified sigma.

Table 6: Treatment of gly-sigma with NH_2OH.

Time (Min)	0	15	60	300	No NH_2OH 300 min.
1 gly/σ	1273	1350	1053	833	820
2 gly/σ	1859	2193	2459	2216	1834
5 gly/σ	5022	5182	5217	5041	4980

5 ug (^3H) gly-sigma (in 20 mM Tris-HCl, pH 7) was incubated with 0.5 M NH_2OH (pH 7) at 25°C for the indicated time and the acid precipitable radioactivity was determined as cpm.

Time (Min)	0	15	60	300
Core + σ	22152	22804	21980	22500
Core + 5 gly/σ	4100	3680	4381	4285

Core polymerase = 4308

Sigma or gly-sigma (2.5 ug) was incubated with hydroxylamine as indicated above. At the end of incubation sigma was assayed using 10 ug core polymerase and T5 DNA assay system. The numbers represent cpm labelled UMP incorporated.

TABLE 7: Cyanogen bromide fragments of sigma. The table is based on the amino acid sequence analysis for sigma subunit (Burton et al., 1981). Molecular weight of the fragments is calculated using a mean residue molecular weight of 114.6. The letters on the right side reffer to the bands on Figure 37.

Length	Size	Mol. Wt.	No. of residues Lys	Arg	Cys	Asp	Glu	
1	1	114.6						
2-47	46	5271.6	2	1		5	5	C_1
48-51	4	458.4				1		
52-56	5	573						
57-66	10	1146				3	2	
67-100	34	3896.4		2		3	5	D_1
101-102	2	229.2						
103-105	3	343.8		1			1	
106-273	168	19252.8	7	9	1	22	24	A
274-276	3	343.8		1				
277	1	114.6						
278-288	11	1260.6		3		1	1	
289-297	9	1031.4	2		2		1	
298-322	25	2865	2			1	1	
323-365	43	4927.8	4	3		2	7	C_3
366-379	14	1604.4	3	2			2	
380-413	34	3896.4	2	2		1	2	D_2
414-456	43	4927.8	3	6		2	1	C_4
457-470	14	1604.4	1	2			1	
471-474	4	458.4					1	
475-487	13	1489.8		2			4	
488-489	2	229.2						
490-507	18	2062.8	4	1		1	2	
508-561	54	6188.4	1	3		7	6	B
562-567	6	687.6		1			1	
568-613	46	5271.6	3	7		5	4	C_2

Table 8: Specific activity of (^3H) NEM.

Procedure#	NEM-cysteine adduct		NEM	
	cpm	cpm*	cpm	cpm*
Expected radioactivity/nmol@.	14667		14667	
1 nmol in Aquasol (NEN).	17122		16106	
1 nmol on GFC filter, counted in Aquasol.	16500	14371	16124	14480
1 nmol on GFC, dried for 30 min., counted in Aquasol.	12697	10418	3004	2500
1 nmol on GFC, dried, counted in Liquifluor.	9621	668	2475	100
1 nmol in a scintillation vial, dried, 100 ul water was added to the dry spot, counted in Aquasol.	16357		3302	

The scintillation vials were kept at room temperature with intermittent shaking for 24 hours and counted for 10 minutes. The labelled NEM solution was prepared as described on page 46.

* cpm remaining after the filter is removed.

@ The efficiency of the machine for (^3H) was 50.4%.

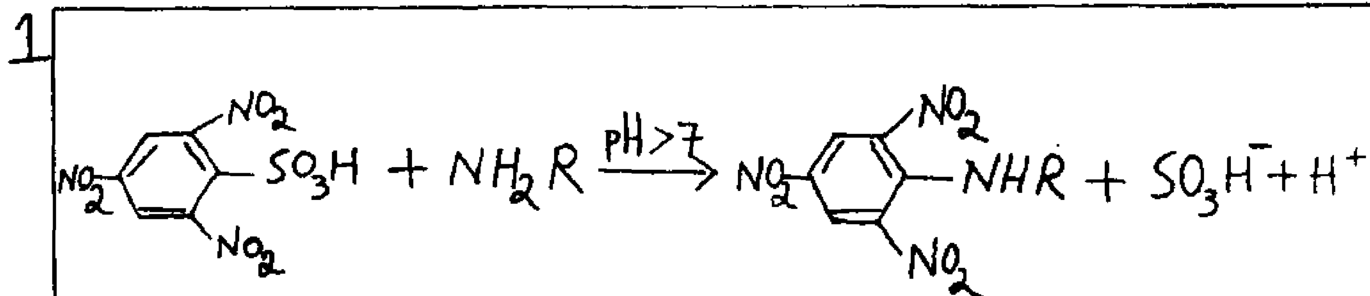
FIGURE A1: Reactions of group specific reagents with amino acid side groups of proteins.

1. Reaction of TNBS with an amine.
2. Reaction of HCNO with an amine.
3. Reaction of succinic anhydride with an amine.
4. Reaction of phenylglyoxal with guanidino group.
5. Reaction of NEM with sulfhydryl group.
6. Reaction of DTNB with sulfhydryl group.

7A. Reaction of a carbodiimide with carboxyl group.

7B. Reaction of an amine with carboxyl group in presence of a carbodiimide.

1



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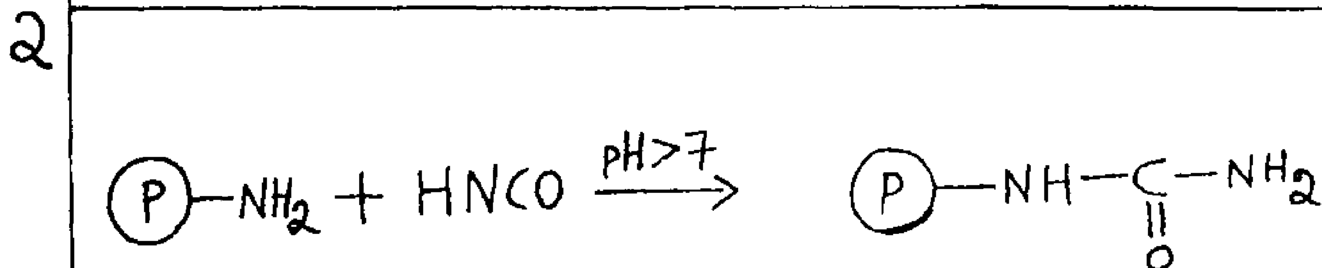

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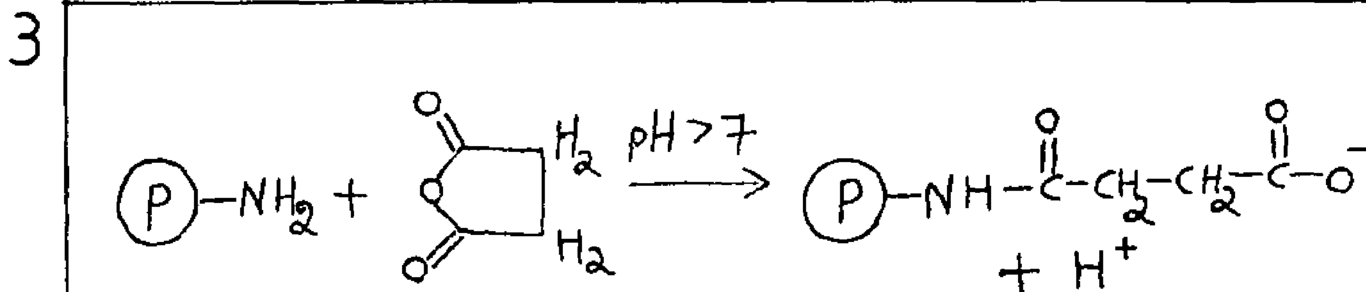

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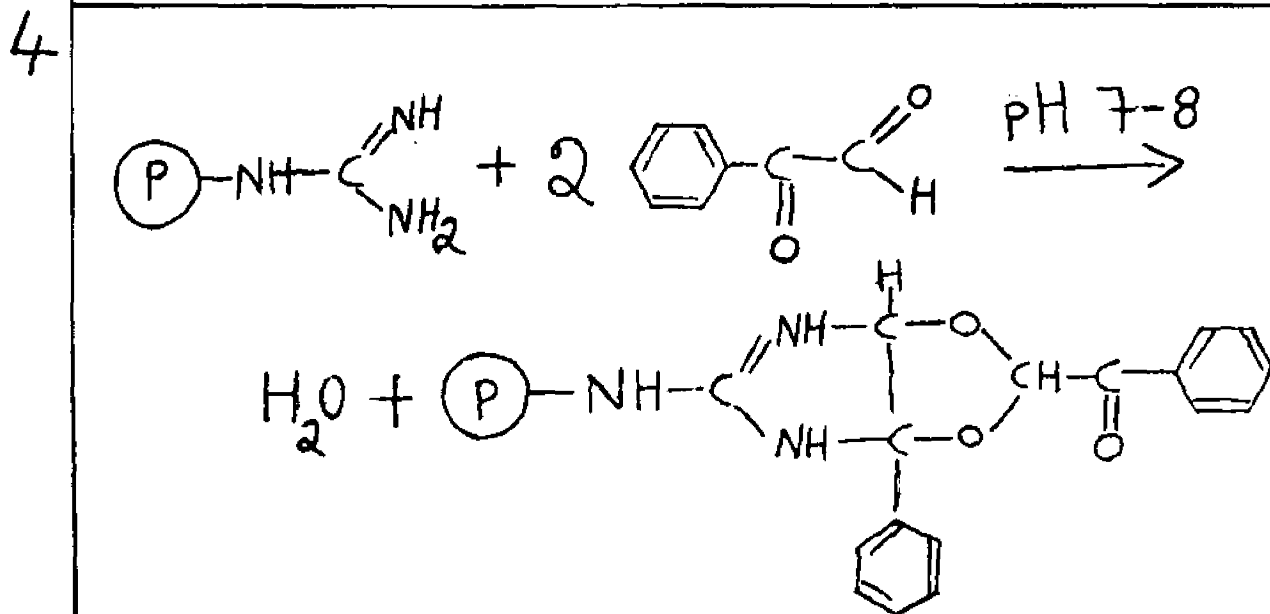


5

$$\text{(P)}{-}SH + \text{N-ethylmaleimide}\ (N\text{-}C_2H_5) \xrightarrow{pH>5} \text{(P)}{-}S{-}CH{-}CH_2\ \text{(succinimide ring, } N\,C_2H_5\text{)}$$

6

$$\text{(P)}{-}SH + NO_2{-}C_6H_3(COO^-){-}S{-}S{-}C_6H_3(COO^-){-}NO_2 \underset{}{\overset{pH \geq 6.8}{\rightleftharpoons}}$$

$$\text{(P)}{-}S{-}S{-}C_6H_3(COO^-){-}NO_2 + {}^-S{-}C_6H_3(COO^-){-}NO_2 + H^+$$

7

A

$$\text{(P)}{-}C(=O){-}O^- + R{-}N{=}C{=}N^+(H){-}R' \longrightarrow \text{(P)}{-}C(=O){-}O{-}C(=NR){-}NHR'$$

B

$$\text{(P)}{-}C(=O){-}O^- + NH_2{-}R \xrightarrow{R'{-}N{=}C{=}N{-}R''} \text{(P)}{-}C(=O){-}NHR$$

FIGURE A2: Agarose gel electrophoresis of the purified restriction endonuclease fragments. HaeIII digest of T7 DNA was electrophoresed on 1% low temperature melting agarose gel. The fragments were isolated from the gel according to the procedure described by Weislander (1981). An aliquot of the purified fragment (0.5 ug DNA) was electrophoresed on 1% agarose gel. A, fragment C; B, fragments B1 and B2; C, fragment A and D, HaeIII digest of T7 DNA.

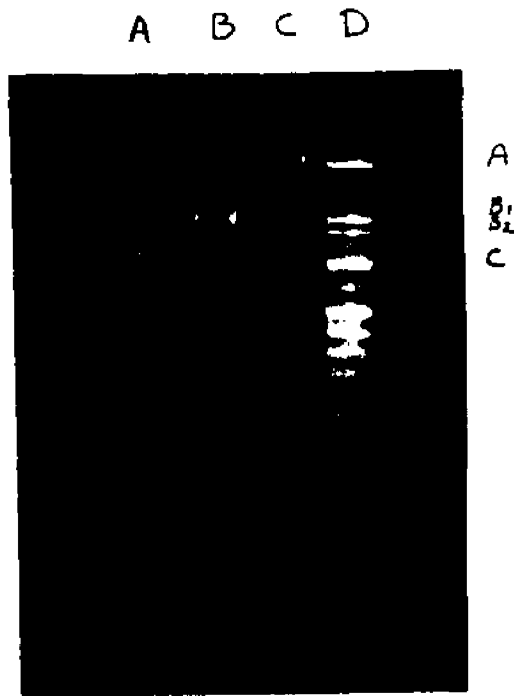


FIGURE A3: Effect of salt, pH and sodium sulfite on the activity of sigma.

A) Sigma (2 ug) was incubated at 37°C in 200 mM borate buffer (pH 9) with various concentrations of KCl for 90 minutes and assayed using 10 ug core polymerase and 20 ug T5 DNA.

B) Sigma (2 ug) was incubated at 37°C with 40 mM BTP buffer at various pH for 30 minutes and assayed.

C) Sigma (2 ug) was at 37°C with 40 mM BTP (pH 9) and various concentrations of sodium sulfite for 30 minutes and assayed.

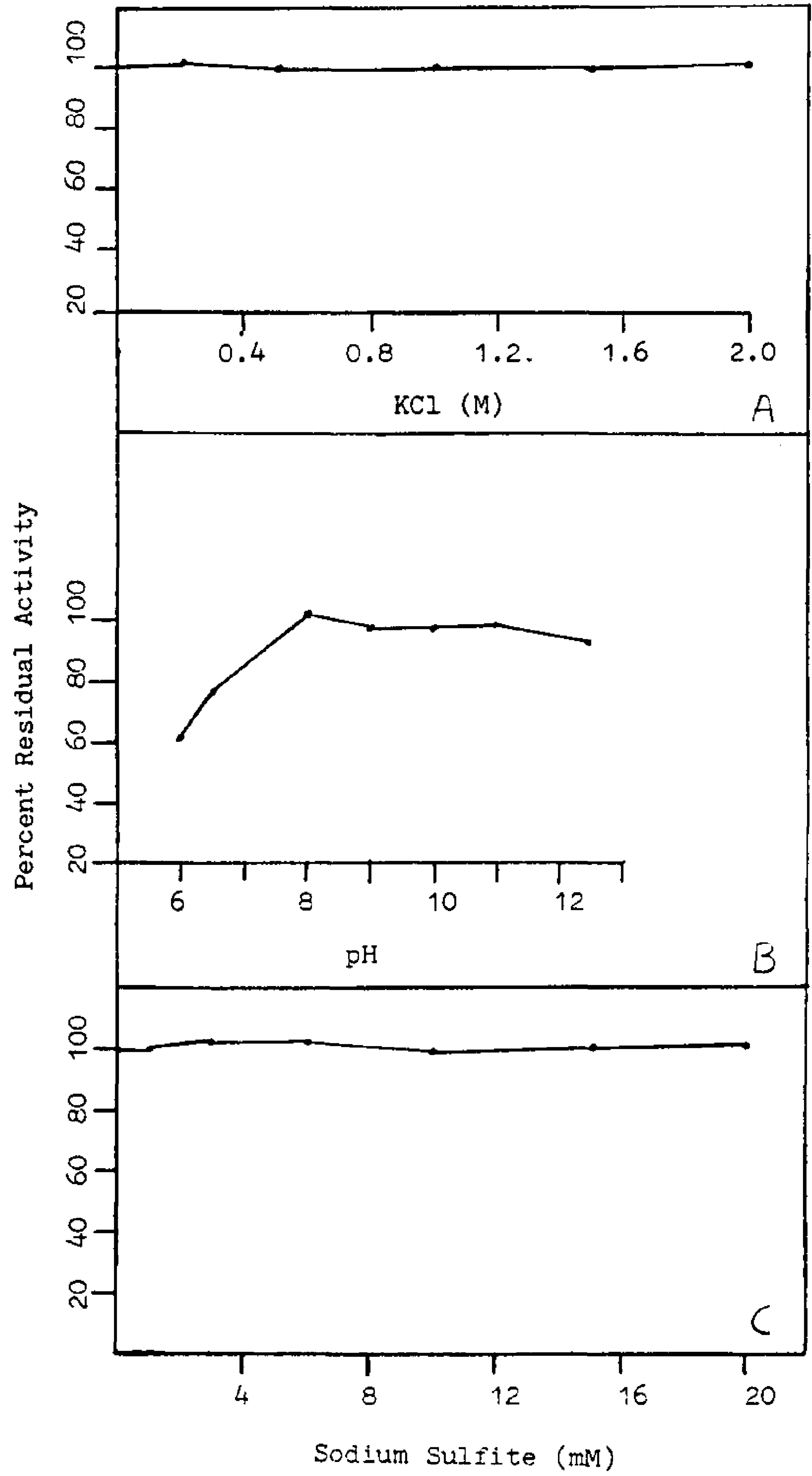
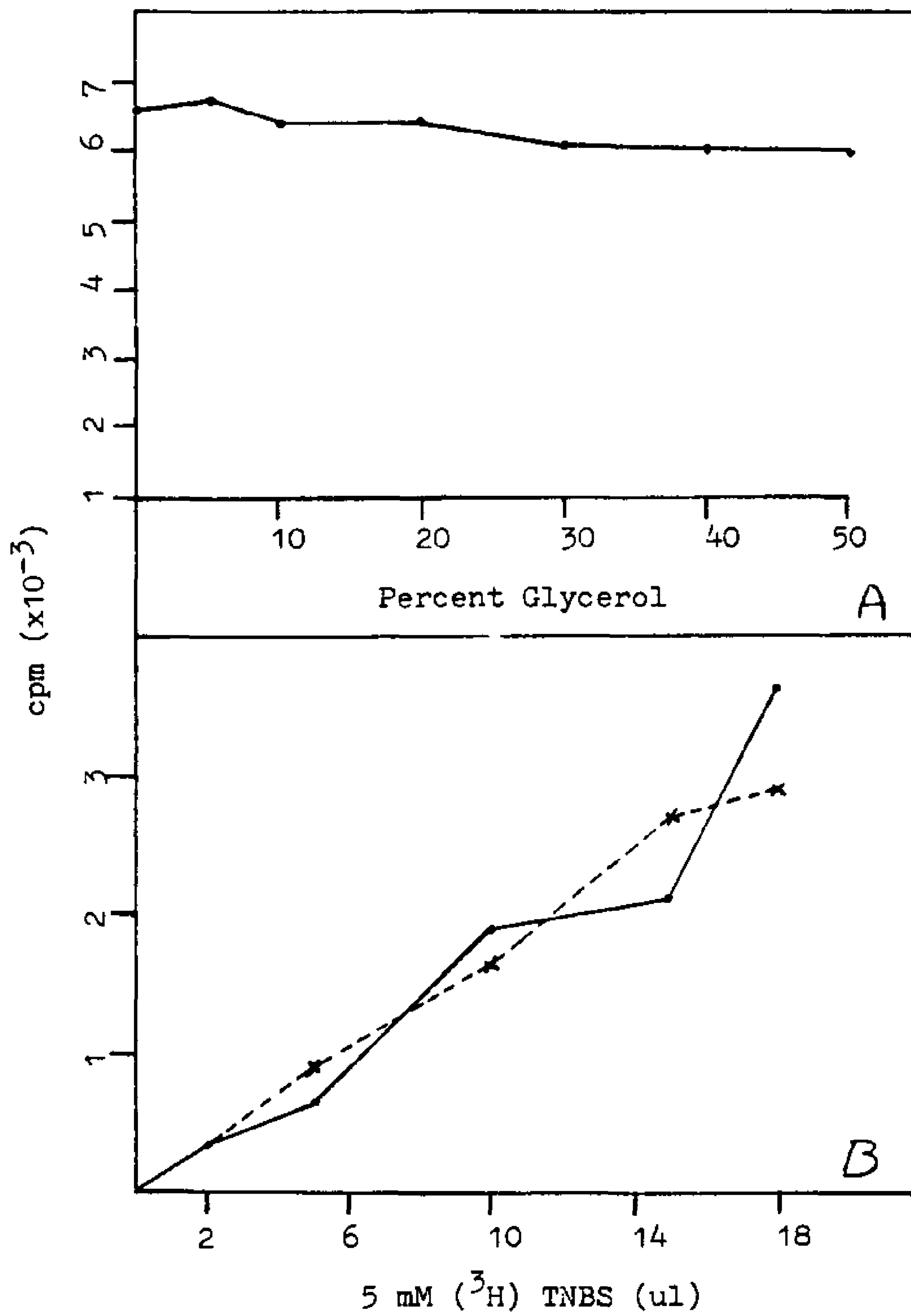

Percent Residual Activity
20
40
60
80
100
0.4
0.8
1.2.
1.6
2.0
KCl (M)
A
6
8
10
12
pH
B
4
8
12
16
20
Sodium Sulfite (mM)
C

FIGURE A4:

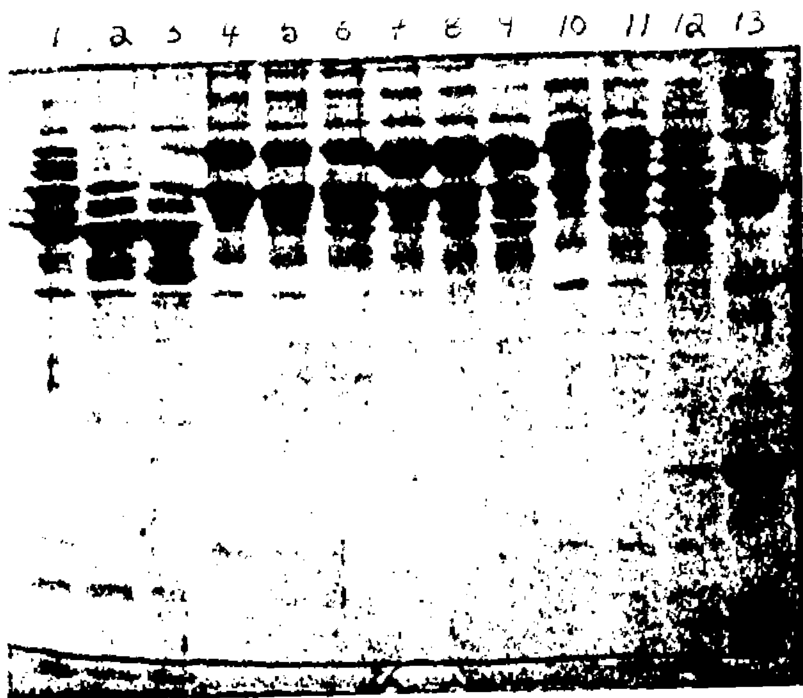
A) Effect of glycerol concentration on trinitrophenylation. Sigma (5 ug) was incubated in 40 mM BTP (pH 9) with various concentrations of glycerol and 2 mM (^{3}H) TNBS at 37°C for 30 minutes. The reaction was stopped with 20 ul of 0.2 M lysine, the mix (70 ul) was dialyzed against 20 mM Tris-HCl (pH 8), 0.1 mM DTT overnight, TCA precipitated, filtered and counted.

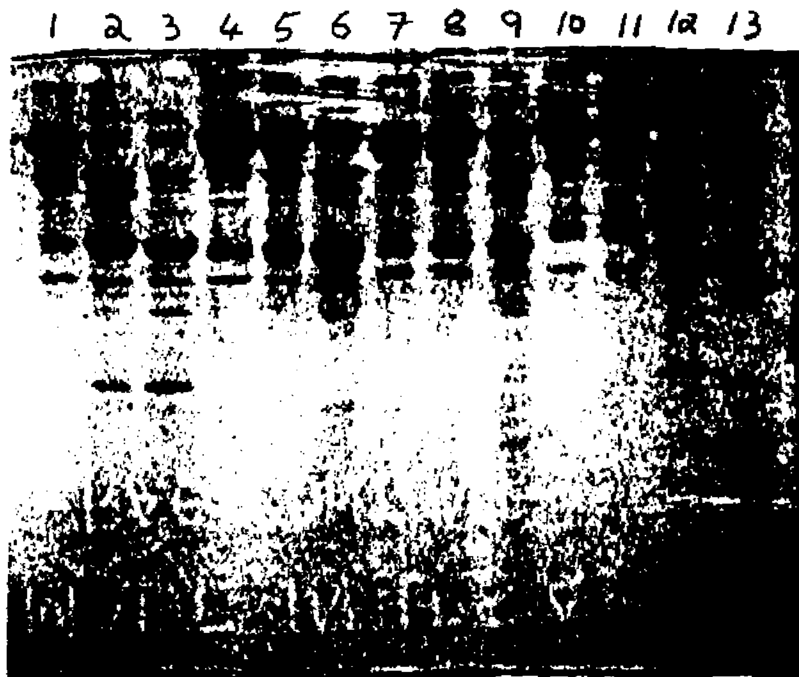
B) Reactivity of (^{3}H) and unlabelled TNBS. Keeping the total concentration of TNBS at 5 mM, the percent of (^{3}H) TNBS was varied from 10 to 90%. Sigma (5 ug) was incubated with TNBS and 40 mM BTP (pH 9) at 37°C for 30 minutes. The mix (20 ul) was TCA precipitated filtered and counted. x------x , expected; •———•, experimental.



cpm (x10^{-3})
1
2
3
4
5
6
7
10
20
30
40
50
Percent Glycerol
A
1
2
3
2
6
10
14
18
5 mM (^{3}H) TNBS (ul)
B

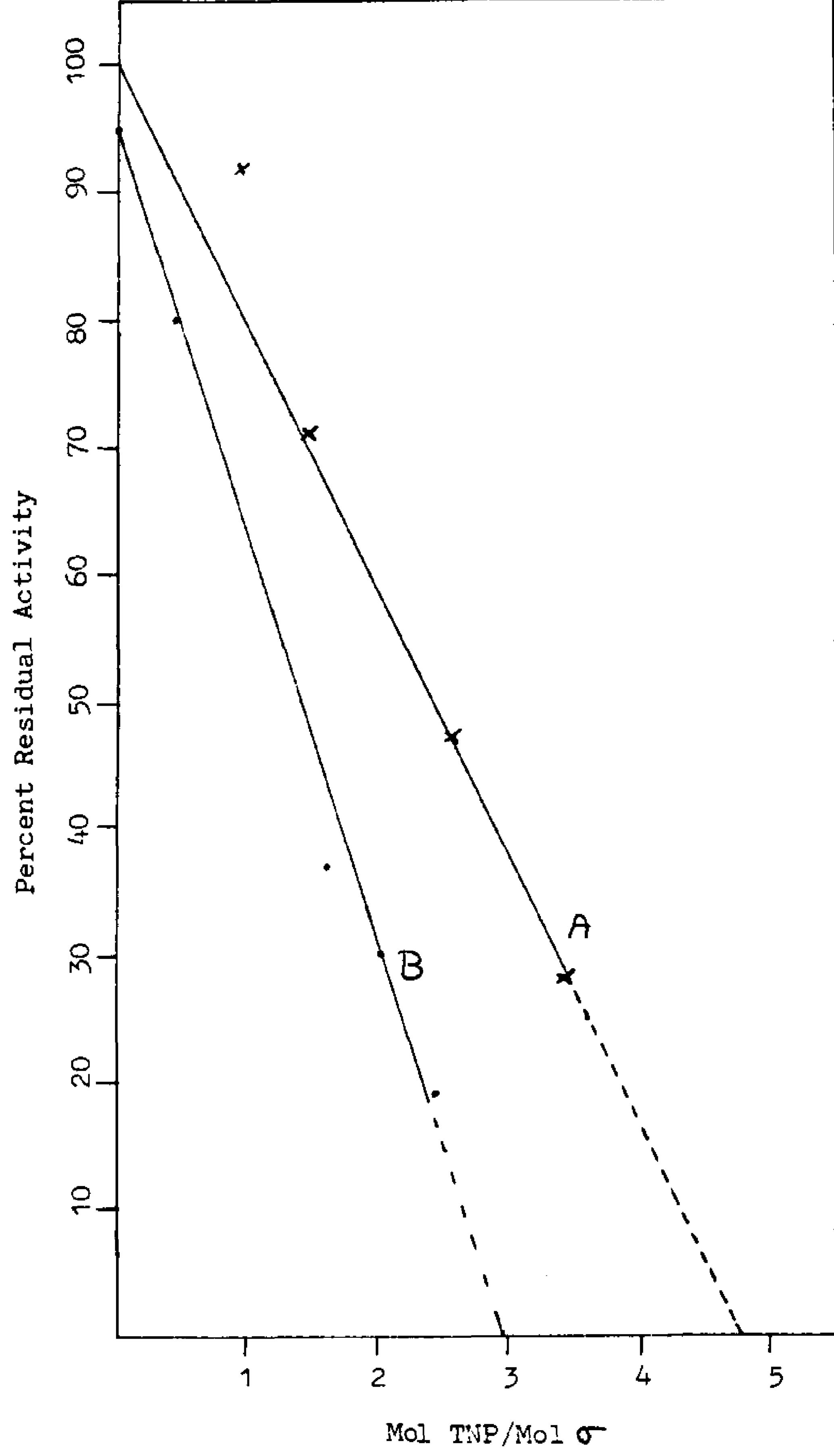
FIGURE A5: Protease digestion of carbamylated and succinylated sigma. In a 50 ul reaction volume, 20 ug sigma or modified sigma was digested with 0.4 ug SAP (A), 0.06 ug chymotrypsin (B) or 0.06 ug trypsin (C) at 37°C for various time intervals. The digests were electrophoresed on polyacrylamide gel (7.5%). The time intervals of digestion were as follows: for SAP, 5, 15 and 30 minutes; for chymotrypsin, 1, 5 and 15 minutes and for trypsin, 1, 10 and 30 minutes. 1,2,3, sigma; 4,5,6, 7-TNP-sigma; 7,8,9, carbamylated sigma; 10,11,12, succinylated sigma and 13, 10 ug each of holoenzyme, BSA, CRP and myoglobin.


1 2 3 4 5 6 7 8 9 10 11 12 13
ββ'
σ
BSA
α
SAP
CRP
MYOGLOBIN


1 2 3 4 5 6 7 8 9 10 11 12 13
CHYMO
TRYPSIN

1 2 3 4 5 6 7 8 9 10 11 12 13
TRYPSIN

FIGURE A6: Relationship of lysine modification to loss of activity of CDC-sigma. Sigma was modified with a 1000 fold molar excess of CDC (solution prepared in water) in 20 mM KP buffer (pH 9) at 37°C for 60 minutes. The unreacted CDC was removed by overnight dialysis against 20 mM KP buffer (pH 9). Relationship of loss of activity to trinitrophenylation of CDC-sigma was determined as described in Figure 5. A, sigma and B, CDC-sigma.



Percent Residual Activity
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
Mol TNP/Mol σ
A
B

FIGURE A7: Determination of pK value of TNBS. Five ml of 200 mM TNBS in water was titrated with 1 M NaOH and the pH was monitored.

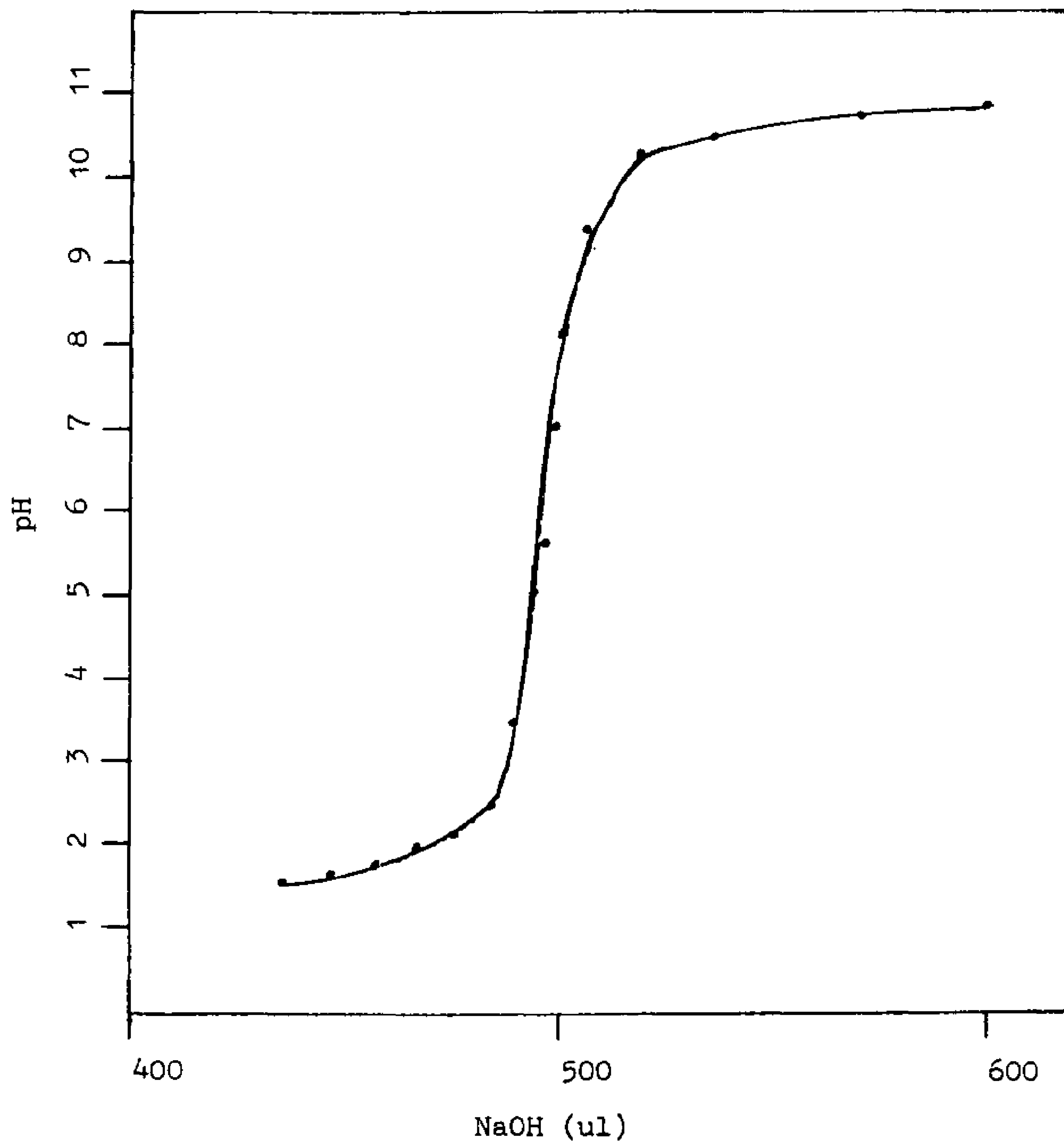
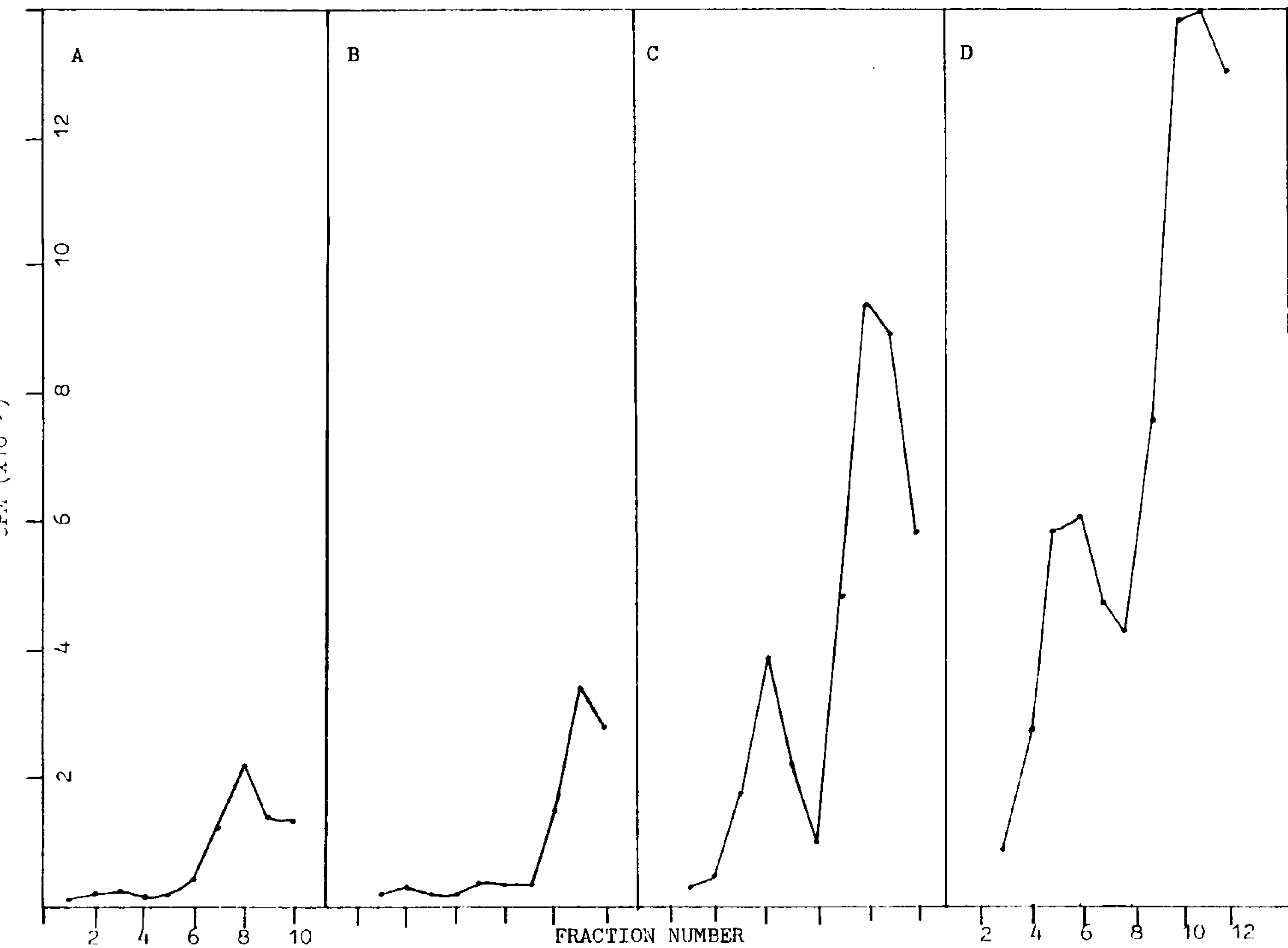


FIGURE A8: Sedimentation profile of holoenzyme modified with various concentrations of TNBS. TNP-holoenzyme was prepared as described under Methods using ^{3}H TNBS at 0.05, 0.2, 1 and 5 mM (figures A, B, C and D respectively). Following dialysis 100 to 300 ug TNP-holoenzyme was layered on a 15 go 35% glycerol gradient in TMS buffer and centrifuged as described under Methods. The gradients were fractionated and fractions were analyzed.


A
B
C
D
12
10
8
6
4
2
2
4
6
8
10
FRACTION NUMBER
2
4
6
8
10
12