THE AFFINITY OF GTP AND ITP FOR THE INITIATION AND ELONGATION SITES OF *AZOTOBACTER VINELANDII* RNA POLYMERASE CORE ENZYME

by

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$d(I\text{-}C)_n$	alternating deoxyribocopolymer of deoxyinosinic and deoxycytidylic acids
$d(I\text{-}\overline{BrC})_n$	alternating deoxyribocopolymer of deoxyinosinic and deoxy 5 bromocytidylic acids
$d(I\text{-}\overline{IC})_n$	alternating deoxyribocopolymer of deoxyinosinic and deoxy 5 iodocytidylic acids
$d(I)_n \cdot d(C)_n$ or poly d(I)·d(C)	double stranded deoxyribohomopolymer of deoxyinosinic and deoxycytidylic acids
$d(G)_n \cdot d(C)_n$ or poly d(G)·d(C)	double stranded deoxyribohomopolymer of deoxyguanylic and deoxycytidylic acids
$d(C)_n$ or poly d(C)	single stranded deoxyribohomopolymer of deoxycytidylic acid
poly d(I)	single stranded deoxyribohomopolymer of deoxyinosinic acid
$r(I\text{-}C)_n$	alternating ribocopolymer of inosinic and cytidylic acids
$r(G\text{-}C)_n$	alternating ribocopolymer of guanylic and cytidylic acids
$r(I)_n$ or poly r(I)	single stranded ribohomopolymer of inosinic acid
$r(G)_n$ or poly r(G)	single stranded ribohomopolymer of guanylic acid
poly r(C)	single stranded ribohomopolymer of cytidylic acid

GpC	guanylyl (3'-5') cytosine
CpG	cytidylyl (3'-5') guanosine
GpG	guanylyl (3'-5') guanosine
CpC	cytidylyl (3'-5') cytosine
Rif	rifampicin
E-DNA	binary complex of *A. vinelandii* RNA polymerase core enzyme with DNA
PP_i	pyrophosphate
PuTP	purine ribonucleoside triphosphate
NTP	ribonucleoside triphosphate (purine or pyrimidine)
TCA	trichloroacetic acid
ME	mercaptoethanol
Tris	TRIZMA base (HCl form) - Sigma Chemical Corporation

THE AFFINITY OF GTP AND ITP FOR THE INITIATION AND ELONGATION SITES OF *AZOTOBACTER VINELANDII* RNA POLYMERASE CORE ENZYME

INTRODUCTION

Azotobacter vinelandii RNA polymerase is an acidic protein whose main function is the transcription of a DNA template from ribonucleoside triphosphates. The enzyme has been isolated from phosphocellulose columns in two forms: the holoenzyme, E^{σ}, and the core enzyme, E (1). The holoenzyme contains the subunits β', β, σ, α_2 of molecular weights 155,000, 145,000, 90,000 and 40,000 respectively (1,2,3) whereas the core enzyme contains only the β', β and α_2 subunits. The subunits have been isolated in pure active form from *E. coli* (4,5) and *M. luteus* (6) and active enzyme reconstituted from the subunits.

The β' and β subunits are involved in the catalytic activity of RNA polymerase. It is believed that the main role of the β' subunit is in binding to DNA (7) for the formation of the binary complex. The β subunit is apparently involved in the binding of purine ribonucleoside triphosphates for initiation of transcription.

It is believed that the sigma subunit confers a conformational change on the core enzyme and that this conformational change is responsible for the difference in properties between these two active forms (1-3, 9-14). The holoenzyme exhibits more activity for transcription of T_4 DNA than the core enzyme (9) and has a higher affinity for promotor regions of T_7 DNA than the core enzyme (10,11). Sigma is not necessary for the formation of an E-DNA binary complex, but does enhance the stability of the complex formed (14). Core enzyme binds loosely and nonspecifically to nonpromotor regions of T_7 DNA (11,12) to form a binary E-DNA complex, whereas the holoenzyme binds

tightly to promotor regions of the template to form very stable binary complexes which support rapid initiation of RNA transcription (13,14).

The conformational changes related to increased affinity for promotor regions of a template do not affect elongation significantly, for sigma has been found to dissociate from RNA polymerase both after initiation of transcription of phage λ DNA *in vivo* (15) as well as following ternary complex formation during RNA synthesis with synthetic templates *in vitro* (1, 16).

Another function of sigma, not well understood with respect to its role in transcription, is its function regarding the aggregation of the enzyme. It has been found that E^{σ} aggregates at ionic strengths less than 0.1 and the core enzyme aggregates at ionic strengths less than 0.2 (2, 3). This may be one of the major factors involved with respect to differences in ease of formation of highly stable binary complexes with holoenzyme vs. core enzyme.

The role of RNA polymerase in transcription may be divided into the following categories subsequent to the formation of a stable E-DNA binary complex:

1. Initiation
2. Elongation
3. Termination

This study is concerned only with the first two steps of RNA synthesis.

Prior to initiation of transcription of a specific DNA template from complementary ribonucleoside triphosphates, a binary complex is formed between RNA polymerase and DNA. That RNA polymerase binds to

a DNA template to form a stable binary complex has been determined following sedimentation of active RNA polymerase - DNA binary complexes in a zonal gradient (17, 18) and by the formation of RNA polymerase - DNA binary complexes which can be retained on a nitrocellulose filter. (19, 20). The natural (19 - 22) or synthetic DNA's (21, 23) used to form the binary complex are not retained on the filter in the absence of the protein (19, 20).

A binary complex formed between RNA polymerase and supercoiled DNA exhibits greater stability than the corresponding complex with non-supercoiled DNA (22). It has also been demonstrated that double stranded DNA's form more stable binary complexes than single stranded DNA's (24).

Heparin resistance has been used as a probe to study the binary complex (8). Heparin is a mucopolysaccharide composed of equimolar amounts of D-glucuronic acid 2 sulfate and D-galactosamine N C-6 disulfate (25) which presumably acts by binding to basic amino acid residues near the template binding site on the β' subunit of the enzyme (8). The mode of action of heparin is not clear; however, it is known that it binds to a site distinct from the initiation site (8, 26), that it dissociates aggregated forms of the enzyme at low salt (26, 27), that it dissociates the enzyme - DNA binary complex (8, 27), but that this dissociation rate is decreased when the sigma subunit is present (8), that it inhibits both free and DNA-bound RNA polymerase (26), but that termination of RNA synthesis is not as abrupt as in the case of rifampicin. Thus, heparin has been a useful probe in the study of binary complex stability (24).

Nevertheless, a binary complex as described above is not necessary for the onset of RNA synthesis. Unprimed synthesis of poly r(A)· poly r(U) (28, 29) and poly r(I-C) (5, 30) have been detected. However, the mechanism of unprimed synthesis is in need of further clarification, and template-directed RNA synthesis is of greater relevance to the present study.

Binding of the enzyme to DNA to form a rapid start complex is a temperature dependent phenomenon. It has been found by Mangel and Chamberlin (31) that the enzyme dissociates more readily from the binary complex at a low temperature implying that a local unwinding of the DNA helix (14) may be involved. A role of sigma with respect to the melting of the DNA helix to form stable complexes at low temperature is not evident for both core and holoenzyme behave in a similar manner in forming binary complexes as a function of temperature (32).

Template-directed initiation of RNA synthesis occurs with the incorporation of a purine ribonucleoside triphosphate to form the 5' terminus of the chain (21, 33) regardless of whether the template is double-stranded DNA, single-stranded DNA or single-stranded RNA. The existence of s single initiation site on the β subunit has been postulated (34, 35). Studies using initial kinetics (36), equilibrium dialysis (35) and fluorescence quenching (34) imply that purine nucleotides have specific binding to the rifampicin sensitive initiation site, the K_s for binding ranging from 133 - 185 μM in the presence or absence of divalent cations.

Pyrophosphate exchange studies by Krakow and Fronk (37)

indicate that the initiation site of A. vinelandii RNA polymerase holoenzyme specifically binds purine ribonucleoside triphosphates as a function of template, has greater affinity for ATP and GTP which are most commonly found at the 5'end of the RNA molecules than for analogs of these substrates, and exhibits greater affinity for ATP and GTP than does the core enzyme.

To further probe the nature of the initiation site, rifampicin, an antibiotic which binds to the β subunit of bacterial RNA polymerase (8, 38) at a site 37 Å from the initiation site (39), is employed. X-ray analysis (40) of rifamycin B and Y as well as two other ansa compounds implies that the aromatic nucleus containing oxygen atoms O(1) and O(2) and/or the hydroxyl groups on C(21) and C(23) are involved in binding to RNA polymerase.

There is a single binding site for rifampicin for the RNA polymerase protomer (41, 42). Rifampicin, in a bimolecular reaction, attacks free RNA polymerase or the RNA polymerase - DNA binary complex (43) and blocks the binding of purine ribonucleoside triphosphates to the initiation site (11, 35, 43). That inhibition is not caused by dissociation of the binary complex has been demonstrated by Ishihama and Hurwitz (44) who showed that rifampicin-treated RNA polymerase can bind DNA.

Rifampicin does not inhibit RNA polymerase involved in RNA synthesis (41, 45) and resistance to rifampicin is a consequence of formation of a rapid starting binary complex (43). Rifampicin sensitivity is greater at low temperatures and under these conditions RNA polymerase is rifampicin sensitive even in the presence of excess

DNA (32). On the other hand, after inhibition of transcription of RNA synthesis with excess rifampicin, there has been found residual RNA synthesis *in vivo* in *E. coli* (46).

Rifampicin competes with purine ribonucleoside triphosphates during the initiation of RNA synthesis. Using a technique in which rifampicin and the ribonucleoside triphosphates are simultaneously added to the E^{σ}-T_7 binary complex, the rate constant for initiation of T_7 RNA transcription has been determined. The rate of initiation is more affected by changes in substrate concentration than the rate of elongation (48); larger concentrations of purine ribonucleoside triphosphates are required for initiation than for elongation (36, 49, 50).

Initiation by purine ribonucleoside triphosphates is not a prerequisite for *in vitro* RNA synthesis. Initiation of RNA synthesis can be bypassed with the use of a dinucleoside monophosphate or oligonucleotide primer (48 - 50). Primers have been shown to be covalently linked to the 5' end of RNA chains (51) and to preform an $E\begin{matrix}\nearrow \text{DNA} \\ \searrow \text{primer}\end{matrix}$ ternary complex by effectively competing with the purine ribonucleoside triphosphate for the initiation site at concentrations of 0.16 mM or greater (48). Formation of the ternary complex stabilizes the enzyme to rifampicin attack (47).

It is not known whether initiation in the absence of a primer proceeds by an ordered or random mechanism; however, an ordered mechanism has been proposed (37, 47, 52) in which the enzyme, which is present as a binary complex with the template, functions by:

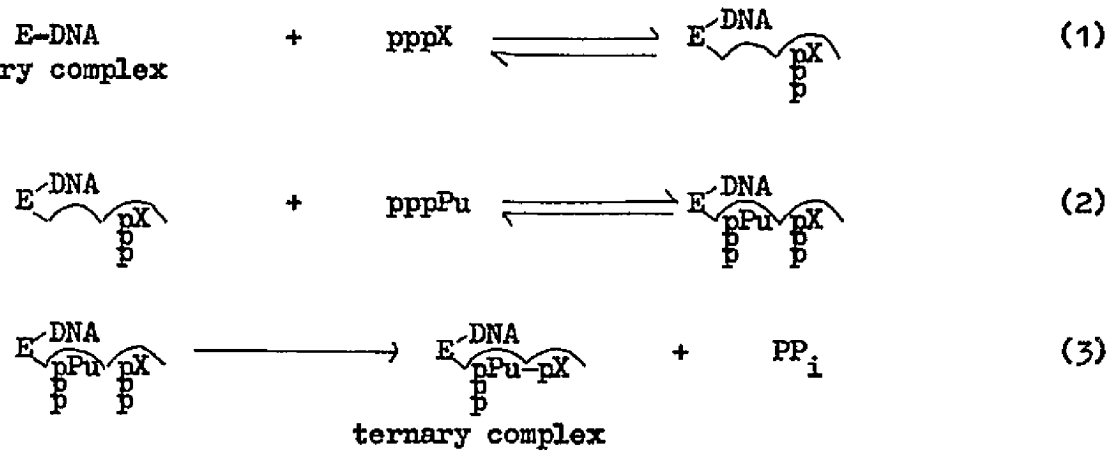
1. binding a ribonucleoside triphosphate (pppX) to the elongation

site, followed by

2. binding a purine ribonucleoside triphosphate (pppPu) to the initiation site, followed by

3. formation of a phosphodiester bond with the release of pyrophosphate (PP_i).

This may be represented as follows:



The ternary complex becomes a stable chain elongation complex following the formation of the second phosphodiester bond. In the case of double stranded templates chain elongation is apparently processive - i.e. elongation occurs without dissociation of the chain elongation complex (53, 54). This may not be the case when single stranded templates are used as will be indicated in further detail in a latter section of this study.

Ternary complexes (i.e. ternary elongation complexes) isolated in vivo (55) or studied in vitro by polyacrylamide gel electrophoresis (1, 16) are stable and lack the sigma subunit. It is believed that during chain elongation, approximately twelve residues of the nascent RNA chain proximal to the 3' OH terminus are bound to the product

binding site (56). The elongating ribonucleoside triphosphate directed by the DNA template binds to the substrate site and polymerization continues with the release of pyrophosphate (37). Chain elongation requires a lower substrate concentration than initiation (37, 57, 58). The direction of chain growth is from the 5' end of the RNA chain to the 3' end (53, 59).

During chain elongation, the ternary complex is insensitive to inhibitors which bind the binary complex only (8, 41, 60), but is inhibited by the RNA formed during the reaction after long periods of synthesis (61 - 64).

It has been found that an increase in ionic strength stimulates RNA synthesis, the optimal stimulation being at ionic strengths of 0.2 - 0.3 (65, 66). The principal effect of salt stimulation is that the rate of elongation increases with increasing ionic strength provided the salt concentration is not so high that the ternary complex dissociates (67, 68).

High salt thus causes rapid termination of RNA chains by the early dissociation of ternary complexes (68, 70). Such early dissociation does not ordinarily occur at low ionic strength under which conditions both ternary and binary complexes have greater stability.

In contrast to the stimulation of elongation by increasing ionic strength, there is inhibition of initiation at high ionic strengths. This is related in part to the dissociation of the binary complex (71). High ionic strength probably has an effect on enzyme conformation and/or on the affinity for binding to the template. For example, increased ionic strength has been noted to change the starting

nucleotide sequence of RNA's synthesized on T_7 DNA (72), a phenomenon which may reflect different promotor affinities.

Due to differences in ionic strength effects (70, 71, 73) upon initiation and elongation, the results of binding studies (35) and affinity labeling (74), the enzyme is postulated to have two sites for the binding of ribonucleoside triphosphates. The initiation site or product terminus site (37) binds the purine ribonucleoside triphosphate specified by the DNA template to become the 5' terminal triphosphate. The second site, called the elongation site or substrate site, binds either purine or pyrimidine ribonucleoside triphosphates directed by the template. Binding to this second site occurs with lower selectivity for purine vs. pyrimidine than the initiation site (75, 76), is rifampicin insensitive and requires the presence of a divalent cation (35).

The role of the divalent cation in initiation and elongation of RNA synthesis is not clear. However, a divalent cation such as Mg^{++} or Mn^{++} is a prerequisite for RNA synthesis. It is known that metal ions bind to the N-7 of bases such as inosine (77) or guanine (78). And, for $d(A\text{-}T)_n$ directed $r(A\text{-}U)_n$ synthesis in the presence of Mg^{++}, Rhodes and Chamberlin (58) identify the substrates as Mg^{++}•ATP and Mg^{++}•UTP, Mg^{++}•ATP being the initiating purine ribonucleoside triphosphate (52).

Divalent cations such as Mg^{++} and Mn^{++} also interact with nucleic acids. Diamagnetic ions such as Mg^{++} cause small chemical shifts in the NMR spectra of nucleic acids, whereas paramagnetic ions such as Mn^{++} cause even greater chemical shifts and line broadening due to

the interaction of the nucleus with unpaired electrons (77). Further, metal ion competition with hydrogen bonding causes destabilization of the double helical structure of the template whereas metal ion binding to phosphate groups apparently causes stabilization of the helix (79). Mg^{++} ions have been shown to stabilize the helix to a greater extent than Mn^{++} ions due to their greater affinity for the phosphate group of the base (79, 80).

Mg^{++} and Mn^{++} also have been found to stabilize the RNA polymerase - poly U complex from dissociation by 1 M urea, Mn^{++} having the greater stabilizing effect (81). Mn^{++} apparently protects the subunit structure of core RNA polymerase against attack by 1 M urea and 1×10^{-5} M phenylmercurisulfonate, whereas Mg^{++} does not. These data indicate that Mn^{++} affects the conformation of RNA polymerase itself in addition to its effects on ribonucleoside triphosphate bases and the template. Nevertheless, that the effects of both divalent cations cannot be considered as similar is further indicated by the difference in concentration range of cation needed for optimal conditions - that concentration range being narrow for Mn^{++} and broad for Mg^{++} (82).

The template also affects transcription in a manner still requiring clarification. To elucidate the role of template during transcription, natural DNA has been frequently employed. RNA is synthesized such that the transcript is antiparallel to the template, and if the template has specific promotor regions and/or termination regions, the product will be affected by the restrictions determined by these regions (57, 83, 84). Secondly, transcription has been studied

using single stranded polyribonucleotides or polydeoxyribonucleotides as templates for ribohomopolymer synthesis (82, 85). Alternating deoxyribonucleotide and ribonucleotide copolymers are also effective as templates; the transcription of RNA templates occurs at a slower rate than that of the DNA templates (23, 30, 86, 87).

The rate of elongation is apparently affected by the base stacking of the template, the rate being slower for the transcription of $d(G\text{-}C)_n$ than $d(I\text{-}C)_n$ (23, 88) and the temperature sensitivity of $r(G\text{-}C)_n$ synthesis being greater than that of $r(I\text{-}C)_n$ synthesis (23). Since base stacking interactions of the template have been noted to affect binding of proteins such as the lac repressor (89) in a study involving a comparison of $d(A\text{-}T)_n$ and an analog $d(A\text{-}\overline{BrU})_n$, there is an additional possibility that the nature of the template affects tight binding of the enzyme.

In addition to template base stacking interactions, the nearest neighbor effects of analogs of ribonucleoside triphosphates have been tested. The variation in K_m for elongation with these substrates has been attributed in part to differences in base stacking interactions in the RNA (90).

The above effects do not comprehensively discuss the interactions of RNA polymerase in prokaryotic systems - a multitude of interactions exist which are not within the scope of this study, e.g., the interaction of RNA polymerase with a termination factor, rho (91, 92), with protein modulators of initiation (89, 93), and with inhibitors (94) both in vivo and in vitro. All of these interactions point to the fact that regulation of transcription is a complicated phenomenon

dependent upon the totality of cellular metabolic functions.

A common means of analyzing some specific properties of any complex system is to isolate parts of the system and use simple models for in vitro studies. This is the approach used in this study.

It is known from pyrophosphate exchange studies by Krakow and Fronk (37) that the initiation site of A. vinelandii RNA polymerase holoenzyme has a significantly greater affinity for GTP than for ITP. Other studies (34 - 37) further indicate that only purines have specific affinity for binding to the rifampicin sensitive initiation site. There also exists a second site, the elongation site, which is rifampicin insensitive, has a divalent cation requirement and to which both purines and pyrimidines bind.

Kinetic studies with RNA polymerase holoenzyme (58) indicate that when preformed initiation complexes are isolated, they may be used to study the K_m for elongation. The K_m for elongation was found to be independent of the template used for transcription and to correlate with the K_s for binding of ribonucleoside triphosphates to the elongation site of RNA polymerase determined by Wu and Goldthwait (34, 35).

The present study has examined the relative affinity of GTP and ITP for the initiation and elongation sites of A. vinelandii RNA polymerase core enzyme (i.e. enzyme in the absence of sigma) and the effect of the divalent cations Mg^{++} and Mn^{++} at various ionic strengths on these affinities. Pyrophosphate exchange studies are not a good probe for studying the relative differences in affinity of the core enzyme for GTP and ITP. There is no significant difference in the

amount of $^{32}PP_i$ exchanged into GTP when GTP or ITP is the initiating nucleoside triphosphate; the exchange rate is very low for both nucleoside triphosphates when compared with that of the holoenzyme (95). Further, a comparison of the effects of the divalent cations Mg^{++} and Mn^{++} cannot be studied in this system due to the precipitation of manganese pyrophosphate. Nevertheless, it is of interest to probe the specificity of the initiation and elongation sites of A. vinelandii RNA polymerase in the absence of the apparent difference in affinity conferred by the presence of the sigma subunit (10 - 13).

Rifampicin inhibits initiation of RNA synthesis and binds to core and holoenzyme (42), to binary enzyme - template complexes of core and holoenzyme (43) but neither binds to nor inhibits the ternary enzyme$\begin{matrix}\nearrow \text{template} \\ \searrow \text{product}\end{matrix}$ complex engaged in RNA synthesis (41). Therefore, it is possible to use rifampicin challenge experiments (47) to study the half time for initiation by preformed binary complexes of RNA polymerase and DNA.

The rifampicin challenge technique can be applied to the core polymerase - $d(I\text{-}C)_n$, $-d(I)_n$, $d(I)_n \cdot d(C)_n$, etc. as a probe to determine the relative affinity of GTP and ITP for the initiation site during ribohomopolymer synthesis of poly r(G) vs. poly r(I) and during alternating ribocopolymer synthesis of poly r(G-C) vs. poly r(I-C) as a function of template, divalent cation and ionic strength.

Since heparin can be used to probe the effects of binary complex stability (8, 24, 26, 27), the role of base stacking interactions of $d(I\text{-}\overline{BrC})_n$ and $d(I\text{-}\overline{IC})_n$ vs. $d(I\text{-}C)_n$ templates during $r(G\text{-}C)_n$ and $r(I\text{-}C)_n$ synthesis may also be determined.

The use of inhibitors of transcription such as rifampicin and heparin combined with an analysis of the relative K_m's of GTP, CTP and ITP determined under elongation conditions may discern the relative affinities of the initiation site of the core enzyme for GTP and ITP as well as to analyze the effects of the divalent cation cofactor and/or ionic strength on the system. These probes may then determine the role of the RNA polymerase core enzyme, RNA polymerase in the absence of sigma and termination factors, in initiation and elongation during transcription.

MATERIALS AND METHODS

Tris HCl, GTP, ITP, mercaptoethanol, mercaptoethylamine, rifampicin, GpC and CpG were obtained from Sigma Chemical Corporation. Rifampicin was prepared as an aqueous solution. A molar extinction of 15.4×10^3 at 475 nm and a molecular weight of 823 was assumed for rifampicin.

Poly dI, poly dC, poly dG•dC were obtained from P. L. Biochemicals. Poly dI•dC was prepared by mixing equimolar quantities of poly dI and poly dC (96). $d(I\text{-}C)_n$ was synthesized using E. coli DNA polymerase I with dITP and dCTP, $d(I\text{-}\overline{BrC})_n$ with dITP and dBromoCTP, $d(I\text{-}\overline{IC})_n$ with dITP and dIodoCTP. (^{3}H)CTP, (^{3}H)GTP, (^{3}H)ATP and ($\alpha\,^{32}$P)GTP were purchased from New England Nuclear. (^{3}H)ITP was prepared by nitrous acid deamination of (^{3}H)ATP. All nucleoside triphosphates were chromatographically purified on Dowex 1X. All labeled nucleoside triphosphates were determined to be free of mono- and diphosphates by chromatography on PEI cellulose (97).

An ϵ^{260} value of 5.35×10^3 was assumed for poly dI (98)[1], ϵ^{260} of 5.3×10^3 for poly dC (98)[1], ϵ^{260} of 6.34×10^3 for poly dI•dC (96), ϵ^{260} of 9.3×10^3 for $d(I\text{-}C)_n$, $d(I\text{-}\overline{BrC})_n$ and $d(I\text{-}\overline{IC})_n$, and ϵ^{260} of 7.95×10^3 for poly dG•dC (98)[1]. Concentrations are in moles of constituent nucleotides.

All other chemicals were reagent grade.

[1] Concentrations were reconfirmed using ϵ^{246} of 9.4×10^3 for poly d(I), ϵ^{273} of 6.8×10^{-3} for poly dC and ϵ^{253} of 7.4×10^3 for poly dG•dC as indicated by literature supplied by P. L. Biochemicals.

A. vinelandii core RNA polymerase

Azotobacter vinelandii RNA polymerase core enzyme was purified by a modification of the published procedure (99, see Appendix A) with chromatography on phosphocellulose (Whatman P 11) as the final step. The RNA polymerase used in this study had a specific activity of 900 - 1400 units/mg. protein (1 unit = 1 nmole (^{3}H) UMP incorporated into $r(A\text{-}U)_n$ in 10 minutes at 37° C with $d(A\text{-}T)_n$ as template) and an $A_{280/260}$ of 1.4 to 1.8. The protein concentration was determined by the method of Schaeffner and Weissmann (100). The polymerase was essentially free of the sigma subunit as determined by SDS polyacrylamide gel electrophoresis in Tris-glycine buffer (101). It is to be noted that K_m determinations, rifampicin resistance and heparin resistance determinations were independent of specific activity of enzyme which varied as indicated above with different enzyme preparations.

Determination of acid precipitable radioactivity

Reactions were terminated by the addition of 0.1 ml. of 0.2 M sodium pyrophosphate and 3 ml. of cold 5% trichloroacetic acid. The mixture was cooled for 10 minutes in an ice bath after which precipitates were filtered onto Whatman GF/C filters and washed with 10 ml. of 5% trichloroacetic acid. Filters were dried and counted with a toluene-PPO-POPOP scintillation solution and radioactivity was determined in a Beckman LS-230 scintillation counter.

Determination of nitrocellulose bound radioactivity

Reactions were terminated by the addition of 0.1 ml. of 0.5 M

EDTA and filtered on nitrocellulose filters. Filters were dried and counted in a toluene-PPO-POPOP scintillation solution. Radioactivity was determined in a Beckman LS-230 scintillation counter.

Nearest neighbor analysis of $r(G\text{-}C)_n$

$r(G\text{-}C)_n$ prepared from ($\alpha^{32}P$) GTP (446 cpm/pmole) and CTP was hydrolyzed overnight with 0.5 N NaOH. The hydrolyzate was applied to a Dowex 1X formate column and eluted with 0 - 2 M NH_4COOH. 98% of the total radioactivity was recovered as 2', 3' CMP.

RESULTS

Characteristics of $r(G\text{-}C)_n$ and $r(I\text{-}C)_n$ synthesis

A. Optimum Conditions

The optimum conditions for elongation of $r(G\text{-}C)_n$ and $r(I\text{-}C)_n$ in the presence of the divalent cations Mg^{++} and Mn^{++} have been determined as indicated in Table Ia. Synthesis of $r(G\text{-}C)_n$ and $r(I\text{-}C)_n$ is linear regardless of buffer for about 10 minutes (Figure 1a, b) after which the rate of synthesis decreases. The maximum percent incorporation of substrate nucleotides after long periods of incubation is indicated in Table Ib.

It will be noted that there is more synthesis (determined as incorporation of (^{3}H)CMP) of $r(I\text{-}C)_n$ than $r(G\text{-}C)_n$ at the maximum. This also holds true for all time periods during synthesis regardless of divalent cation in the reaction mix. 100% incorporation of input nucleotides is never obtained.

Fidelity of transcription has been shown by nearest neighbor analysis.

B. Restimulation of Enzyme Activity

Due to the fact that only about 50% of input nucleotides were incorporated even after long periods of synthesis, the following questions were asked:

1. Is the enzyme inactivated as a result of long incubations?
2. Is there sufficient template in the system?
3. Is enzyme stimulation possible by further addition of equimolar quantities of ribonucleoside triphosphates as is the case for $r(A\text{-}U)_n$ synthesis (41)?

TABLE I

a. Determination of optimum conditions

RNA Synthesized	Divalent Cation	Optimum [Cation]
$r(G-C)_n$	Mg^{++}	36 - 44 mM
$r(I-C)_n$	Mg^{++}	40 mM
$r(G-C)_n$	Mn^{++}	0.8 mM
$r(I-C)_n$	Mn^{++}	0.8 - 1.6 mM

b. % synthesis at optimum (120 minutes incorporation)

RNA Synthesized	Divalent Cation	Maximum % Incorporation of Input Nucleotides
$r(G-C)_n$	Mg^{++}	42
$r(I-C)_n$	Mg^{++}	54
$r(G-C)_n$	Mn^{++}	43
$r(I-C)_n$	Mn^{++}	57

LEGEND TO TABLE I

a. 10 μmoles Tris pH 8.0, 5 μmoles 1 M mercaptoethylamine, 10 nmoles $d(I-C)_n$, 5 μg. RNA polymerase and 0 - 1.5 μmoles $MnSO_4$ or 0 - 10 μmoles $MgSO_4$ in a total volume of 115 μl were preincubated for 10 minutes at 37° C to form the $E-d(I-C)_n$ binary complex. Following preincubation, 10 μl aliquots containing 50 nmoles (^3H)CTP (2800 - 3100 cpm/nmole) and 50 nmoles GTP or ITP were added. Incubations were terminated after 10 minutes and acid precipitable radioactivity determined.

b. <u>Synthesis when Mg^{++} is the divalent cation</u>

40 mM $MgSO_4$, 80 mM Tris pH 8.0, 40 mM mercaptoethylamine, 100 nmoles $d(I-C)_n$ and 50 μg. RNA polymerase in a total volume of 1.15 ml was preincubated at 37° C for 10 minutes. Following preincubation, 0.5 μmoles (^3H)CTP (2800 - 3100 cpm/nmole) and 0.5 μmoles GTP or ITP in a total volume of 100 μl was incubated with the enzyme - template complex. 100 μl samples were removed into 0.1 ml of 0.2 M sodium pyrophosphate pH 6.0 to terminate the reaction and subsequently acid precipitable radioactivity was determined.

<u>Synthesis when Mn^{++} is the divalent cation</u>

Conditions are the same as for Mg^{++} except 0.8 mM $MnSO_4$ is used in place of 40 mM $MgSO_4$.

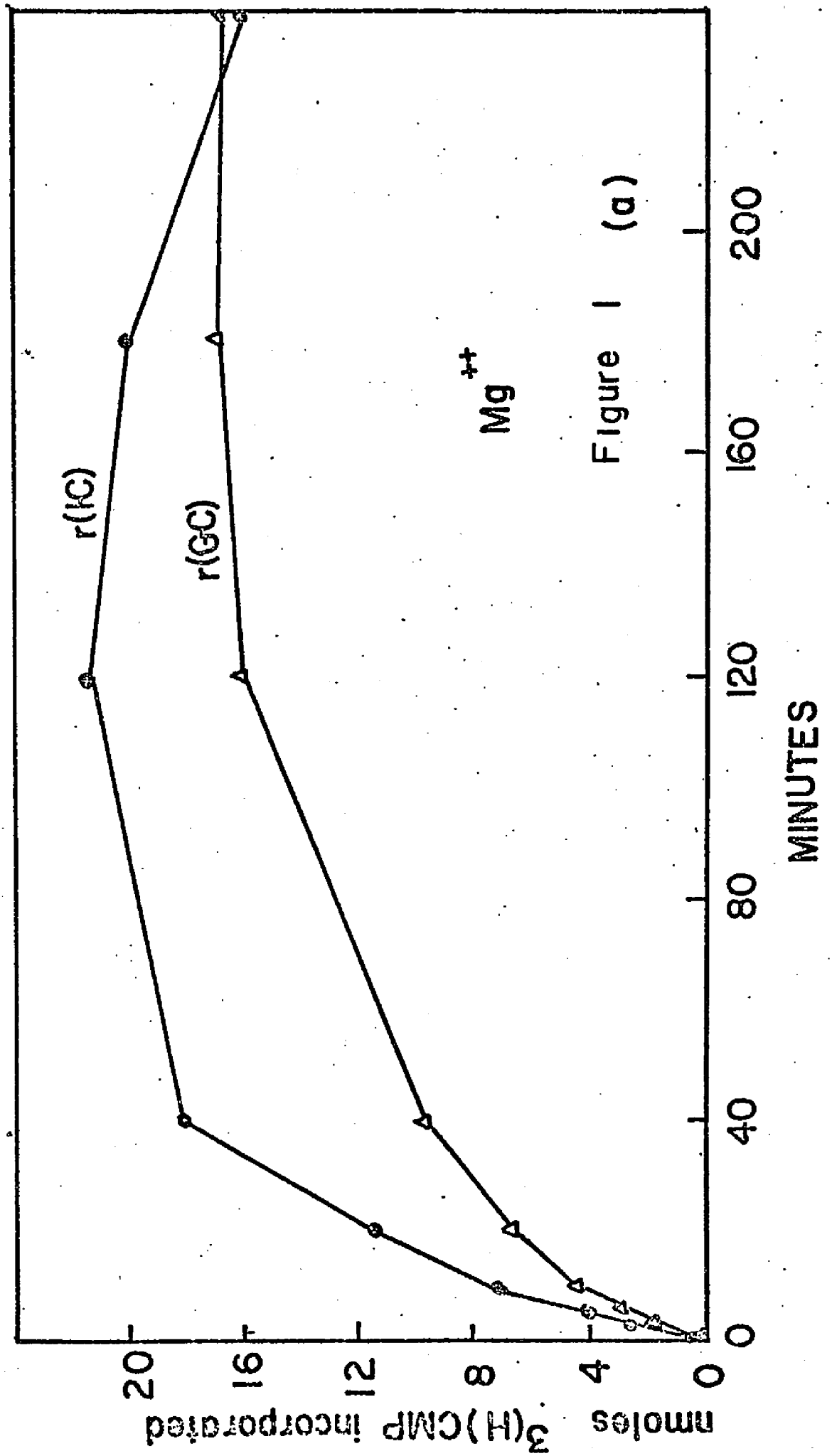


Figure 1 (a)

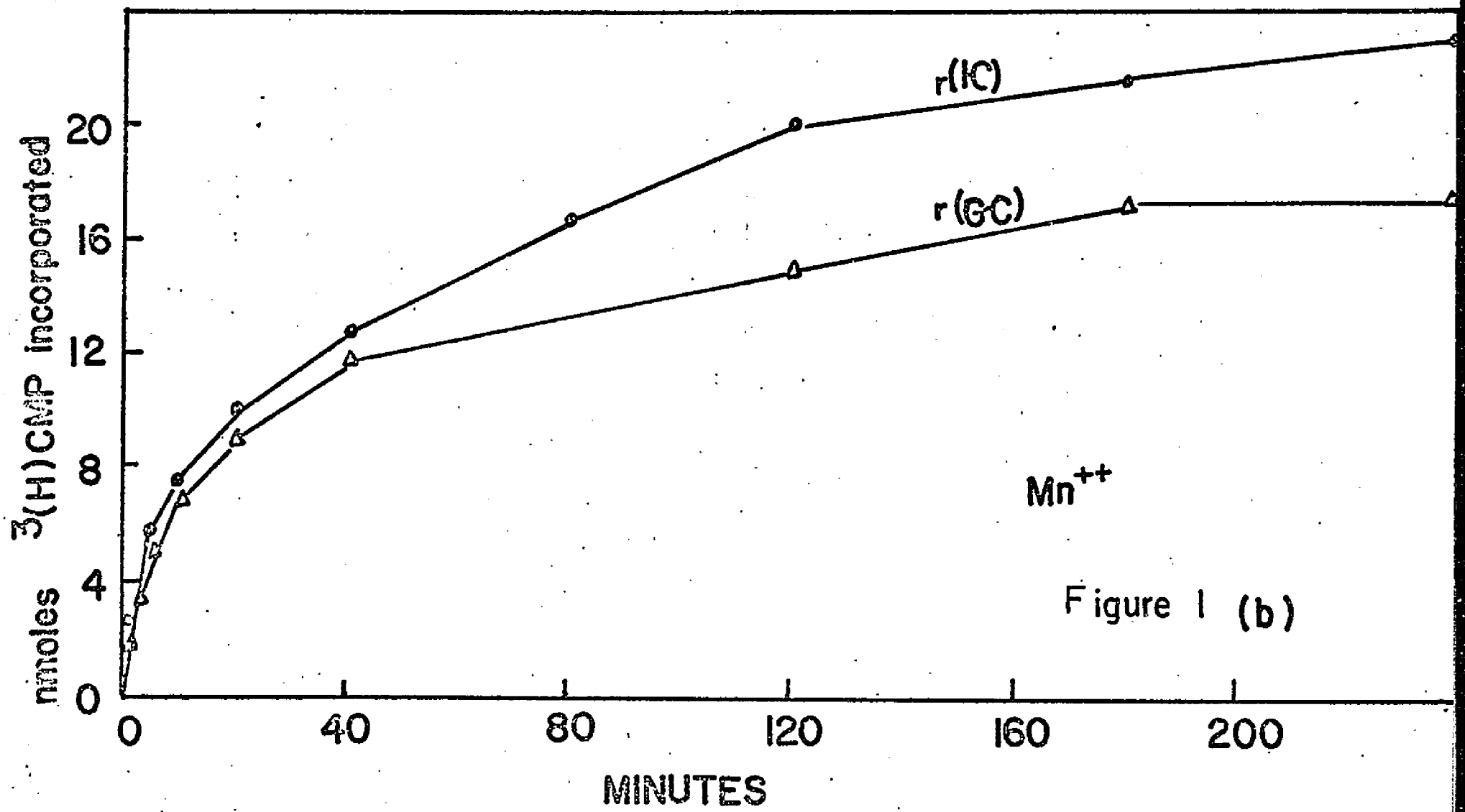


Figure 1 (b)

LEGEND TO FIGURE 1

Comparison of $r(G-C)_n$ and $r(I-C)_n$ synthesis in Mg^{++} and Mn^{++} containing buffers

a. Incorporation of (^3H) CMP into $r(G-C)_n$ and $r(I-C)_n$ when Mg^{++} is the divalent cation

See legend to Table Ib

b. Incorporation of (^3H)CMP into $r(G-C)_n$ and $r(I-C)_n$ when Mn^{++} is the divalent cation

See legend to Table Ib

Table II a and b shows the results of experiments attempting to answer these questions. It is evident from the data that the addition of further enzyme, template or ribonucleoside triphosphates does not lead to further incorporation of input nucleotides into RNA.

Therefore, the template and ribonucleoside triphosphates are apparently present at saturating levels. Product inhibition of synthesis, previously demonstrated by Krakow (61) is a likely cause of the early plateau levels. However, an adequate explanation for cessation of synthetic activity of the enzyme after 120 minutes of incubation although in the presence of saturating levels of template and substrates would require further studies not within the scope of this paper.

C. Effect of Ionic Strength on Elongation

It has been previously demonstrated by Fuchs *et al.* (67) that high ionic strength causes a decrease in RNA synthesis with T_4 DNA as template and by Mangel and Chamberlin (73) that initiation with T_7 DNA as template is decreased even in 0.2 M KCl. However, in both systems, the initiated complexes show a stimulation of RNA synthesis at elevated salt concentrations. The binary E-d$(I-C)_n$ complex is found to be stable to nitrocellulose filtration at $\mu = 0.1$ when preincubation of E-d$(I-C)_n$ occurs for 10 - 20 minutes. The binary E-d$(I-C)_n$ complex has been found to dissociate by increasing the salt concentration above ionic strength 0.1 in the presence or absence of divalent cations (Figure 2). The presence of 0.8 mM Mn^{++} is found to stabilize the binary complex to salt to a greater degree than the absence of divalent cation, and the presence of

TABLE II

Attempts to restimulate enzyme activity

a. Effect of increasing concentration of input ribonucleoside triphosphates on $r(G-C)_n$ and $r(I-C)_n$ synthesis

RNA Synthesized	Divalent Cation	nmoles (^{3}H)CMP Incorporated		
		A	B	C
$r(I-C)_n$	Mn^{++}	20.3	22.6	18.8
$r(G-C)_n$	Mn^{++}	14.3	15.2	14.5
$r(I-C)_n$	Mg^{++}	21.2	20.8	21.7
$r(G-C)_n$	Mg^{++}	12.6	12.0	12.6

b. Attempts to restimulate $r(I-C)_n$ synthesis in Mn^{++} containing buffers after 40 minutes or 120 minutes synthesis

Incubation Time (minutes)	Additions	Additional Incubation (minutes)	nmoles (^{3}H)CMP Incorporated
40	none	none	16.7
40	0.4 mM NTP's	40	16.1
120	none	none	20.6
120	10 nmoles $d(I-C)_n$	40	18.1
120	none	40	22.6
120	20 µg. RNA polymerase	40	20.7

LEGEND TO TABLE II

a. 300 µl of a reaction mix containing 25 mM $MgCl_2$, 50 mM Tris pH 8.0, 50 mM ME, 30 nmoles $d(I-C)_n$ and 15 µg. RNA polymerase or containing 1 mM $MnSO_4$, 50 mM Tris pH 8.0, 50 mM ME, 30 nmoles $d(I-C)_n$, 15 µg. RNA polymerase and 7.5 mM KCl were preincubated for 10 minutes at 37° C. Following preincubation, 30 µl aliquots containing 150 nmoles (^{3}H)CTP (2800 - 3100 cpm/nmole) and 150 nmoles GTP or ITP were added to the preincubation mix.

A. After 120 minutes at 37° C, 100 µl samples were removed into 100 µl 0.2 M sodium pyrophosphate to terminate the reaction

B. After 120 minutes at 37° C, 100 µl samples were incubated an additional 40 minutes and the reaction terminated as in A.

C. After 120 minutes at 37° C, 100 µl samples were incubated an additional 40 minutes with a 10 µl aliquot containing 50 nmoles (^{3}H)CTP and 50 nmoles GTP or ITP and the reaction terminated as in A.

Acid precipitable counts were determined for all samples.

b. Preincubation conditions as in a.

Following preincubation, 30 µl aliquots containing 150 nmoles (^{3}H)CTP (2800 - 3100 cpm/nmole) and 150 nmoles GTP or ITP were added to the preincubation mix.

For the 40 minute incubations: After 40 minutes at 37° C, 100 µl samples were removed into 100 µl of 0.2 M sodium pyrophosphate to terminate the reaction; 100 µl samples were withdrawn and incubated an additional 40 minutes with a 10 µl aliquot containing 50 nmoles

(^{3}H)CTP (2800 - 3100 cpm/nmole) and 50 nmoles GTP or ITP and the reaction then terminated.

For the 120 minute incubations: After 120 minutes at 37° C, 100 µl samples were withdrawn into 100 µl of 0.2 M sodium pyrophosphate to terminate the reaction, 100 µl samples were withdrawn and incubated an additional 40 minutes and the reaction terminated, 100 µl samples were incubated an additional 40 minutes after the addition of a 10 µl aliquot containing 10 nmoles $d(I-C)_n$ and the reaction terminated, and 100 µl samples were incubated an additional 40 minutes with a 20 µl aliquot containing 20 µg. RNA polymerase.

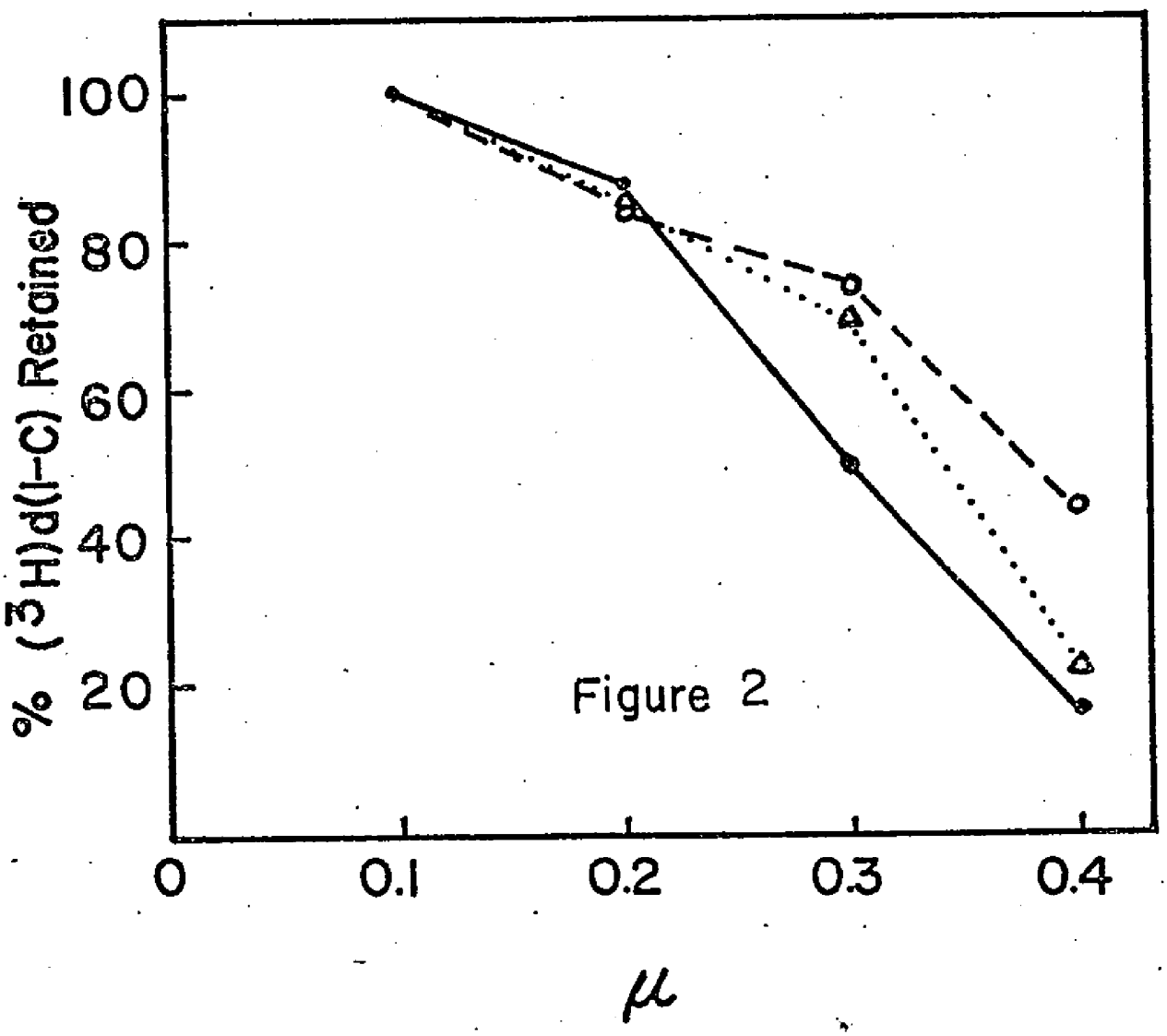


Figure 2

LEGEND TO FIGURE 2

Effect of ionic strength on retention of the E-d(I-C)$_n$ binary complex

400 µl of a buffer containing: Δ -- Δ 50 mM Tris pH 8.0, 50 mM ME, 75 mM KCl; • — • 50 mM Tris pH 8.0, 50 mM ME, 25 mM $MgCl_2$, o -- o 50 mM Tris pH 8.0, 50 mM ME, 1 mM $MnSO_4$ 75 mM KCl was added to 60 µl of a mix containing 40 nmoles (^{3}H)d(I-C)$_n$ (1635 cpm/nmole) and 20 µg A. vinelandii RNA polymerase core enzyme in a total volume of 460 µl. After 15 minutes preincubation at 37° C, 100 µl of each sample was filtered on a nitrocellulose filter (previously soaked in 0.1 N KOH and stored in 0.05 M NaCl) (102) and washed with 1 ml of the preincubation buffer containing Δ -- Δ 40 mM Tris pH 8.0, 40 mM ME; • — • 40 mM Tris pH 8.0, 40 mM ME, 20 mM $MgCl_2$; o -- o 40 mM Tris pH 8.0, 40 mM ME, 1 mM $MnSO_4$ with the ionic strength of each solution adjusted with KCl to 0.1, 0.2, 0.3 or 0.4 as indicated. Samples were analyzed for filter bound radioactivity. The % (^{3}H)d(I-C)$_n$ retained was calculated relative to nmoles (^{3}H)d(I-C)$_n$ retained at µ = 0.1 which was considered as 100%.

Δ -- Δ 100% = 3.8 nmoles (^{3}H)d(I-C)$_n$ retained

• — • 100% = 4.1 nmoles (^{3}H)d(I-C)$_n$ retained

o -- o 100% = 4.6 nmoles (^{3}H)d(I-C)$_n$ retained

20 mM Mg^{++} to have a destabilizing effect. However, Mg^{++} has an effect in increasing the % retention of E-(^{3}H)d(I-C)$_n$ on nitrocellulose filters after 0.4 ionic strength salt washes when GTP or ITP are added to the preincubation mixture (Table III). Table III indicates that the addition of nucleoside triphosphates to the binary complex enhances the stability of the complex in the presence of a primer only when a divalent cation is present during preincubation and that this stability is not totally lost when the filtered complex is washed with a $\mu = 0.4$ buffer in the absence of divalent cation. It is further to be noted that in the presence of a GpC primer + GTP the % retention of (^{3}H)d(I-C)$_n$ is significantly more enhanced in a Mn^{++} containing buffer than is the % retention of (^{3}H)d(I-C)$_n$ in the presence of a GpC primer + ITP. The enhancement of retention is also evident in Mg^{++} buffers, but not to the extent of that noted in Mn^{++} buffers implying a greater affinity of the enzyme for GTP than ITP which is divalent cation-dependent and which suggests the addition of the ribonucleoside monophosphate to the primer. Stabilization of the binary complex in the presence of GTP and ITP ± primers implies formation of stable elongation ternary complexes since when synthesis of r(I-C)$_n$ or r(G-C)$_n$ is allowed to occur for 2.5 minutes the ternary complex formed is found to be retained on nitrocellulose filters (data not shown). This ternary complex is more salt stable than the binary complex as indicated in Table III.

If synthesis is allowed to proceed for long periods of time in buffers at elevated ionic strength, it is of interest to determine the effect on: a. rate of synthesis; b. ease of dissociation of

TABLE III

% $(^3H)d(I-C)_n$ retained in 0.4 ionic strength wash buffers

Preincubated Components	No Me^{++} [a]	Mg^{++} [b]	Mn^{++} [c]	Mn^{++} [d] preincubated No Me^{++} wash	Mg^{++} [e] preincubated No Me^{++} wash
E-$(^3H)d(I-C)_n$	21.8	16.5	44.0	28.8	28.7
E-$(^3H)d(I-C)_n$ + CTP	20.3	18.6	44.0	21.4	24.1
E-$(^3H)d(I-C)_n$ + GpC	22.7	22.5	41.5	40.0	27.5
E-$(^3H)d(I-C)_n$ + CpG	25.0	21.8	34.9	41.5	26.6
E-$(^3H)d(I-C)_n$ + GTP	17.2	41.5	48.0	43.6	47.0
E-$(^3H)d(I-C)_n$ + ITP	17.3	30.2	44.0	33.6	17.4
E-$(^3H)d(I-C)_n$ + GpC + GTP	30.8	54.9	95.8	70.6	49.5
E-$(^3H)d(I-C)_n$ + GpC + ITP	22.6	47.5	54.6	38.0	30.2
E-$(^3H)d(I-C)_n$ + CpG + CTP	19.9	52.0	52.0	46.6	33.5
E-$(^3H)d(I-C)_n$ + GpC + CTP	----	45.7	51.1	----	----
E-$(^3H)d(I-C)_n$ + CpG + GTP	----	59.7	62.4	----	----
E-$(^3H)d(I-C)_n$ + GpG + CTP	----	33.2	44.1	----	----
E-$(^3H)d(I-C)_n$ + CpC + GTP	----	42.4	49.5	----	----
E-$(^3H)d(I-C)_n$ + CpG + ITP	----	43.1	47.5	----	----
E-$(^3H)d(I-C)_n$ + CpC + ITP	----	26.0	46.0	----	----
E-$(^3H)d(I-C)^*_n$ + GTP + CTP	----	89.1	94.2	----	----
E-$(^3H)d(I-C)^*_n$ + ITP + CTP	----	83.7	98.8	----	----

LEGEND TO TABLE III

% $(^3H)d(I-C)_n$ retained in 0.4 ionic strength wash buffers

200 µl of buffer containing:

a. 50 mM Tris pH 8.0, 50 mM ME, 75 mM KCl

b. 50 mM Tris pH 8.0, 50 mM ME, 25 mM $MgCl_2$

c. 50 mM Tris pH 8.0, 50 mM ME, 1 mM $MnSO_4$, 75 mM KCl

d. same as c

e. same as b

were added to 30 µl of a mix containing 20 nmoles $(^3H)d(I-C)_n$ (1635 cpm/nmole), 10 µg. RNA polymerase and 0.4 mM GTP, CTP, ITP as indicated and/or 0.32 mM GpC, CpG, CpC, GpG as indicated, in a total volume of 230 µl. After 15 minutes preincubation at 37° C, 100 µl of each sample was filtered on a nitrocellulose filter and washed with 1 ml of buffer containing:

a. 40 mM Tris pH 8.0, 40 mM ME

b. 40 mM Tris pH 8.0, 40 mM ME, 20 mM $MgCl_2$

c. 40 mM Tris pH 8.0, 40 mM ME, 0.8 mM $MnSO_4$

d. 40 mM Tris pH 8.0, 40 mM ME, 1 mM EDTA

e. 40 mM Tris pH 8.0, 40 mM ME, 25 mM EDTA

with the ionic strength of each solution adjusted to 0.1 or 0.4 with KCl as necessary. Samples were analyzed for filter bound radioactivity. Nmoles $(^3H)d(I-C)_n$ retained in 0.1 ionic strength solution was considered as 100% retention. 100% = 3.8 - 5.0 nmoles $(^3H)d(I-C)_n$ retained.

* In this case preincubation of the binary complex was allowed to occur for 15 minutes. Then, initiation of RNA synthesis occurred with the addition of 0.4 mM GTP + 0.4 mM CTP or 0.4 mM ITP + 0.4 mM

CTP as indicated. Samples were filtered after 2.5 minutes incubation.

terminated chains from the ternary complex. Figures 3a and b indicate that the amount of nitrocellulose-bound RNA at any given time, i.e. $E\begin{smallmatrix} d(I\text{-}C)_n \\ r(I\text{-}C)_n \end{smallmatrix}$ ternary complex formed, is less at ionic strength 0.3 than at ionic strength 0.16 although the rate of $r(I\text{-}C)_n$ synthesis is elevated as indicated by a greater amount of TCA precipitable material. This implies that RNA chains are released from the ternary complex at a more rapid rate at elevated ionic strengths as in the case of T_4 DNA transcription (67).

Secondly, from Figure 3a, it will be noted that salt-stimulated synthesis at brief time intervals is not as enhanced as salt stimulation after longer periods of synthesis.

Thirdly, from Figure 3b, it will be noted that both Mn^{++} and Mg^{++} buffers support $r(I\text{-}C)_n$ synthesis at elevated ionic strength, but that neither buffer enhances retention of $r(G\text{-}C)_n$ on nitrocellulose filters after 5 minutes of synthesis. It was noted, however, that $r(G\text{-}C)_n$ and $r(I\text{-}C)_n$ synthesis determined as TCA precipitable material was enhanced more significantly at elevated ionic strengths when Mn^{++} rather than Mg^{++} was employed as the buffer (Figure 3c). There is significantly greater salt stimulation of synthesis to ionic strength 0.3 than there is when Mg^{++} is the divalent cation employed. Neither Mg^{++} nor Mn^{++} as divalent cation support synthesis at ionic strength 0.4. Reactions were allowed to occur for only 90 sec. during which a lag was noted at high salt concentrations (Figure 3a) and during which all TCA precipitable material is capable of being retained on a nitrocellulose filter. During this period of time, it is suspected that initiation is the rate determining step, elongation being

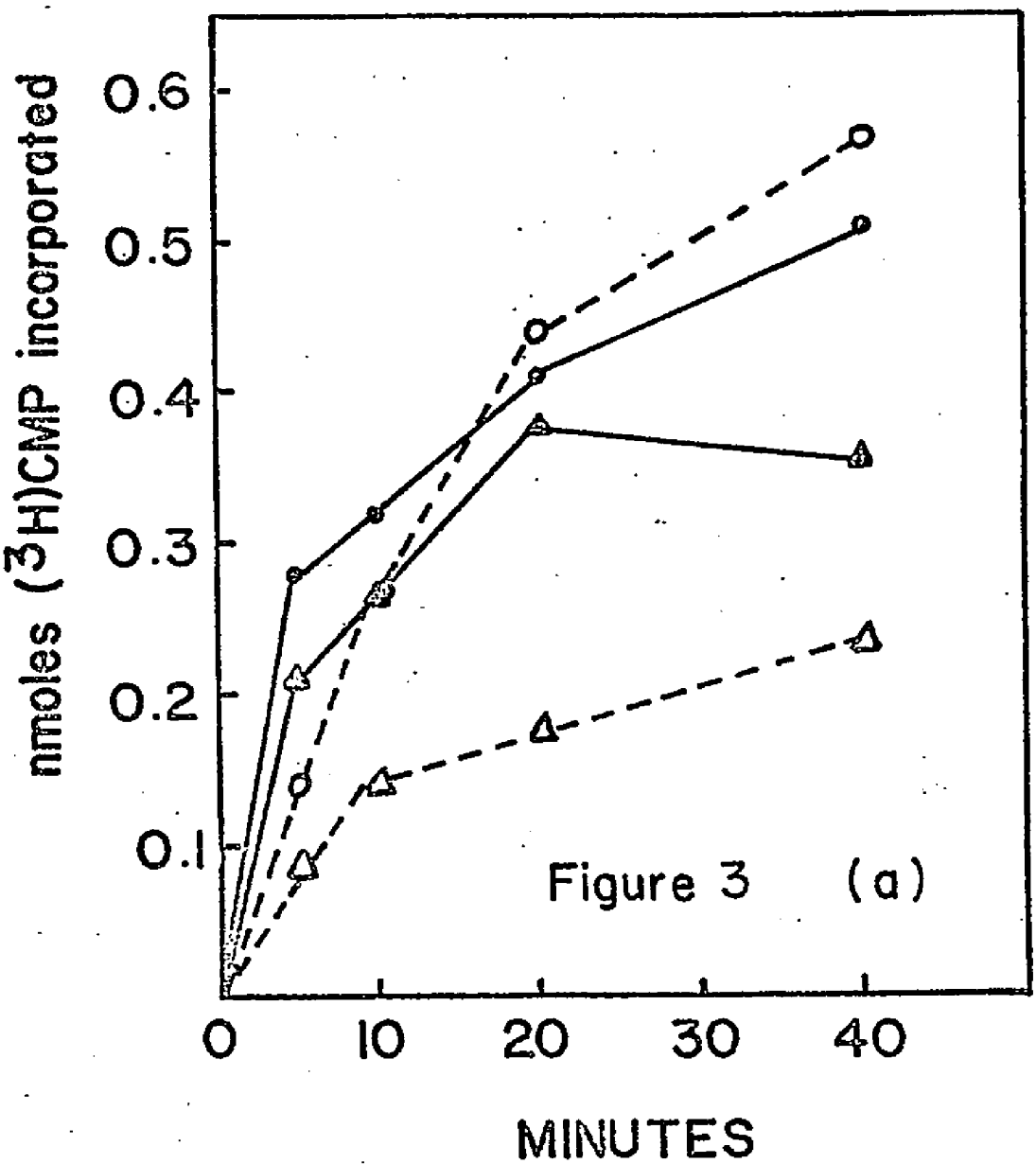


Figure 3 (a)

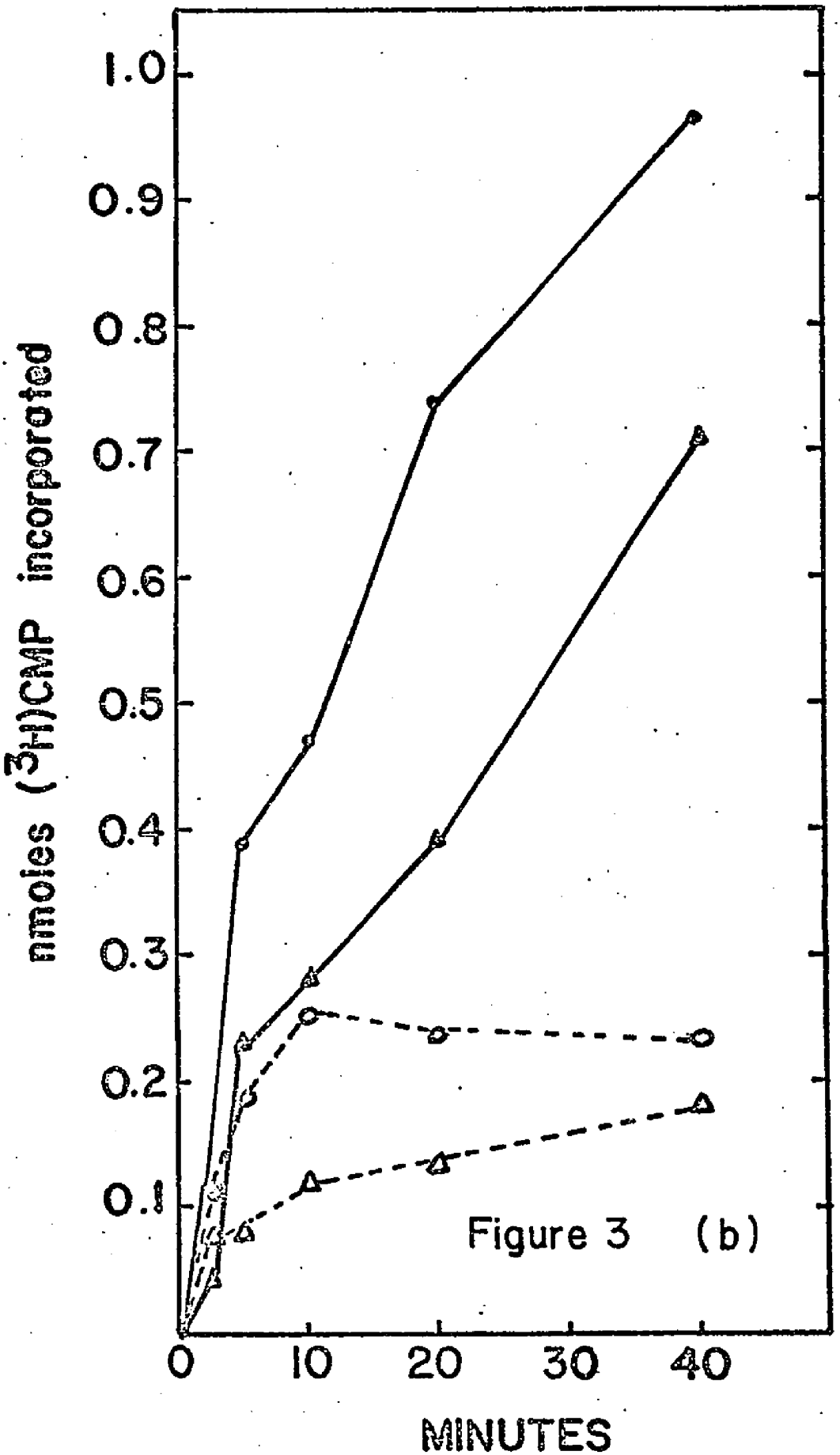


Figure 3 (b)

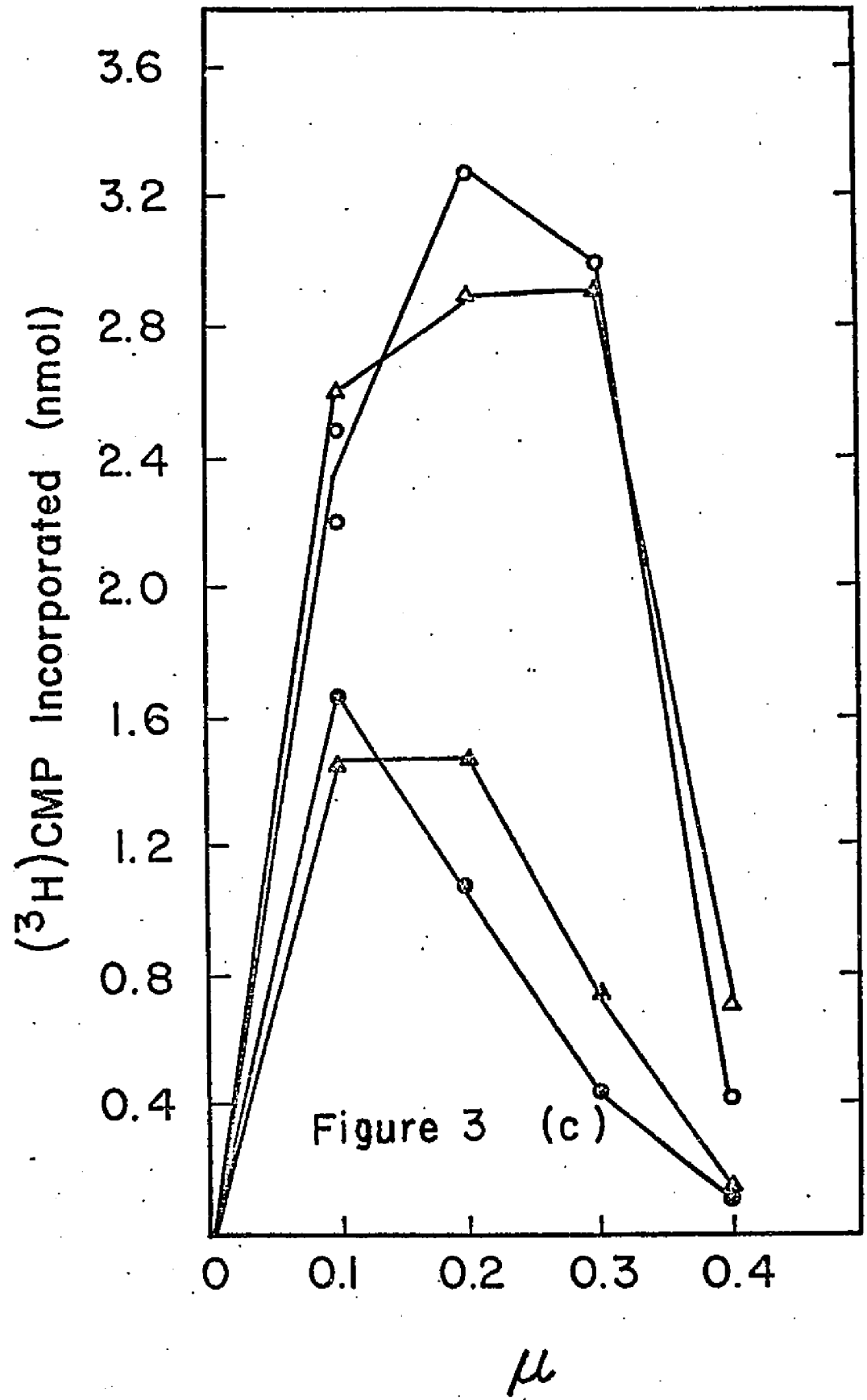


Figure 3 (c)

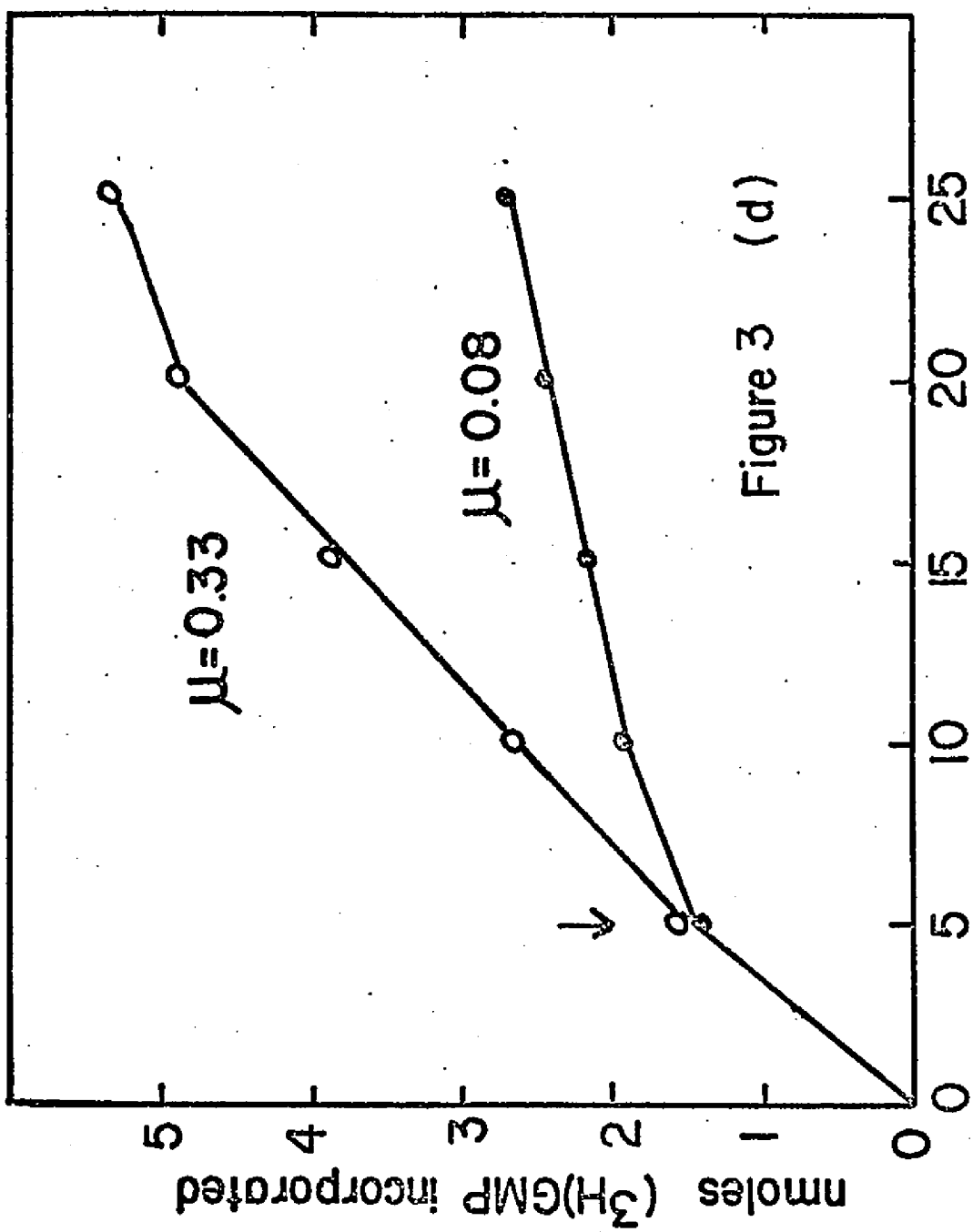


Figure 3 (d)

LEGEND TO FIGURE 3

Role of ionic strength

a. Effect of Ionic Strength on $r(I\text{-}C)_n$ Synthesis in Buffers Containing Mg^{++}

100 µl of a buffer containing 25 mM $MgSO_4$, 100 mM Tris pH 8.0, 50 mM ME, 187 mM KCl, 10 nmoles $d(I\text{-}C)_n$, 5 µg. RNA polymerase were preincubated for 10 minutes at 37° C in a total volume of 115 µl. Following preincubation, 10 µl aliquots containing 50 nmoles (^3H)CTP (3100 cpm/nmole) and 50 nmoles ITP were added. After incubating at 37° C for the times indicated, 10 µl aliquots were added to 100 µl of 0.2 M sodium pyrophosphate, precipitated with 3 ml of 5% TCA and filtered on Whatman GF/C filters (TCA) or added to 100 µl of 0.5 M EDTA and filtered on nitrocellulose filters (nitrocellulose).

o — o TCA, final μ = 0.33

Δ — Δ nitrocellulose, final μ = 0.33

● — ● TCA, final μ = 0.16

▲ — ▲ nitrocellulose, final μ = 0.16

b. Effect of Divalent Cation on Nitrocellulose Bound $E\begin{matrix}d(I\text{-}C)_n \\ r(G\text{-}C)_n\end{matrix}$ at Elevated Ionic Strength

100 µl of a buffer containing 25 mM $MgSO_4$, 100 mM Tris pH 8.0, 50 mM ME and 187 mM KCl or 1 mM $MnSO_4$, 100 mM Tris pH 8.0, 50 mM ME and 250 mM KCl; and 10 nmoles $d(I\text{-}C)_n$, 5 µg. RNA polymerase in a total volume of 115 µl were preincubated for 10 minutes at 37° C. Following

preincubation, 10 µl aliquots containing 50 nmoles (^{3}H)CTP (3100 cpm/nmole) and 50 nmoles GTP were added. After incubating at 37° C for the times indicated, 10 µl aliquots were added to 100 µl of 0.2 M sodium pyrophosphate or 0.5 M EDTA and filtered as in a.

● – ● Mn^{++}, TCA

○ – ○ Mn^{++}, nitrocellulose

▲ – ▲ Mg^{++}, TCA

△ – △ Mg^{++}, nitrocellulose

c. Effect of Ionic Strength on $r(I\text{-}C)_n$ and $r(G\text{-}C)_n$ Synthesis in Mg^{++}- and Mn^{++}-Containing Buffers

600 µl of buffers containing 25 mM $MgCl_2$, 50 mM Tris pH 8.0, 50 mM ME; or 1 mM $MnSO_4$, 50 mM Tris pH 8.0, 50 mM ME were preincubated for 15 minutes at 37° C with 60 nmoles $d(I\text{-}C)_n$ and 30 µg. RNA polymerase with KCl to adjust the ionic strength as indicated, in a total volume of 690 µl. 100 µl of each mixture was incubated for 90 sec. with a 15 µl aliquot containing 50 nmoles (^{3}H)CTP (4480 cpm/nmole) and 50 nmoles GTP or ITP as indicated. Reactions were terminated by the addition of 100 µl of 0.2 M sodium pyrophosphate and acid precipitable radioactivity determined.

○ – ○ ITP, Mn^{++}

△ – △ GTP, Mn^{++}

● – ● ITP, Mg^{++}

▲ – ▲ GTP, Mg^{++}

d. Effect of Salt Stimulation on Preformed $E^{d(I-C)_n}_{r(G-C)_n}$ Ternary Complexes

300 µl of a buffer containing 1 mM $MnSO_4$, 100 mM Tris pH 8.0, 50 mM ME, 30 nmoles $d(I-C)_n$ and 15 µg. RNA polymerase were preincubated for 10 minutes at 37° C. Following preincubation, a 30 µl aliquot containing 150 nmoles (^{3}H)GTP (1900 cpm/nmole) and 150 nmoles of CTP was added. After 5 minutes, either 25 µl of water or 25 µl containing 93.75 µmoles KCl was added to the mixture. 50 µl aliquots were added to test tubes containing 100 µl of 0.2 M sodium pyrophosphate at the times indicated and acid precipitable radioactivity determined.

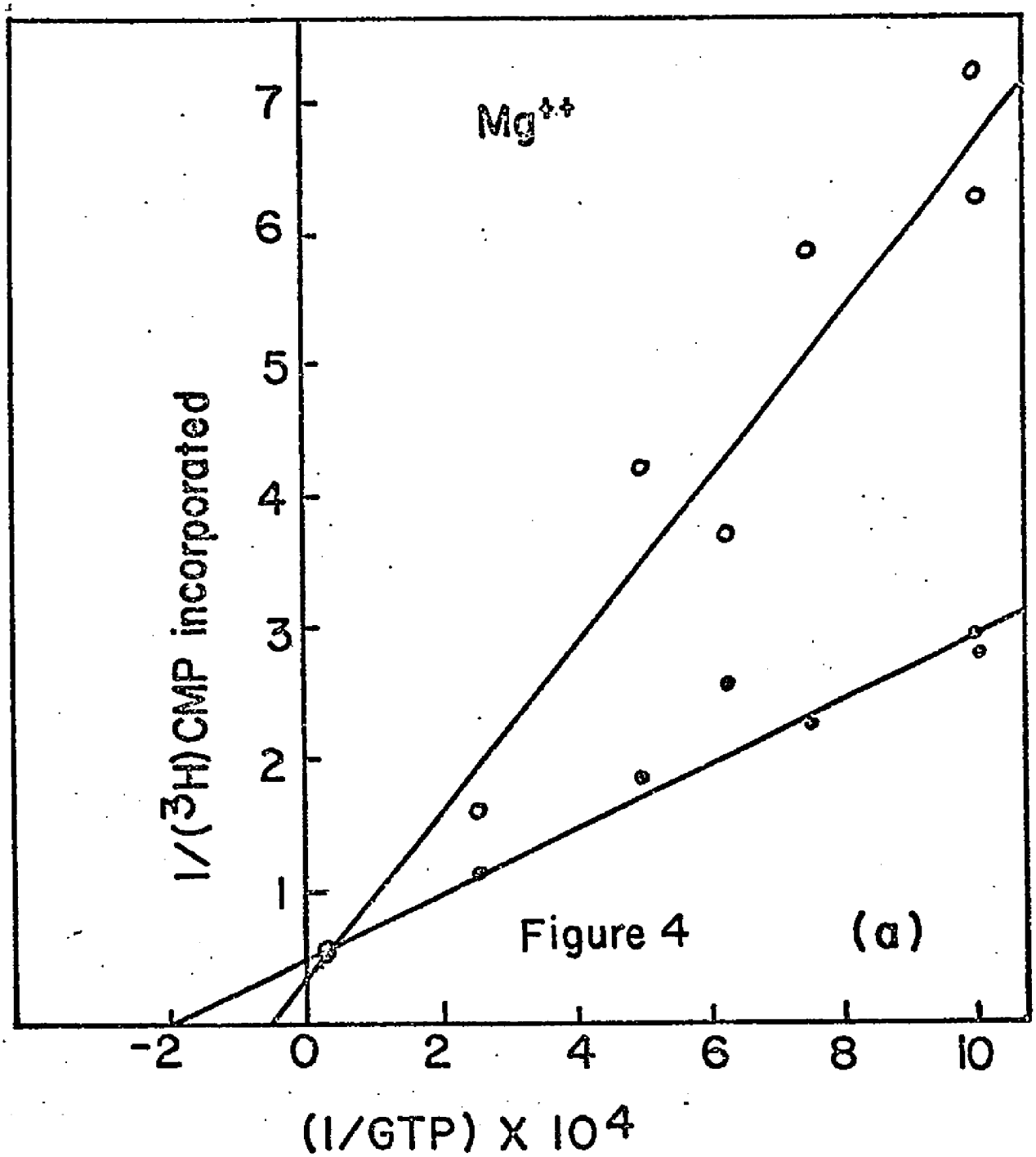
stimulated once initiation has occurred as long as there is formation of a stable elongation ternary complex (Table III).

To demonstrate this effect, initiation complexes were preformed by allowing synthesis of $r(I\text{-}C)_n$ to occur for 5 minutes, followed by the addition of salt, increasing the ionic strength from 0.08 to 0.33 (Figure 3d). There is a marked stimulation of synthesis and a larger period of time before the plateau region of synthesis occurs implying that elongation is stimulated at high ionic strength once a stable elongation ternary complex has been formed.

D. Apparent K_m values as a probe for relative affinities of GTP and ITP for RNA polymerase

The apparent K_m values for GTP and CTP in $r(G\text{-}C)_n$ synthesis and ITP and CTP in $r(I\text{-}C)_n$ synthesis in Mg^{++} containing buffers were determined from Lineweaver Burk plots. (Figures 4a, b, c, d give examples of the plots). The apparent K_m data are listed in Table IV. It will be noted that the K_m for CTP and ITP in $r(I\text{-}C)_n$ synthesis and the K_m for GTP in $r(G\text{-}C)_n$ synthesis is 200 μM, a value approximating that determined by Wu and Goldthwait for the K_s of binding of purine triphosphates to the initiation site of RNA polymerase by equilibrium dialysis (35) and fluorescence quenching (34). However, the K_m for CTP is reduced four-fold in $r(G\text{-}C)_n$ synthesis. Further, the apparent K_m for all three nucleoside triphosphates in the Mn^{++}-containing buffers is 25 μM.

It has been suggested from previous studies (103) that Mn^{++} enhances the rate of initiation by RNA polymerase core enzyme. Therefore, the lowered K_m value is indicative that initiation has



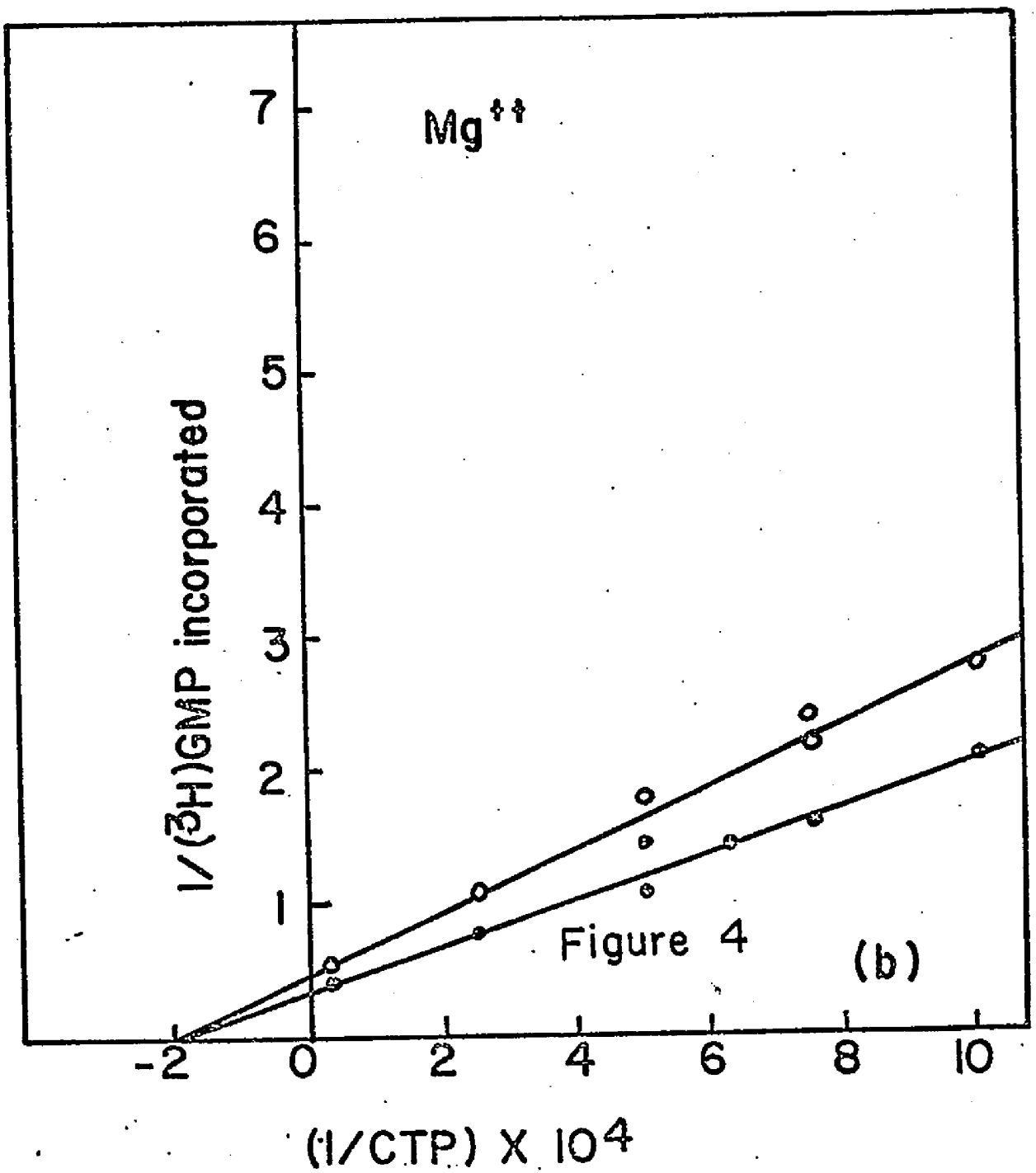
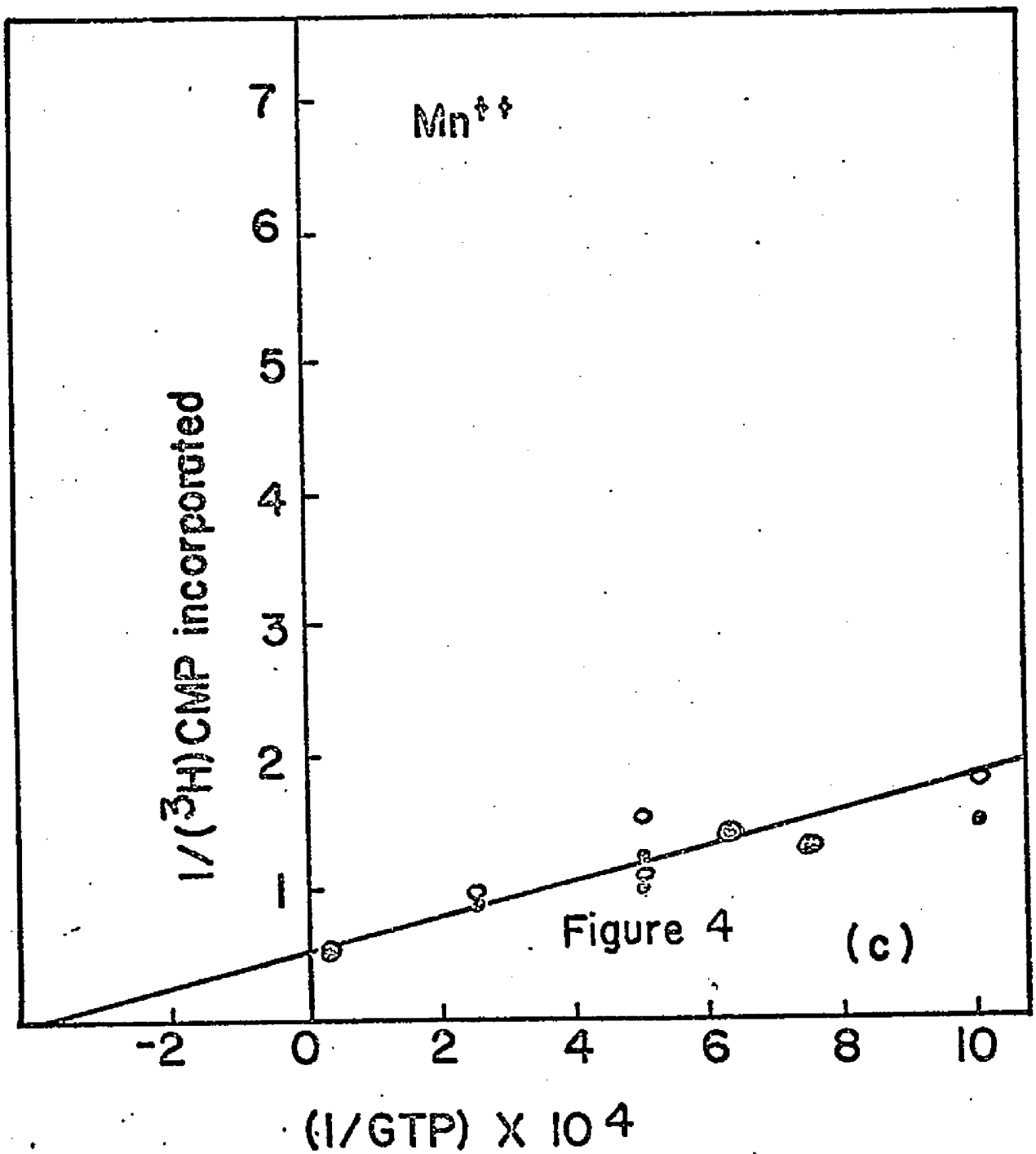


Figure 4



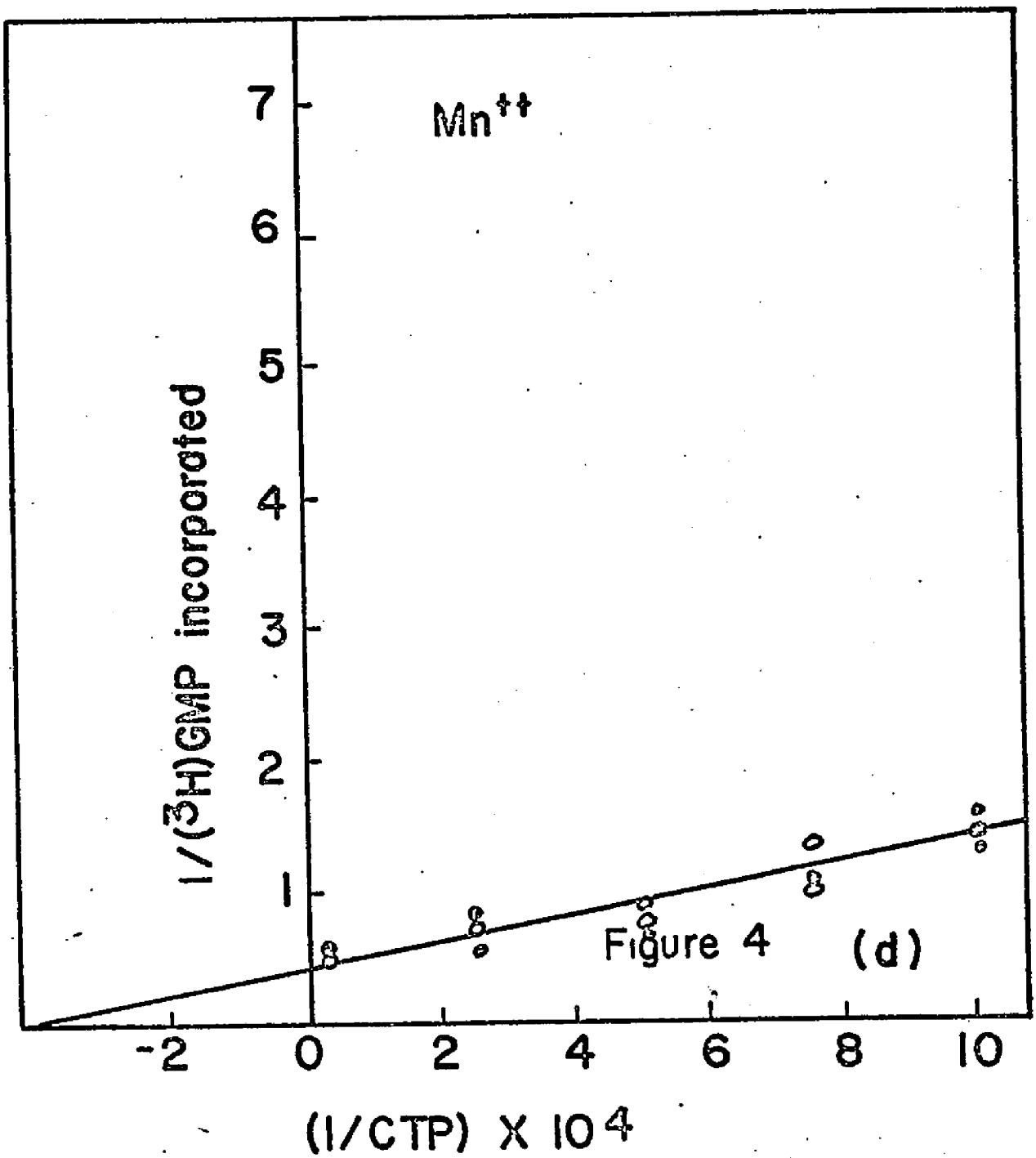


Figure 4 (d)

LEGEND TO FIGURE 4

Apparent K_m determinations by Lineweaver-Burk plots

100 µl of buffers containing 25 mM $MgCl_2$, 50 mM Tris pH 8.0, 50 mM ME or 1 mM $MnSO_4$, 50 mM Tris pH 8.0, 50 mM ME and 75 mM KCl were preincubated with 10 nmoles $d(I-C)_n$ and 5 µg. RNA polymerase in the presence or absence of 40 nmoles of GpC in a total volume of 115 µl for 15 minutes at 37° C. RNA synthesis was initiated by the addition of a 10 µl aliquot containing 50 nmoles (^{3}H)CTP (9016 cpm/nmole) and 1.67 to 50 nmoles GTP or 50 nmoles (^{3}H)GTP (8533 cpm/nmole) and 1.67 to 50 nmoles CTP. Reactions were terminated after 2.5 minutes at 37° C by the addition of 100 µl of 0.2 M sodium pyrophosphate and the amount of acid insoluble $r(G-C)_n$ determined.

a. o - o Mg^{++}, (^{3}H)CTP + GTP, no dinucleoside monophosphate

● - ● Mg^{++}, (^{3}H)CTP + GTP + GpC

b. o - o Mg^{++}, (^{3}H)GTP + CTP, no dinucleoside monophosphate

● - ● Mg^{++}, (^{3}H)GTP + CTP + GpC

c. o - o Mn^{++}, (^{3}H)CTP + GTP, no dinucleoside monophosphate

● - ● Mn^{++}, (^{3}H)CTP + GTP + GpC

d. o - o Mn^{++}, (^{3}H)GTP + CTP, no dinucleoside monophosphate

● - ● Mn^{++}, (^{3}H)GTP + CTP + GpC

TABLE IV

Apparent K_m determinations for $r(G-C)_n$ and $r(I-C)_n$ synthesis

Apparent K_m Determined	RNA Synthesized	K_m for Unprimed Synthesis as Function of Divalent Cation (μM)		K_m for GpC Primed Synthesis as Function of Divalent Cation (μM)		K_m for CpG Primed Synthesis as Function of Divalent Cation (μM)	
		Mn^{++}	Mg^{++}	Mn^{++}	Mg^{++}	Mn^{++}	Mg^{++}
K_G	$r(G-C)_n$	25	200	25	50	25	67
K_C	$r(G-C)_n$	25	50	25	50	25	25
K_I	$r(I-C)_n$	25	200	25	50	25	67
K_C	$r(I-C)_n$	25	200	25	50	25	125

LEGEND TO TABLE IV

Apparent K_m determinations for $r(G-C)_n$ and $r(I-C)_n$ synthesis

K_m's were determined from Lineweaver-Burk plots as described in Figure 4. Conditions: 100 µl of Mg^{++} or Mn^{++} buffer as described in Figure 4, 10 nmoles of $d(I-C)_n$ and 5 µg. RNA polymerase in the presence or absence of 40 nmoles GpC or CpG primer in a total volume of 115 µl was preincubated for 15 minutes at 37° C. RNA synthesis was initiated by the addition of a 10 µl aliquot containing 50 nmoles (^{3}H)CTP (9016 cpm/nmole) and 1.67 to 50 nmoles GTP or ITP (as indicated) or 50 nmoles (^{3}H)GTP (8533 cpm/nmole) or (^{3}H)ITP (4166 cpm/nmole) and 1.67 to 50 nmoles CTP. Reactions were terminated after 2.5 minutes at 37° C and the amount of acid insoluble $r(I-C)_n$ or $r(G-C)_n$ determined.

occurred more easily or more effectively, allowing the true K_m for elongation to be reflected. Also, when GTP is present in saturating concentrations the K_m for CTP is reduced in Mg^{++} containing buffers. This does not occur when CTP or ITP are present in saturating concentrations in Mg^{++}-containing buffers. This implies that GTP is more efficient than ITP for initiation, and can support rapid initiation when present in the reaction mix at saturating concentrations. The K_m for CTP is not reduced in $r(I\text{-}C)_n$ synthesis since ITP is a less efficient initiator. Increasing the concentration of ITP 3.3 fold has no effect in lowering the K_m of CTP suggesting that the necessary conformation of the initiation site for effective initiation is not provided when ITP is the initiating ribonucleoside triphosphate.

E. Use of primers to bypass initiation

Primers such as ApU and UpA have been shown to initiate $r(A\text{-}U)_n$ synthesis effectively (48). To demonstrate that this is the case for $r(G\text{-}C)_n$ synthesis, it has been shown (Figure 5) that at concentrations of nucleoside triphosphates of 7.4 μM (below the apparent K_m for initiation for $r(G\text{-}C)_n$ synthesis), both CpG and GpC stimulate synthesis when preincubated with the binary complex.

To demonstrate that formation of a preinitiation complex with a primer can reduce the K_m to more effectively represent the K_m for elongation, GpC and CpG have been preincubated with the binary complex and K_m's determined.

Table IV indicates that K_m values for all the nucleoside triphosphates in a Mg^{++} containing buffer are 50 μM in the presence of the GpC primer, whereas the K_m values determined in the Mn^{++} buffer remain at

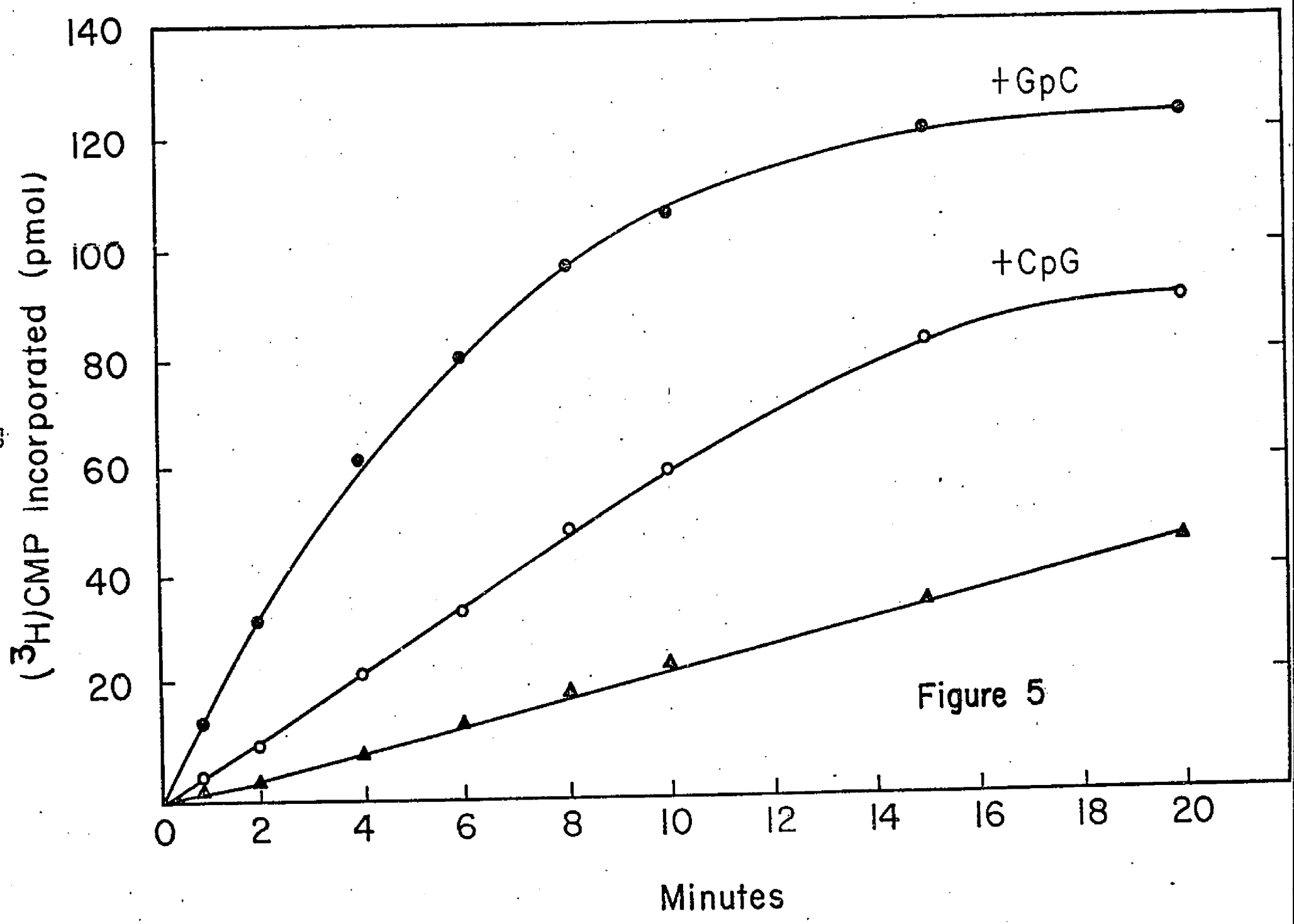


Figure 5

LEGEND TO FIGURE 5

Effect of primers on the rate of $r(G-C)_n$ synthesis

200 µl of a buffer containing 50 mM Tris pH 8.0, 50 mM ME, 25 mM $MgCl_2$, 20 nmoles $d(I-C)_n$ and 10 µg. RNA polymerase were preincubated at 37° C for 15 minutes in the presence or absence of 80 nmoles GpC or CpG in a total volume of 230 µl. RNA synthesis was initiated by the addition of 40 µl of a mix containing 2 nmoles (^3H)CTP (335 cpm/pmole) and 2 nmoles GTP. After the times indicated, 30 µl aliquots were withdrawn into 100 µl of 0.2 M sodium pyrophosphate and the amount of acid insoluble $r(G-C)_n$ determined.

▲ - ▲ no dinucleoside monophosphate

○ - ○ + CpG

● - ● + GpC

the formerly determined value of 25 μM in the absence of the primer. Nevertheless, the CpG primer is not as effective. However, this confirms the previous assumption that reduction in K_m occurs when initiation occurs and that initiation in the presence of a Mn^{++} buffer is more effective than that in a Mg^{++} buffer for both $r(I-C)_n$ and $r(G-C)_n$ synthesis. That the K_m for elongation is lower than the K_m for initiation in Mg^{++} containing buffers is suggested by the lowered values for the apparent K_m determined in the primed system.

F. Effect of salt on K_m

When attempts were made to determine the K_m values for $r(I-C)_n$ and $r(G-C)_n$ synthesis at ionic strengths of 0.33 in Mn^{++} buffers or in Mg^{++} buffers, deviations from linearity in Lineweaver-Burk plots are pronounced, the deviations being greatest in the Mg^{++} buffer (Figure 6a, b). Nevertheless, when one considers a plot of 1/CTP vs. $1/GTP^2$ (Figure 6c), it is apparent that the plots are linear at low concentrations of GTP. The linearity of this plot suggests that the reaction reflects both the binding of GTP to the product terminus site as well as the binding of GTP to the substrate site with formation of a phosphodiester bond (48), the rate determining step. The graph of 1/GTP vs. $1/CTP^2$ is nonlinear suggesting that it is GTP binding to the product terminus site, not CTP to the substrate site, which controls the initiation rate. These data also confirm the work of Fuchs *et al.* (67) and the interpretation of the data of Figures 3a, b and c, d of this paper which indicate that high ionic strength hampers initiation while stimulating elongation.

It is apparent that the K_m values of Table IV give an indication

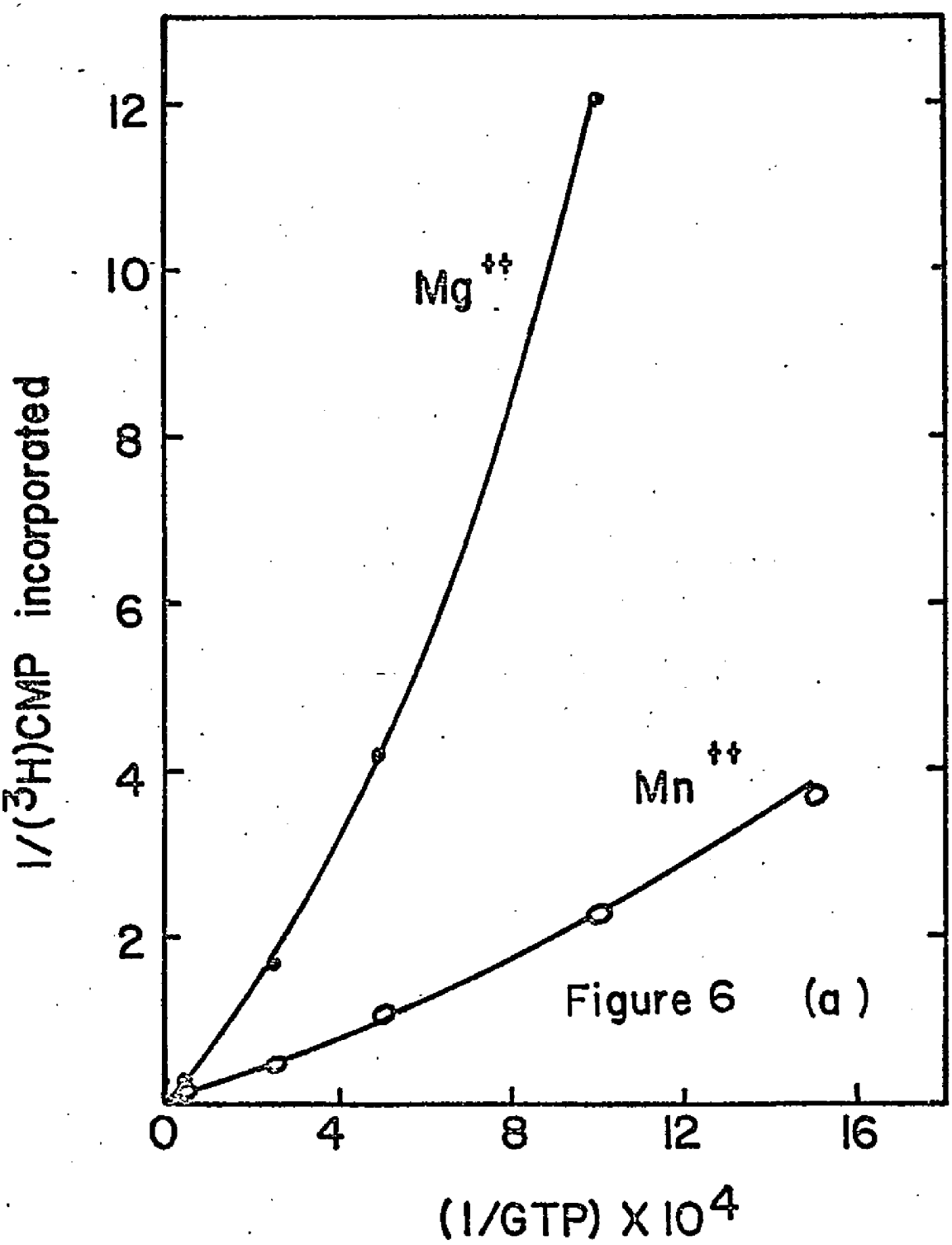
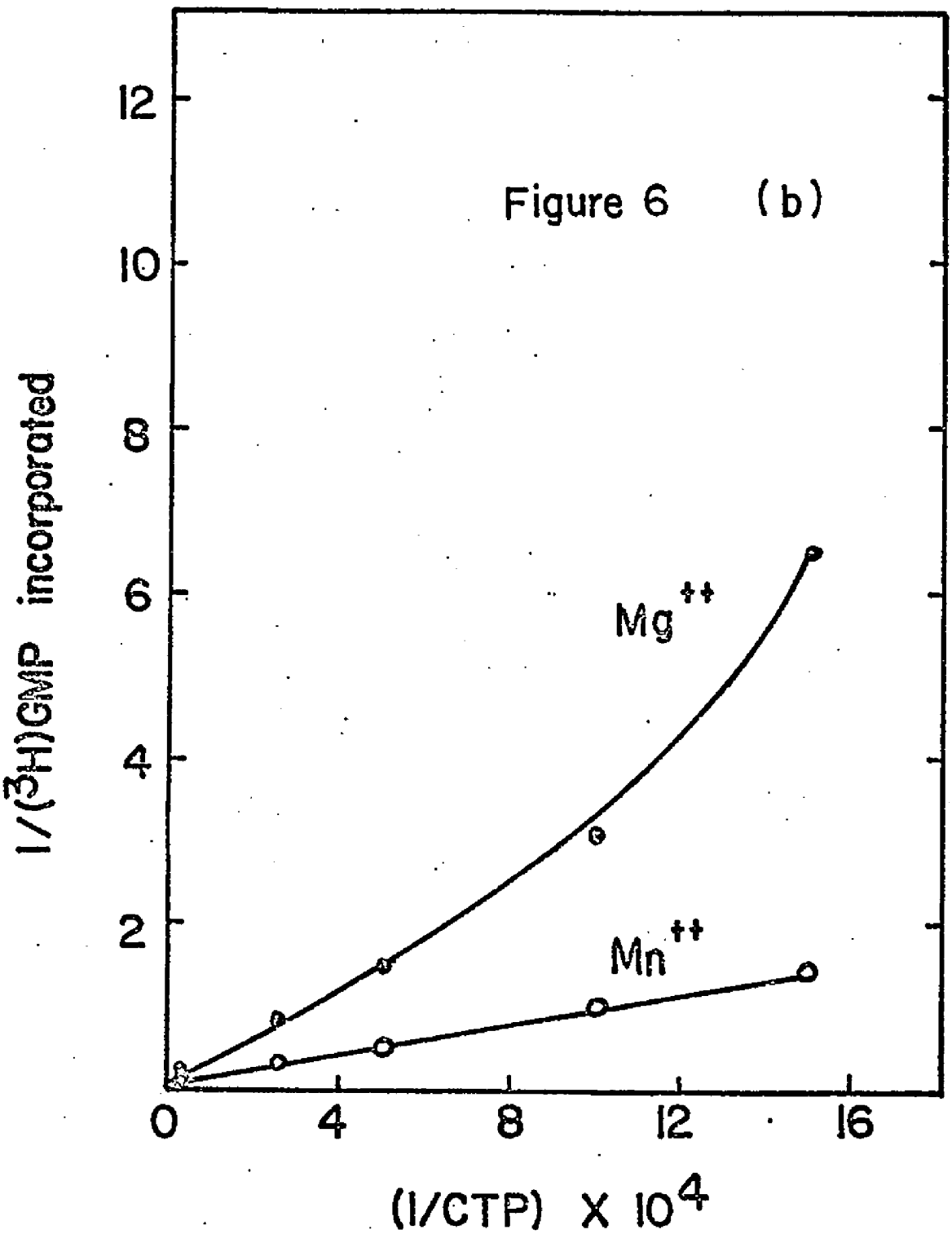
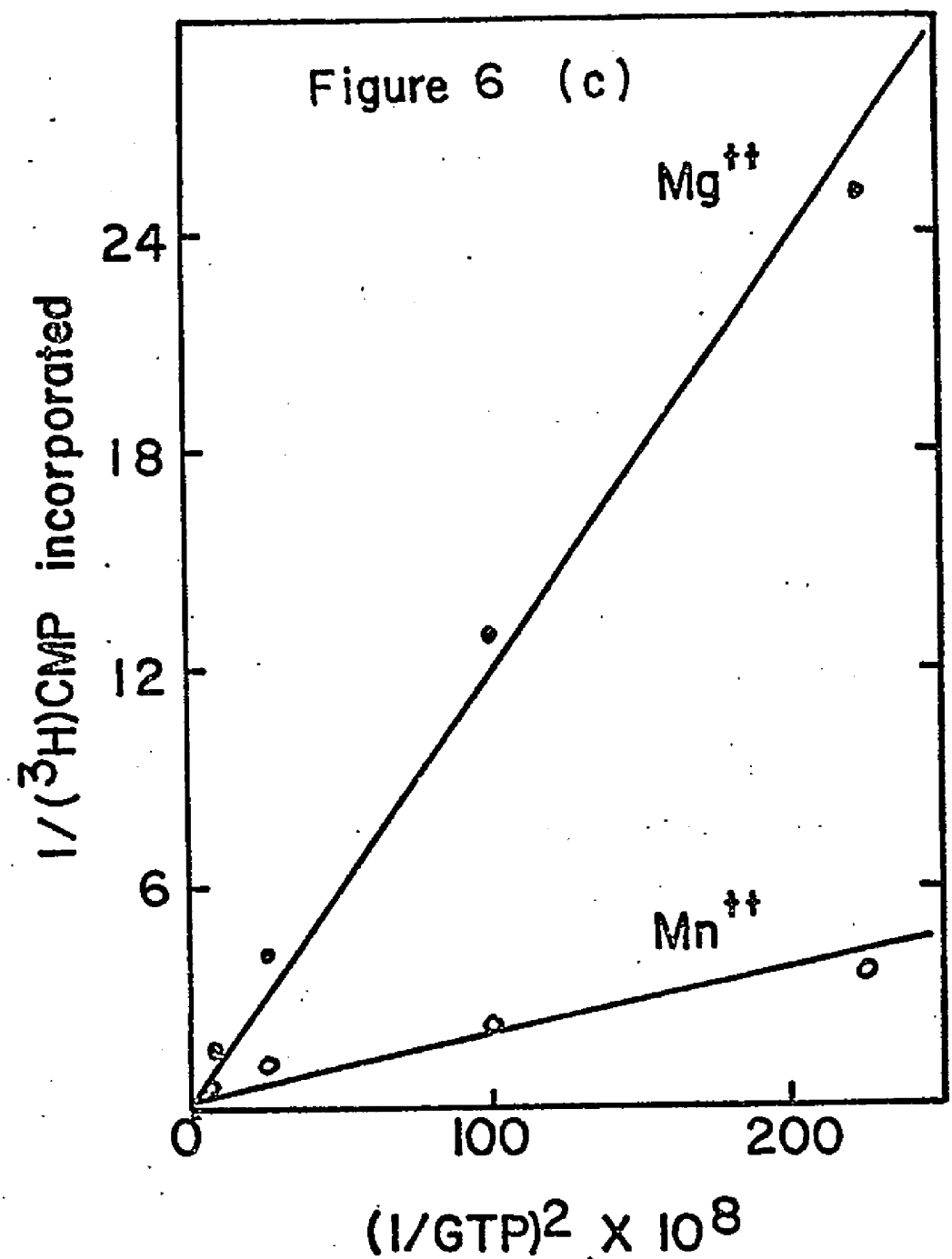
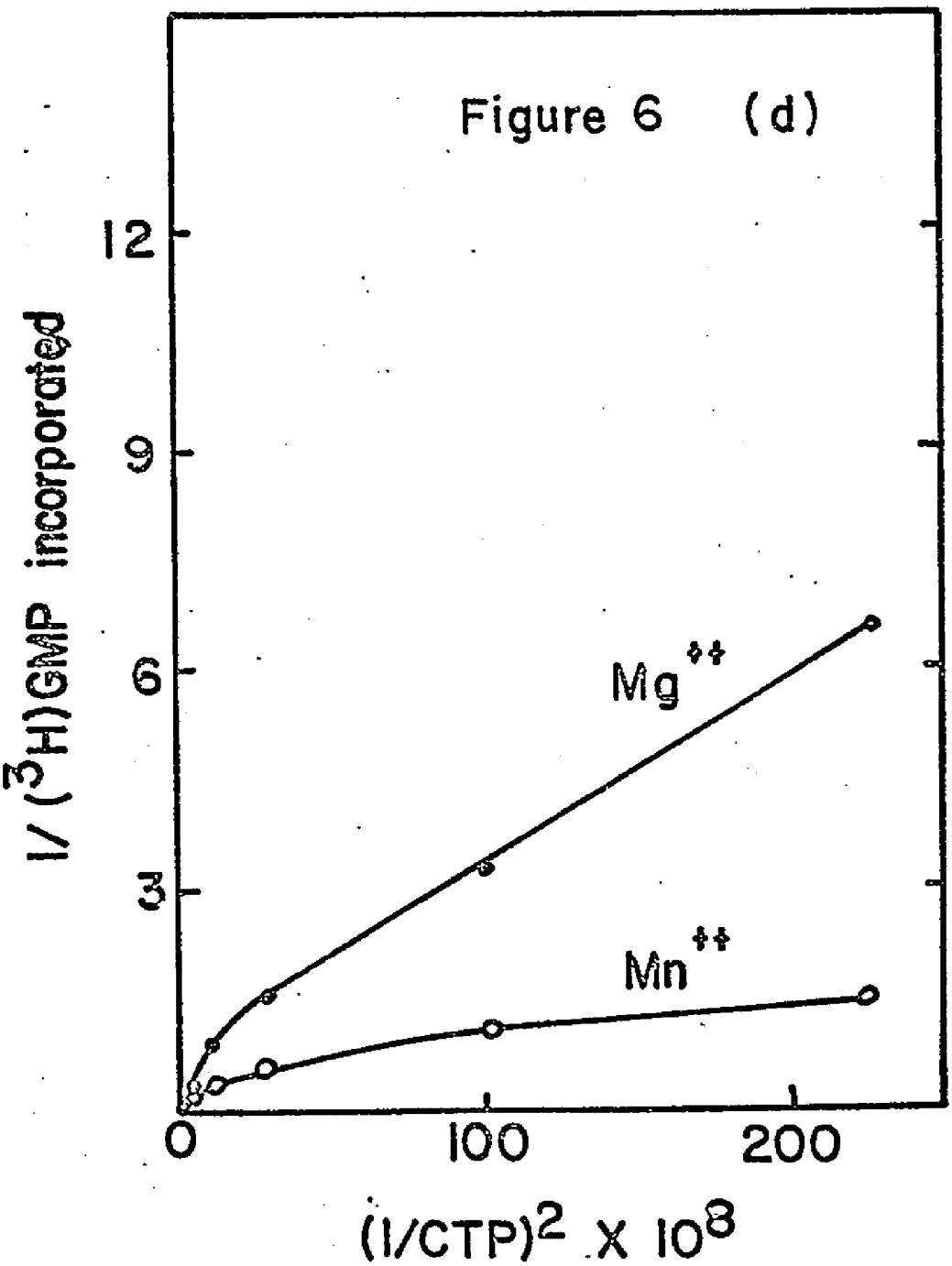


Figure 6 (a)







LEGEND TO FIGURE 6

Effect of increasing the ionic strength on Lineweaver-Burk plots

100 µl of a mixture containing 25 mM $MgSO_4$, 100 mM Tris pH 8.0, 50 mM ME and 187 mM KCl or 1 mM $MnSO_4$, 100 mM Tris pH 8.0, 50 mM ME and 250 mM KCl; 10 nmoles $d(I-C)_n$ and 5 µg. RNA polymerase were preincubated for 10 minutes at 37° C in a total volume of 115 µl. RNA synthesis was initiated as in Table IV. Reactions were terminated after 5 minutes at 37° C and the amount of acid insoluble $r(I-C)_n$ or $r(G-C)_n$ determined.

a. ● - ● (^3H)CTP + GTP, Mg^{++}

○ - ○ (^3H)CTP + GTP, Mn^{++}

b. ● - ● (^3H)GTP + CTP, Mg^{++}

○ - ○ (^3H)GTP + CTP, Mn^{++}

c. data from a. replotted

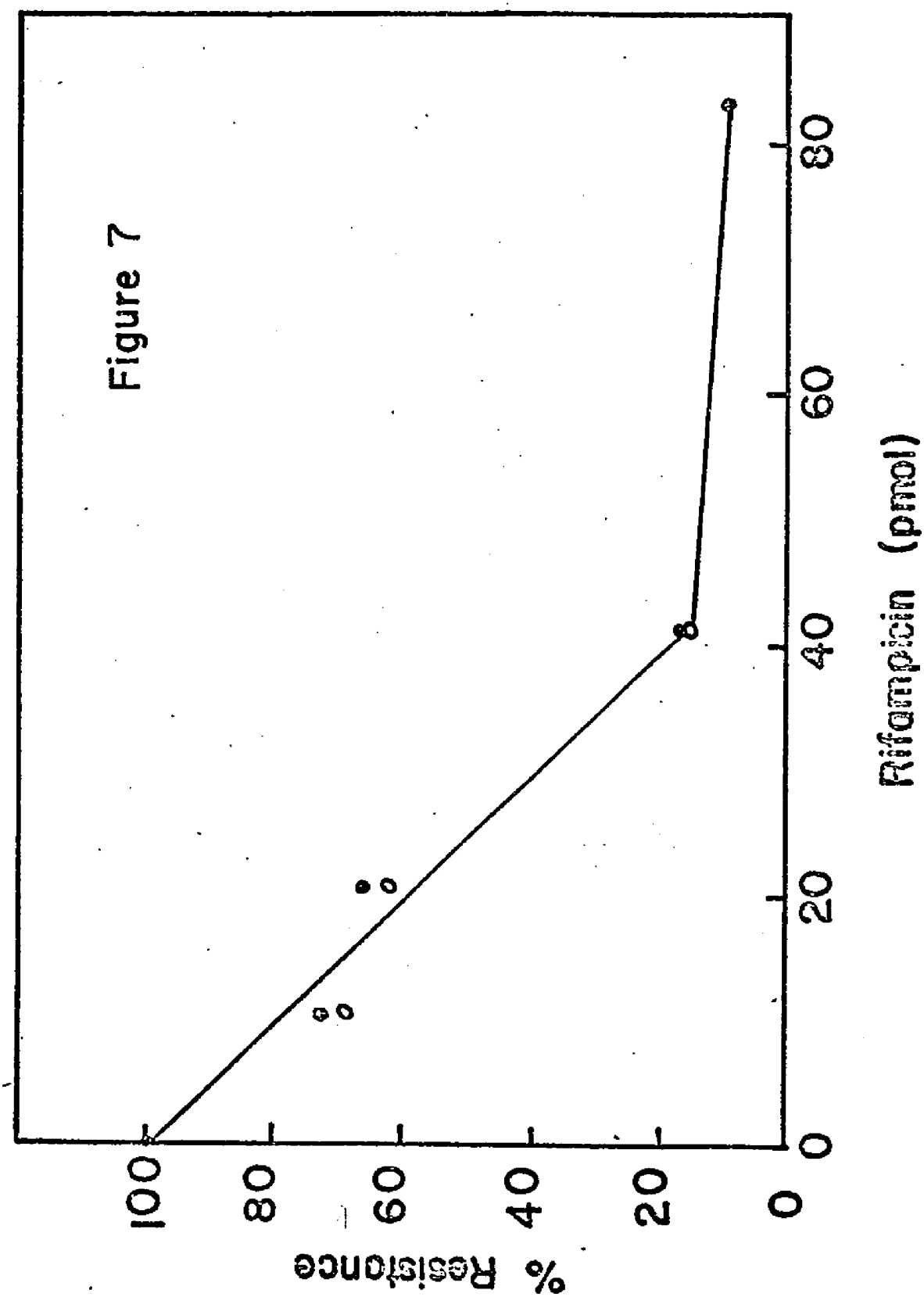
d. data from b. replotted

that GTP has a greater affinity for the initiation site than ITP. This affinity is enhanced by the use of the Mn^{++} buffer. Further, effective initiation complexes can be made using a GpC primer, less effective complexes with a CpG primer. Thirdly, high ionic strength does not stimulate initiation. The above assumptions have been tested using rifampicin challenge experiments.

G. Use of rifampicin to determine relative affinities of GTP and ITP for the initiation site of RNA polymerase

Rifampicin is known to bind to the β subunit of E. coli RNA polymerase (8, 38) at a site approximately 37 Å from the initiation site (39). If rifampicin is preincubated for 10 minutes with A. vinelandii RNA polymerase core enzyme, an input ratio of 4 rifampicin molecules per molecule of enzyme abolishes approximately 90% of the synthesizing ability of the enzyme (Figure 7); however, doubling the rifampicin concentration such that the rifampicin/enzyme ratio is 8 (or greater) does not completely abolish enzyme activity suggesting that there is some rifampicin resistant activity in the enzyme preparation. This is a characteristic of all enzyme preparations to date. The effect of rifampicin on the 'naked' enzyme has been found to be independent of divalent cation or ionic strengths from 0.08 to 0.20 (Figure 7).

Rifampicin attack on the binary core RNA polymerase-$d(I\text{-}C)_n$ complex has been shown to occur at the same rate in Mg^{++} and Mn^{++} buffers and is independent of whether $r(G\text{-}C)_n$ or $r(I\text{-}C)_n$ synthesis is used as a probe for rifampicin inactivation of the enzyme (Figure 8). Since previous data of this paper implies that GTP initiates more rapidly than ITP, these data could be interpreted by assuming that



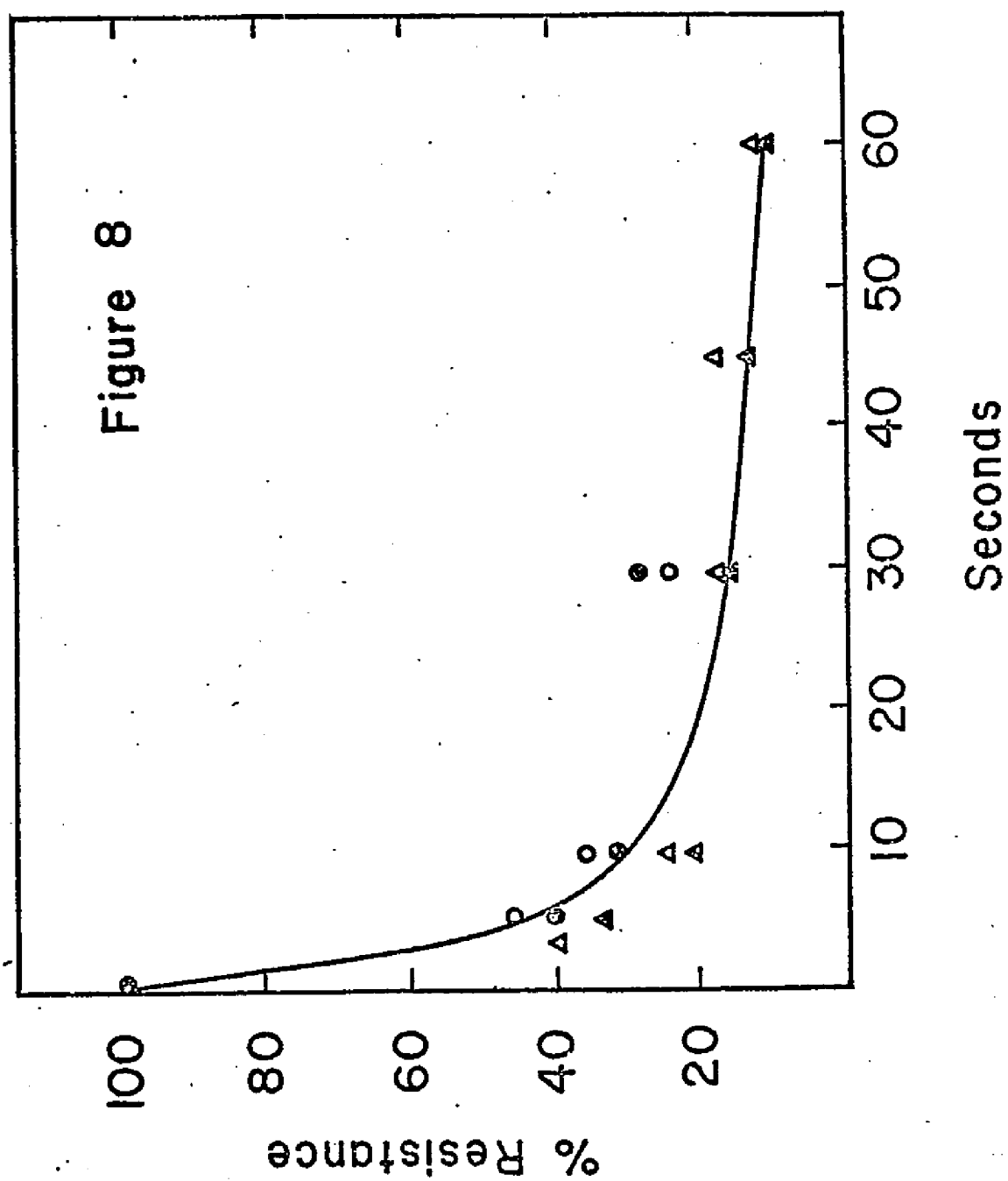
LEGEND TO FIGURE 7

Effect of preincubation of rifampicin with RNA polymerase on synthesis of $r(G-C)_n$

100 µl of a mix containing 25 mM $MgSO_4$, 100 mM Tris pH 8.0 and 50 mM ME or 1 mM $MnSO_4$, 100 mM Tris pH 8.0 and 50 mM ME were preincubated with 5 µg. RNA polymerase and 0 to 83 pmoles rifampicin for 10 minutes at 37° C in a total volume of 115 µl. Synthesis of $r(G-C)_n$ was initiated by the addition of 10 nmoles $d(I-C)_n$, 50 nmoles (^{3}H)GTP (1900 cpm/nmole) and 50 nmoles CTP in a total volume of 130 µl. The reaction was terminated after 5 minutes of synthesis at 37° C and acid precipitable radioactivity determined.

● - ● Mn^{++} buffer, 100% = 4.9 nmoles (^{3}H)GMP incorporated

○ - ○ Mg^{++} buffer, 100% = 3.2 nmoles (^{3}H)GMP incorporated



LEGEND TO FIGURE 8

Effect of preincubation of rifampicin with the E-d(I-C)$_n$ binary complex on the synthesis of r(I-C)$_n$ and r(G-C)$_n$

600 µl of a mixture containing 25 mM $MgCl_2$, 50 mM Tris pH 8.0 and 50 mM ME or 1 mM $MnSO_4$, 50 mM Tris pH 8.0, 50 mM ME and 75 mM KCl were preincubated for 15 minutes at 37° C with 5 µg. RNA polymerase and 10 nmoles d(I-C)$_n$ in a total volume of 690 µl. Following preincubation, 480 pmoles of rifampicin were added to the mix in a total volume of 720 µl. After incubation with rifampicin for 10, 20, 30, 45 or 60 seconds, synthesis of r(G-C)$_n$ or r(I-C)$_n$ was initiated by the removal of 100 µl aliquots of the incubation mix into a 10 µl aliquot containing 50 nmoles (^{3}H)CTP (4480 cpm/nmole) and 50 nmoles GTP or ITP. Reactions were terminated after 90 seconds at 37° C and acid precipitable radioactivity determined. % rifampicin resistance was calculated relative to nmoles acid precipitable r(G-C)$_n$ or r(I-C)$_n$ obtained in the absence of rifampicin.

o - o r(I-C)$_n$, Mg^{++} 100% = 0.7 nmoles (^{3}H)CMP incorporated

● - ● r(I-C)$_n$, Mn^{++} 100% = 1.9 nmoles (^{3}H)CMP incorporated

Δ - Δ r(G-C)$_n$, Mn^{++} 100% = 2.2 nmoles (^{3}H)CMP incorporated

▲ - ▲ r(G-C)$_n$, Mg^{++} 100% = 1.1 nmoles (^{3}H)CMP incorporated

rifampicin attacks the binary enzyme-d(I-C)$_n$ complex at the same rate at which the 'naked' enzyme is attacked.

To determine the second order rate constant for the attack of rifampicin on the E-d(I-C)$_n$ binary complex, it was necessary to alter the original method of Mangel and Chamberlin (47) who determined the k_2 for rifampicin attack on the holoenzyme-T_7 binary complex by pre-incubation with molar excesses of rifampicin to enzyme. In the core enzyme-d(I-C)$_n$ system, use of rifampicin in excess to justify application of pseudo first order kinetics was impossible since 90 - 95% of enzyme activity was abolished in a period of 5 seconds.

Figure 9 shows data for the attack of rifampicin on the E-d(I-C)$_n$ binary complex by a modification of the method of Mangel and Chamberlin (47):

The data were obtained as follows:

Assuming the active binary complex capable if initiating the formation of the first phosphodiester bond, the concentration of the E-d(I-C)$_n$ complex is equal to the concentration of the enzyme in the reaction mix. The attack of rifampicin on the binary complex may then be represented by equation 4:

$$\underset{\text{binary complex}}{E\text{-}d(I\text{-}C)_n} + Rif \xrightarrow{k_2} \underset{\text{inactive}}{E\begin{matrix} d(I\text{-}C)_n \\ Rif \end{matrix}} \qquad (4)$$

and results in the inactivation of the binary complex. It is assumed that all enzyme which is not rifampicin-inactivated is capable of initiating synthesis. Therefore:

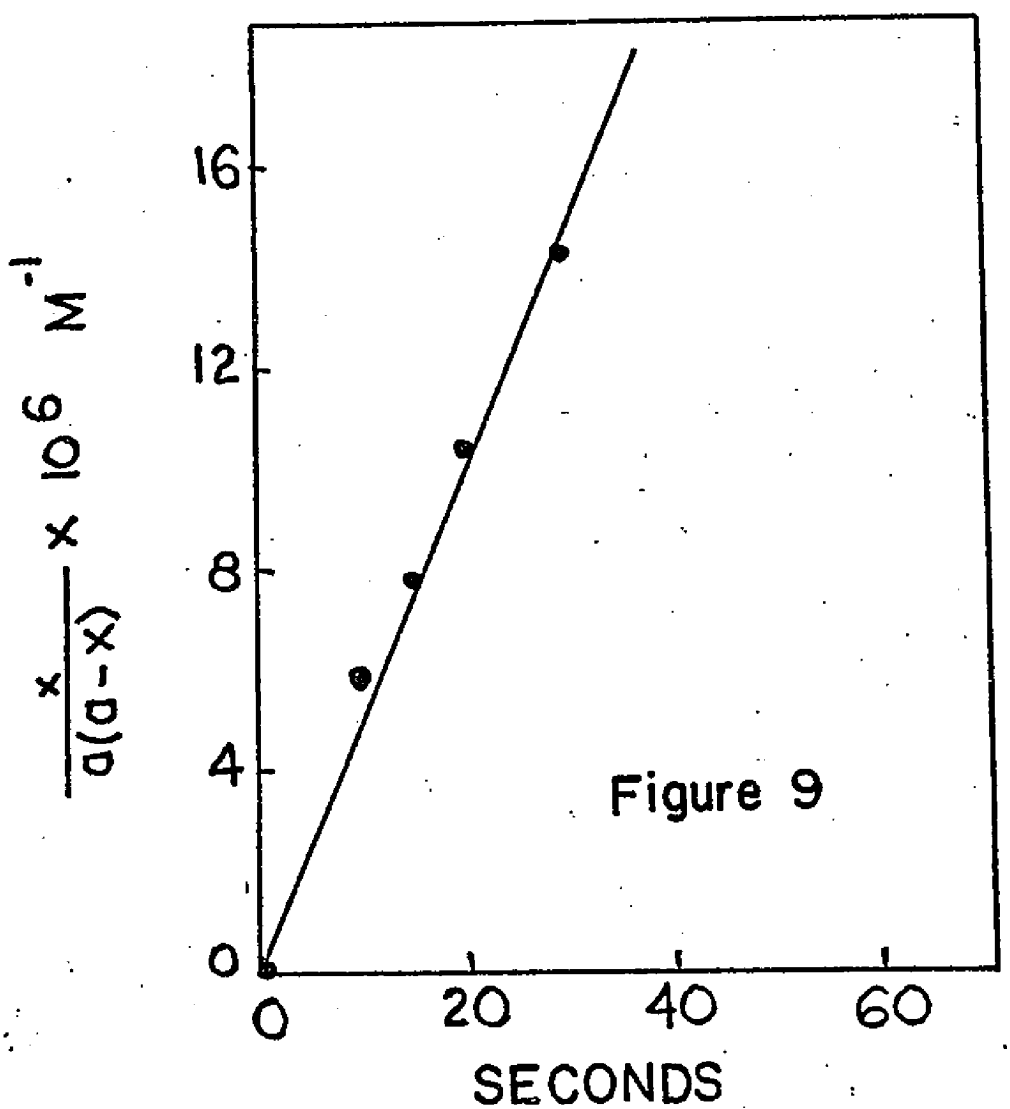


Figure 9

LEGEND TO FIGURE 9

Determination of the second order rate constant, k_2, for inactivation of the E-d(I-C)$_n$ binary complex by rifampicin

100 µl of a buffer containing 25 mM $MgCl_2$, 50 mM Tris pH 8.0 and 50 mM ME were preincubated with 10 nmoles d(I-C)$_n$ and 5 µg. RNA polymerase in a total volume of 115 µl for 15 minutes at 37° C. These samples were then preincubated at 37° C with 0.096 µM rifampicin in a total volume of 120 µl after which synthesis was initiated by the addition of 50 nmoles (^{3}H)CTP (5092 cpm/nmole) and 50 nmoles GTP. The complete mixture was incubated for 90 seconds at 37° C.

100% synthesis = 1.12 nmoles (^{3}H)CMP incorporated

$a = [E\text{-}d(I\text{-}C)_n] = [E]$

x = (100 - %(^{3}H)CMP incorporated in the presence of Rif)(a)

$$-\frac{d[E\text{-}d(I\text{-}C)_n]}{dt} = k_2([E\text{-}d(I\text{-}C)_n] - x)([Rif] - x) \quad (5)$$

where x is the concentration of rifampicin inactivated binary complex and k_2 is the second order rate constant for rifampicin attack on the binary complex. When $[Rif] = [E\text{-}d(I\text{-}C)_n]$, equation 5 becomes:

$$-\frac{d[E\text{-}d(I\text{-}C)_n]}{dt} = k_2([E\text{-}d(I\text{-}C)_n] - x)^2 \quad (6)$$

Integrating equation 6, we obtain:

$$\frac{x}{[E\text{-}d(I\text{-}C)_n]([E\text{-}d(I\text{-}C)_n] - x)} = k_2 t \quad (7)$$

It is possible to determine x if we make the following assumptions:

a. All enzyme capable of $r(G\text{-}C)_n$ synthesis initiates formation of the first phosphodiester bond at the same rate regardless of the site of initiation on the template. (A possible means of validating this assumption would be to work with $d(I\text{-}C)_n$ templates of various lengths. However, the variation of length of the template from various $d(I\text{-}C)_n$ preparations to date has had no significant effect on rifampicin experiments). This may affect the length of elongated chains, but does not affect the rate of initiation.

b. Enzyme to which rifampicin is bound is incapable of $r(G\text{-}C)_n$ synthesis (41) for rifampicin inhibits formation or function (106) of the first phosphodiester bond.

c. All enzyme to which rifampicin is not bound is capable of initiation of the first phosphodiester bond - i.e. synthesis may be used as a probe for phosphodiester bond formation assuming 100%

enzyme activity represents % synthesis in the absence of rifampicin.

d. The formation of the first phosphodiester bond is the rate determining step in $r(G\text{-}C)_n$ synthesis. This has been suggested by K_m data of Table IV, from binding studies of Wu and Goldthwait (35) and from bypass of initiation studies by Downey and So (48).

e. There is no reinitiation during the period of analysis. This is validated by the fact that rifampicin challenge experiments give the same $t_{1/2}$ data whether the experiments are run for 30 seconds, 90 seconds or 150 seconds (data not shown).

f. The second order rate constant for the attack of rifampicin on the $E\text{-}d(I\text{-}C)_n$ binary complex is the same regardless of whether $r(I\text{-}C)_n$ or $r(G\text{-}C)_n$ synthesis is used as a measure of concentration of enzyme which is not rifampicin inactivated. Further, it is assumed that the constant, k_2, is not dependent upon the divalent cations used. This assumption is supported by the data of Figure 7.

Using the above assumptions and equation 7, it can be shown in Figure 9 that the second order rate constant for the attack of rifampicin on the $E\text{-}d(I\text{-}C)_n$ binary complex is 4.9×10^5 M^{-1} sec^{-1}. This value is close to the value obtained by Hinkle, Mangel and Chamberlin (43) for the second order rate constant, k_2, for the attack of rifampicin on the naked E. coli core enzyme (3×10^5 M^{-1} sec^{-1}) and to the value for the rate constant for the association of rifampicin with the core RNA polymerase-T_7 DNA binary complex (1.1×10^5 M^{-1} sec^{-1}) determined by Wehrli, Handschin and Wunderli (105).

H. Rifampicin challenge experiments

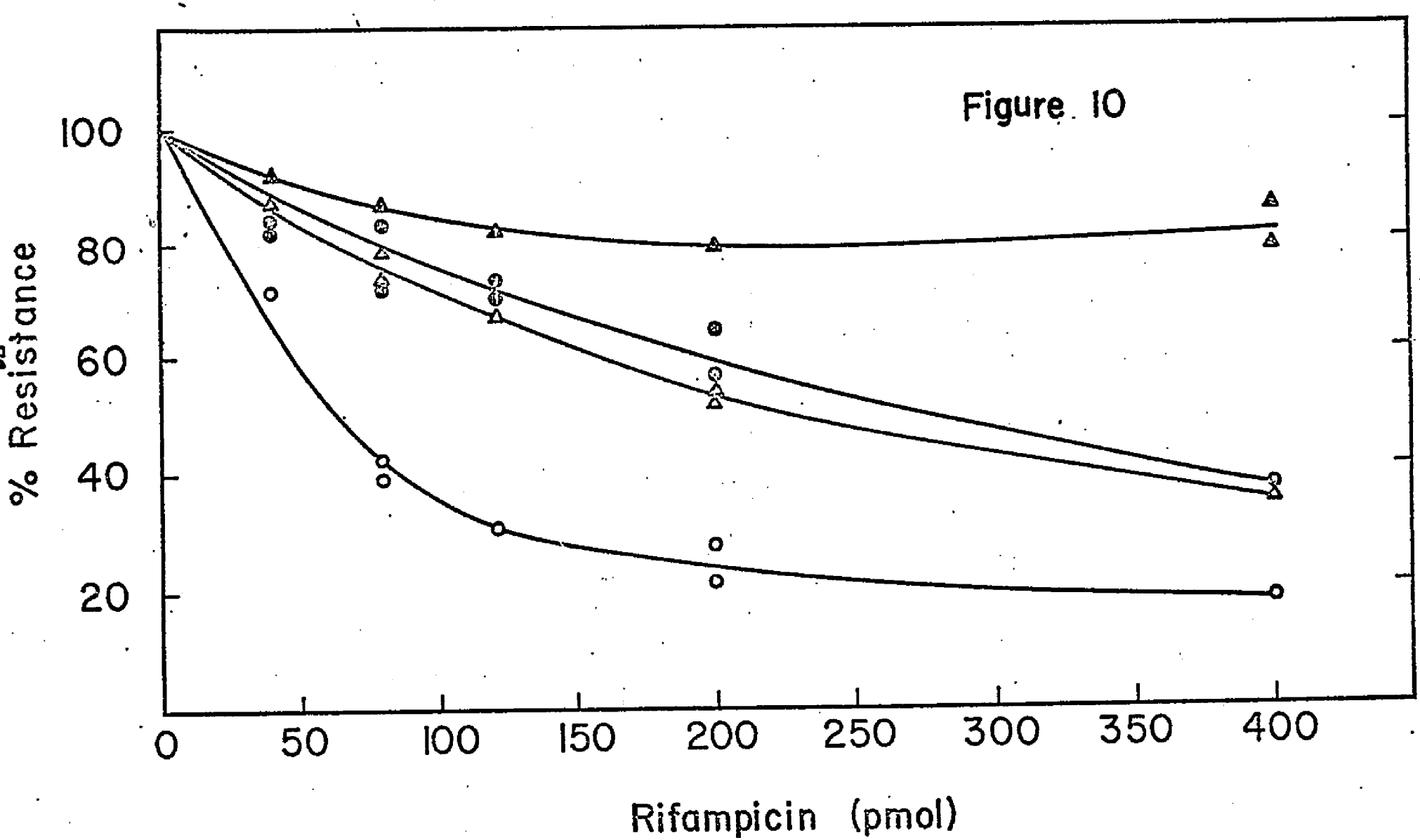
When rifampicin and nucleoside triphosphates are added simultaneous-

to a preformed binary complex, the relative rates of formation of the first phosphodiester bond in the presence of GTP vs. ITP with Mg^{++} or Mn^{++} as the divalent cation may be compared. Figure 10 shows clearly that $r(G-C)_n$ synthesis is more rifampicin resistant than $r(I-C)_n$ synthesis, the rifampicin resistance being greater when Mn^{++} buffers of ionic strength 0.1 were used instead of Mg^{++} buffers of ionic strength 0.1. The effect of primers in preforming preinitiation ternary complexes for $r(I-C)_n$ synthesis is as predicted from the apparent K_m data of Table IV: GpC $\rangle$ CpG $\rangle$ no primer (Figure 11). The significant effect of the primer is not as evident in the case of $r(G-C)_n$ synthesis when Mn^{++} is the divalent cation (Figure 12), demonstrating as in Table IV the hypothesis that GTP has a greater ability to support formation of the first phosphodiester bond than ITP and that the binding of GTP to the initiation site for this purpose is enhanced by the use of Mn^{++} as the divalent cation.

Since it is known that high ionic strength favors elongation and does not support initiation effectively, the effect of rifampicin on $r(G-C)_n$ and $r(I-C)_n$ synthesis from ionic strength 0.1 to 0.4 was considered as a support for the use of rifampicin challenge as an effective probe of relative initiation affinities of GTP vs. ITP. As would be expected, Figure 13 indicates that rifampicin resistance for both $r(I-C)_n$ and $r(G-C)_n$ synthesis is greatest at an ionic strength of 0.1 regardless of whether Mn^{++} or Mg^{++} is the divalent cation.

I. Rate constants for initiation and half times for initiation

Using the data from rifampicin challenge experiments and the value of k_2 for the attack of rifampicin on the $E-d(I-C)_n$ binary



LEGEND TO FIGURE 10

Rifampicin challenge experiment with $r(G-C)_n$ and $r(I-C)_n$ synthesis as a function of Mg^{++} and Mn^{++}- containing buffers

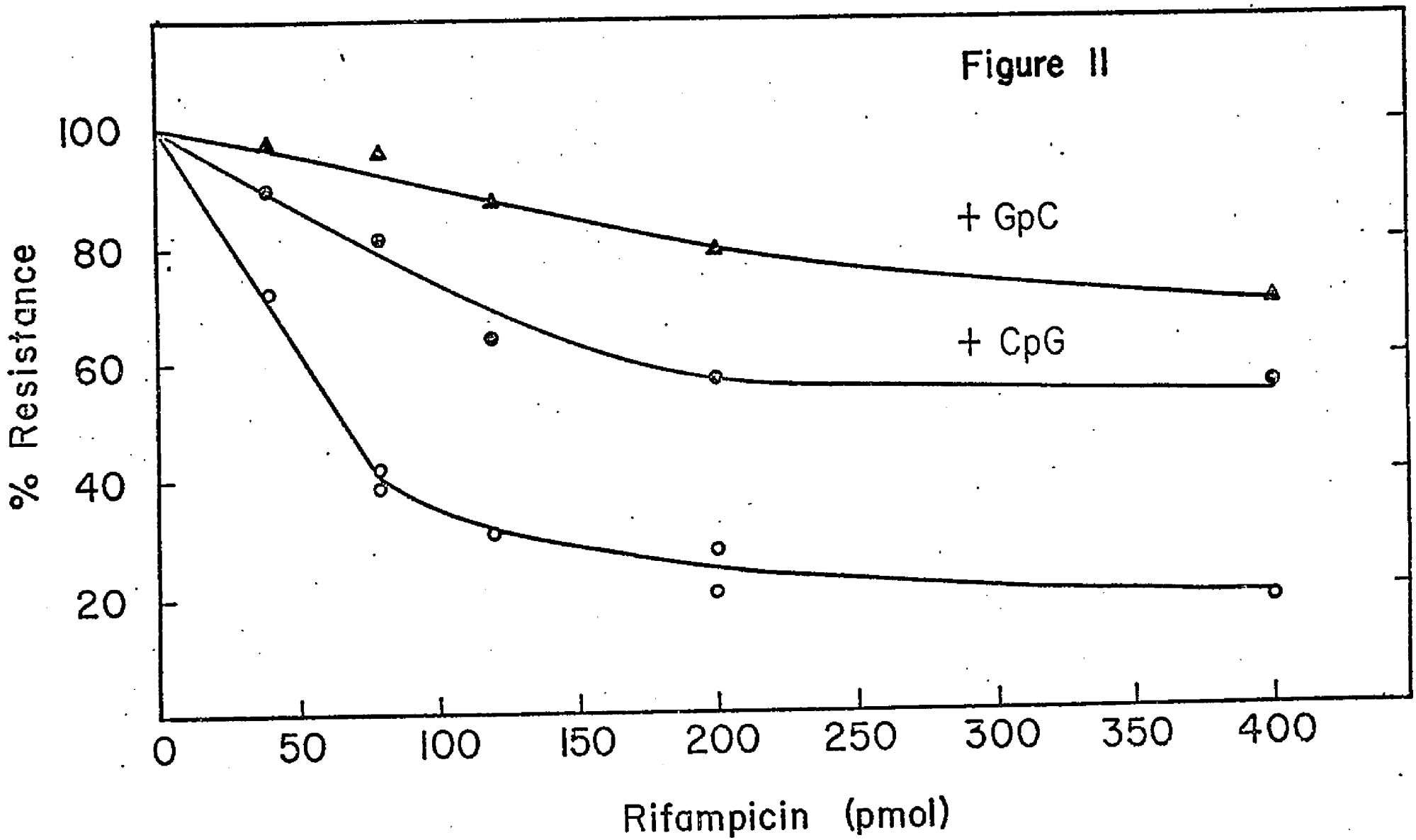
600 µl of a buffer containing 25 mM $MgCl_2$, 50 mM Tris pH 8.0 and 50 mM ME or 1 mM $MnSO_4$, 50 mM Tris pH 8.0, 50 mM ME and 75 mM KCl, was preincubated with 60 nmoles $d(I-C)_n$ and 30 µg. RNA polymerase for 15 minutes at 37° C in a total volume of 690 µl. RNA synthesis was initiated by removal of a 100 µl aliquot of the preincubation mix into a 15 µl aliquot containing 50 nmoles (^{3}H)CTP (4480 cpm/nmole), 50 nmoles GTP or ITP and 0 to 400 pmoles of rifampicin (preincubated for 3 minutes at 37° C). Synthesis was terminated after 90 seconds at 37° C. Radioactivity incorporated into acid precipitable $r(G-C)_n$ or $r(I-C)_n$ was determined. % resistance was determined for each buffer system by setting nmoles (^{3}H)CMP incorporated in the absence of rifampicin as 100% rifampicin resistance.

▲ - ▲ $r(G-C)_n$, Mn^{++} 100% = 2.5 nmoles (^{3}H)CMP incorporated

● - ● $r(G-C)_n$, Mg^{++} 100% = 1.5 nmoles (^{3}H)CMP incorporated

Δ - Δ $r(I-C)_n$, Mn^{++} 100% = 2.4 nmoles (^{3}H)CMP incorporated

○ - ○ $r(I-C)_n$, Mg^{++} 100% = 1.7 nmoles (^{3}H)CMP incorporated



LEGEND TO FIGURE 11

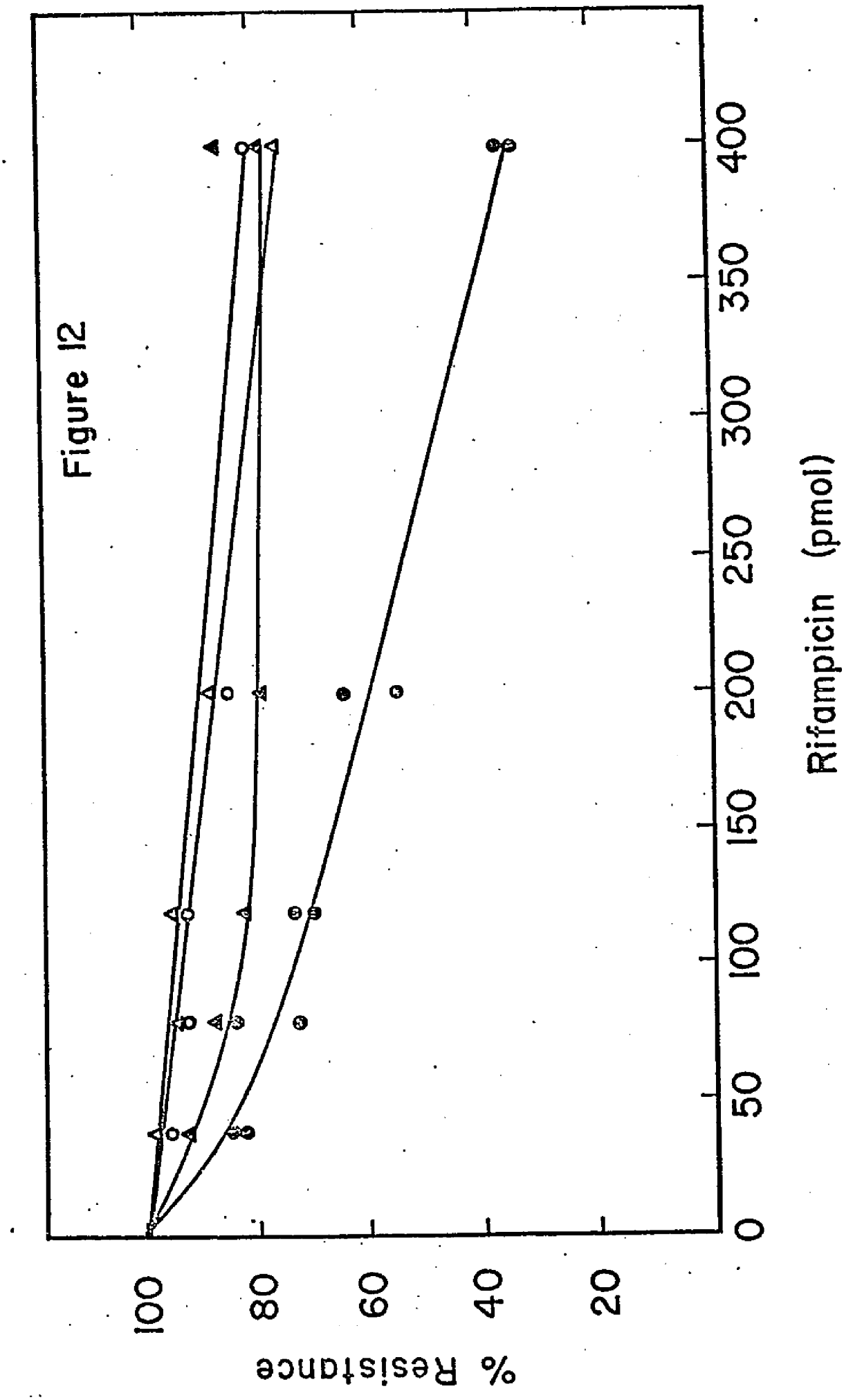
Role of dinucleoside monophosphate primers in enhancing rifampicin resistance of $r(I\text{-}C)_n$ synthesis in Mg^{++}- containing buffers

600 µl of a buffer containing 25 mM $MgCl_2$, 50 mM Tris pH 8.0 and 50 mM ME was preincubated with 60 nmoles $d(I\text{-}C)_n$ and 30 µg. RNA polymerase for 15 minutes at 37° C in the presence or absence of 240 nmoles GpC or CpG in a total volume of 690 µl. RNA synthesis was initiated by addition of a 100 µl aliquot of the mixture to a 15 µl aliquot containing 50 nmoles (^{3}H)CTP (4480 cpm/nmole), 50 nmoles ITP and 0 to 400 pmoles rifampicin. (The nucleoside triphosphates and rifampicin were preincubated for 3 minutes at 37° C.) Synthesis was terminated after 90 seconds at 37° C and % rifampicin resistance determined as in Figure 10.

● - ● + CpG, 100% = 2.6 nmoles (^{3}H)CMP incorporated

▲ - ▲ + GpC, 100% = 2.6 nmoles (^{3}H)CMP incorporated

○ - ○ no dinucleoside monophosphate, 100% = 1.7 nmoles (^{3}H)CMP incorporated



LEGEND TO FIGURE 12

Comparison of the effect of GpC on rifampicin resistance of $r(G-C)_n$ synthesis in Mg^{++}- containing vs. Mn^{++}- containing buffers

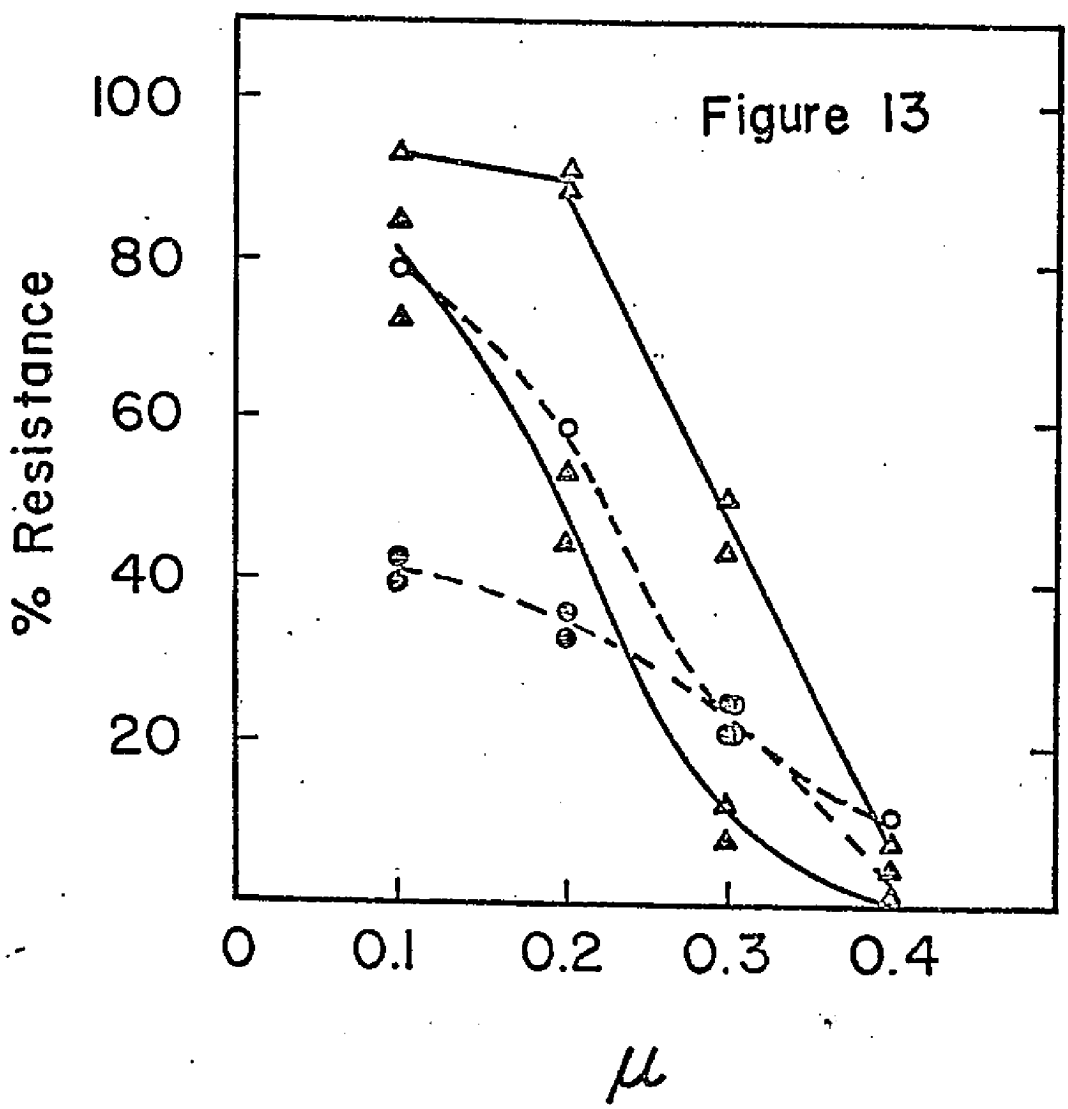
600 µl of a mixture containing 25 mM $MgCl_2$, 50 mM Tris pH 8.0 and 50 mM ME or 1 mM $MnSO_4$, 50 mM Tris pH 8.0, 50 mM ME and 75 mM KCl were preincubated with 30 µg. RNA polymerase and 60 nmoles $d(I-C)_n$ in the presence or absence of 240 nmoles GpC for 15 minutes at 37° C in a total volume of 690 µl. RNA synthesis was initiated by the addition of a 100 µl aliquot of the mixture to a 15 µl aliquot containing 50 nmoles (^{3}H)CTP (4480 cpm/nmole), 50 nmoles GTP and 0 to 400 pmoles rifampicin (the nucleoside triphosphates and rifampicin were preincubated for 3 minutes at 37° C). Synthesis was terminated after 90 seconds at 37° C and % resistance to rifampicin determined as in Figure 10.

Δ - Δ Mn^{++}, + GpC 100% = 2.6 nmoles (^{3}H)CMP incorporated

O - O Mg^{++}, + GpC 100% = 1.5 nmoles (^{3}H)CMP incorporated

▲ - ▲ Mn^{++}, no dinucleoside monophosphate 100% = 2.5 nmoles (^{3}H)CMP incorporated

● - ● Mg^{++}, no dinucleoside monophosphate 100% = 1.5 nmoles (^{3}H)CMP incorporated



LEGEND TO FIGURE 13

Effect of ionic strength on rifampicin resistance

600 µl of buffers containing 25 mM $MgCl_2$, 50 mM Tris pH 8.0 and 50 mM ME or 1 mM $MnSO_4$, 50 mM Tris pH 8.0, and 50 mM ME were preincubated for 15 minutes at 37° C with 60 nmoles $d(I-C)_n$ and 30 µg. RNA polymerase and KCl (to adjust the ionic strength as indicated) in a total volume of 690 µl. 100 µl of each mixture was incubated for 90 seconds at 37° C with a 15 µl aliquot containing 50 nmoles (^3H)GTP (4480 cpm/nmole), 50 nmoles GTP or ITP and 80 pmoles rifampicin. Reactions were terminated by the addition of 100 µl of 0.2 M sodium pyrophosphate and acid precipitable radioactivity determined. The % rifampicin resistance was calculated using the data from Figure 3c as 100% rifampicin resistance.

○ - ○ ITP, Mn^{++}

△ - △ GTP, Mn^{++}

● - ● ITP, Mg^{++}

▲ - ▲ GTP, Mg^{++}

complex, 4.9×10^5 M^{-1} sec^{-1}, it is possible to calculate the rate constants for initiation and the half times for initiation of $r(I\text{-}C)_n$ and $r(G\text{-}C)_n$ synthesis as follows:

From the conservation equation, the total concentration of binary complex at initiation upon simultaneous addition of rifampicin and nucleoside triphosphates to the binary $E\text{-}d(I\text{-}C)_n$ complex is given by:

$$\left[E\text{-}d(I\text{-}C)_n\right] = \left[E^{d(I\text{-}C)_n}_{Rif}\right] + \underbrace{\left[E^{d(I\text{-}C)_n}_{pppPu}\right]}_{\text{active initiation complex}} \qquad (8)$$

The relative concentration of active initiation complex may be determined from the % resistance of the active enzyme to attack by rifampicin.

Since k_2 approximates the value determined by Hinkle, Mangel and Chamberlin (43) for the rate of attack of rifampicin on the naked enzyme, and since the binary complex is not apparently stabilized to a great extent by the presence of a primer until the addition of the first nucleoside triphosphate (Table III), it will be assumed that k_2 is also the constant for rifampicin attack on the $E^{d(I\text{-}C)_n}_{GpC}$ or the $E^{d(I\text{-}C)_n}_{CpG}$ ternary initiation complex as well as for the binary $E\text{-}d(I\text{-}C)_n$ complex. Then:

$$\% \text{ Rifampicin Resistance} = \frac{\left[E^{d(I\text{-}C)_n}_{pppPu}\right]}{\left[E\text{-}d(I\text{-}C)_n\right]} \times 100 \qquad (9)$$

where Pu = GTP or ITP. Similar equations may be written for % rifampicin resistance of the ternary initiation complex for rifampicin can bind to complexes before the formation of a phosphodiester

bond with a third nucleoside triphosphate (104).

It is not possible to obtain the % rifampicin resistance of the enzyme-template binary complex directly. However, assuming all binary complex (or ternary initiation complex) to which a purine ribonucleoside triphosphate has bound is capable of synthesizing RNA:

$$\% \text{ Rifampicin Resistance} = \frac{\text{nmoles } (^3\text{H})\text{NMP incorporated in presence of rifampicin}}{\text{nmoles } (^3\text{H})\text{NMP incorporated in absence of rifampicin}} \times 100 \qquad (10)$$

Assuming no reinitiation occurs within the period of the 90 second incubations, initiation of $r(I\text{-}C)_n$ or $r(G\text{-}C)_n$ synthesis (formation of the first rifampicin-resistant phosphodiester bond - this is possibly the second phosphodiester bond (104), a bond which also exhibits greater stability for the ternary complex when subjected to high ionic strengths (Table III) obeys the following equation (47):

$$\frac{\left[d(I\text{-}C)_n\right]}{\left[E\overset{d(I\text{-}C)_n}{\diagdown r(Pu\text{-}C)_n}\right]} = \frac{k_2}{k^*}\left[Rif\right] + 1 \qquad (11)$$

where $[Rif]$ is the concentration of rifampicin, $\left[E\overset{d(I\text{-}C)_n}{r(Pu\text{-}C)_n}\right]$ is the concentration of complex still active after rifampicin attack, $[E\text{-}d(I\text{-}C)_n]$ is the total concentration of active complex before rifampicin attack; $k_2 = 4.9 \times 10^5\ M^{-1}\ sec^{-1}$ and k^* is the first order rate constant for $r(G\text{-}C)_n$ or $r(I\text{-}C)_n$ chain initiation.

Equation 11 may be written as:

$$0.01\ \%\ \text{Rifampicin Resistance}^{-1} = \frac{k_2}{k^*}\left[Rif\right] + 1 \qquad (12)$$

One may calculate the rate constant for initiation from the slope of the graph of % Rifampicin Resistance^{-1} vs. rifampicin concentration, and $t_{½}$ for initiation may be calculated from k*.

The values for k* are calculated from plots similar to Figure 14 which indicates that initiation obeys first order kinetics for both $r(I\text{-}C)_n$ and $r(G\text{-}C)_n$ synthesis in both Mg^{++} and Mn^{++}- containing buffers. Values for k* and $t_{½}$ for initiation (formation of the first rifampicin resistant phosphodiester bond) are shown in Table V. The half time for initiation of unprimed $r(G\text{-}C)_n$ synthesis is of the order of 0.2 to 0.6 seconds using the core A. vinelandii RNA polymerase. This value is very similar to the value of 0.23 seconds obtained by Mangel and Chamberlin for the half time of initiation by the E. coli RNA polymerase for T_7 RNA synthesis. The data also indicate that the half time for initiation for $r(I\text{-}C)_n$ synthesis is four to five times larger than for $r(G\text{-}C)_n$ synthesis supporting the hypothesis that ITP has a lower affinity for the initiation site than GTP. The half time for initiation for $r(G\text{-}C)_n$ and $r(I\text{-}C)_n$ synthesis is affected by the presence of a primer in Mn^{++}- containing buffers as it is in Mg^{++}-containing buffers for either $r(I\text{-}C)_n$ or $r(G\text{-}C)_n$ synthesis as would be expected from the binary complex stability data of Table III or the K_m data of Table IV. The data also shows a slightly greater ability of GpC than CpG to initiate the formation of the first rifampicin resistant phosphodiester bond in Mg^{++}- containing buffers. This supports the more efficient apparent K_m lowering for K_C by GpC vs CpG as well. It is apparent that Mn^{++} plays a role in initiation, for the ratio of $t_{½}$ for initiation of primed and unprimed RNA synthesis

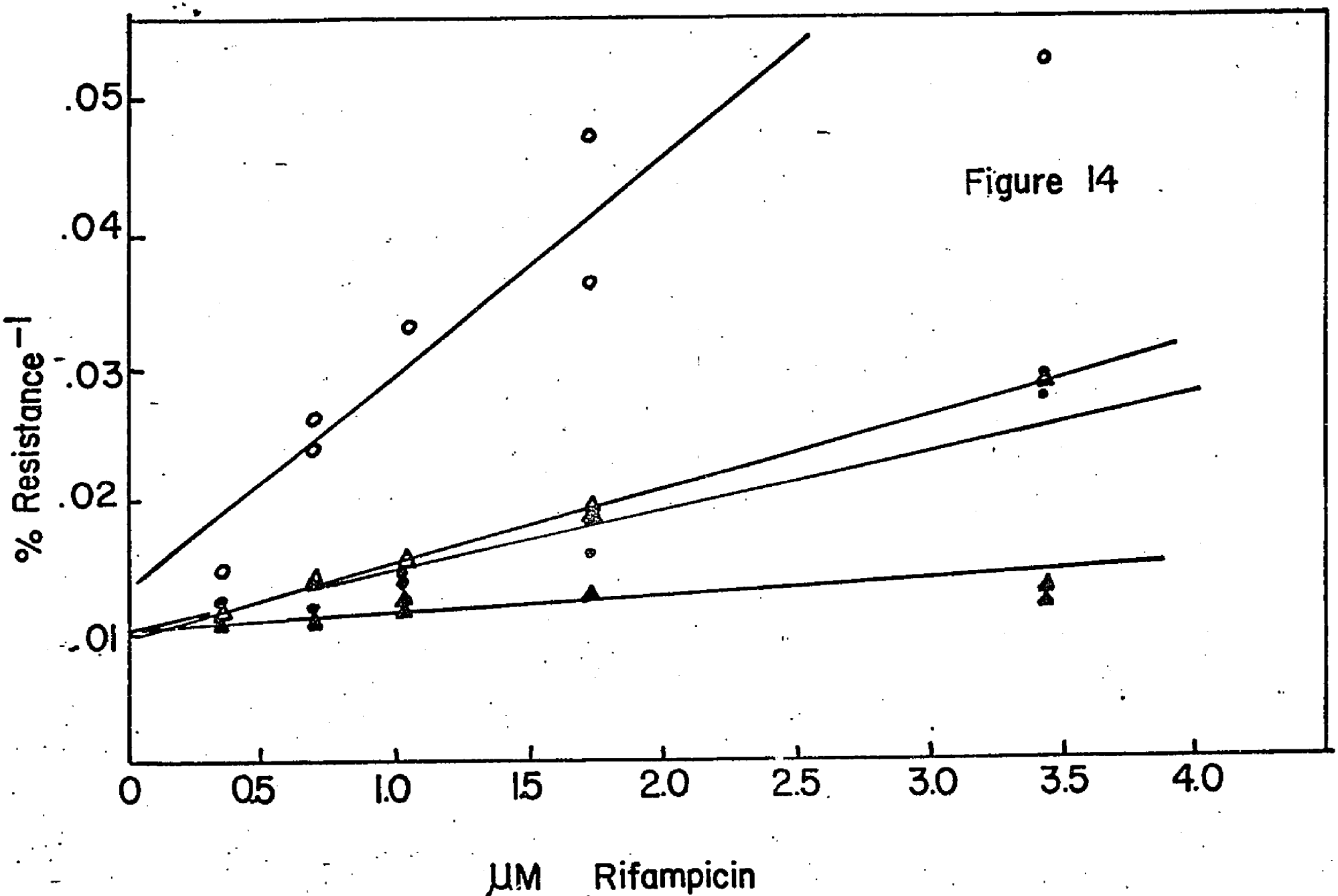


Figure 14

LEGEND TO FIGURE 14

Application of equation 11 to rifampicin challenge experiments for $r(G\text{-}C)_n$ and $r(I\text{-}C)_n$ synthesis

Data from Figure 10 are replotted according to equation 11.

TABLE V

Estimated values of k* and $t_{1/2}$ for initiation of RNA synthesis using a preformed E-d(I-C)$_n$ binary complex

RNA Synthesized	Primer	Divalent Cation	k* sec^{-1}	$t_{1/2}$ sec
r(G-C)$_n$	none	Mg^{++}	1.16	0.59
r(G-C)$_n$	none	Mn^{++}	4.41	0.16
r(I-C)$_n$	none	Mg^{++}	0.31	2.27
r(I-C)$_n$	none	Mn^{++}	0.83	1.09
r(G-C)$_n$	CpG	Mg^{++}	2.44	0.28
r(G-C)$_n$	CpG	Mn^{++}	5.19	0.13
r(I-C)$_n$	CpG	Mg^{++}	1.02	0.67
r(I-C)$_n$	CpG	Mn^{++}	1.07	0.64
r(G-C)$_n$	GpC	Mg^{++}	6.76	0.13
r(G-C)$_n$	GpC	Mn^{++}	6.76	0.13
r(I-C)$_n$	GpC	Mg^{++}	3.75	0.18
r(I-C)$_n$	GpC	Mn^{++}	1.20	0.57

LEGEND TO TABLE V

Estimated values of k* and $t_{1/2}$ for initiation of RNA synthesis using preformed E-d(I-C)$_n$ binary complexes or preformed $E^{d(I-C)_n}_{GpC}$ or $E^{d(I-C)_n}_{CpG}$ ternary complexes

Data were determined by application of equation 11 to rifampicin challenge experiments such as illustrated in Figures 10 - 12 and 14.

approaches unity in Mn^{++}- containing buffers (Table V), and the ratios of half times for initiation determined in Mg^{++} buffers for primed synthesis to that determined in Mn^{++} buffers for primed synthesis also approaches unity. As would be predicted, the effect of the primer in reducing the half time for initiation in Mg^{++}- containing buffers is less for GTP than ITP due to the greater affinity of GTP for the initiation site.

Affinity of GTP and ITP for the initiation site of the A. vinelandii RNA polymerase core enzyme in the transcription of ribohomopolymers

That GTP initiates more effectively than ITP in the transcription of the alternating copolymer $d(I\text{-}C)_n$ has been shown. It is of interest to determine the relative affinity of GTP and ITP for the initiation site during ribohomopolymer synthesis and to what extent initiation is affected by the presence of Mn^{++} and Mg^{++} as the divalent cation.

The apparent K_m's for poly r(G) and poly r(I) synthesis were determined (Table VI). As previously indicated for the $d(I\text{-}C)_n$ template, the apparent K_G is lower than K_I regardless of the buffer used and the apparent K_m of both GTP and ITP is lowered in the presence of Mn^{++} without increasing the maximum velocity. As was the case for $d(I\text{-}C)_n$ transcription, the apparent K_m lowering reflects more effective initiation conditions favorable for subsequent elongation rather than being a representation of the relative affinities of GTP and ITP for the elongation site. That this is the case is demonstrated by K_m data in the presence of the GpG primer which indicates K_m lowering where binding of GTP or ITP to the initiation site is bypassed. However,

TABLE VI

a. Apparent K_m determinations for poly r(G) and poly r(I) synthesis

Apparent K_m	Template	RNA Synthesized	Unprimed K_m μM		Primed K_m μM	
			Mn^{++}	Mg^{++}	Mn^{++}	Mg^{++}
K_G	$d(G)_n \cdot d(C)_n$	$r(G)_n$	11	66	--	--
K_G	$d(I)_n \cdot d(C)_n$	$r(G)_n$	29	77	25	25
K_G	$d(C)_n$	$r(G)_n$	50	200	50	50
K_I	$d(G)_n \cdot d(C)_n$	$r(I)_n$	29	77	--	--
K_I	$d(I)_n \cdot d(C)_n$	$r(I)_n$	67	111	56	100
K_I	$d(C)_n$	$r(I)_n$	200	500	90	90

b. V_{max} for poly r(G) and poly r(I) synthesis

Apparent V_{max}	Template	RNA Synthesized	Unprimed V_{max} pmoles/ sec/2.5 μg enzyme		Primed V_{max} pmoles/ sec/2.5 μg enzyme	
			Mn^{++}	Mg^{++}	Mn^{++}	Mg^{++}
V_G	$d(I)_n \cdot d(C)_n$	$r(G)_n$	20	27	13	13
V_G	$d(C)_n$	$r(G)_n$	48	33	56	56
V_I	$d(I)_n \cdot d(C)_n$	$r(I)_n$	19	22	56	56
V_I	$d(C)_n$	$r(I)_n$	67	111	51	51

LEGEND TO TABLE VI

a. Apparent K_m determinations for poly r(G) and poly r(I) synthesis

100 µl of Mg^{++} or Mn^{++} buffers as described in Figure 4, 10 nmoles poly d(C), 20 nmoles poly d(I)·d(C) or 20 nmoles poly d(G)·d(C) and 2.5 µg. RNA polymerase in the presence or absence of 40 nmoles GpG primer in a total volume of 115 µl was preincubated for 15 minutes at 37° C. RNA synthesis was initiated by the addition of a 10 µl aliquot containing 1.67 to 50 nmoles (α-^{32}P)GTP (10,000 - 12,000 cpm/nmole) or (^{3}H)ITP (3510 cpm/nmole). Reactions were terminated after 2.5 minutes at 37° C and the amount of acid insoluble poly r(I) or poly r(G) determined. Apparent K_m values were determined from Lineweaver-Burk plots such as illustrated in Figure 4.

b. V_{max} for poly r(G) and poly r(I) synthesis

Conditions as in a.

it could be hypothesized from the effect of the primer that the elongation site has a greater affinity for GTP. However, conclusive data in this regard would involve knowledge of the relative stability of the $E\begin{matrix}dC_n \\ GpG\end{matrix}$ or $E\begin{matrix}dI_n \cdot dC_n \\ GpG\end{matrix}$ ternary complex.

Support for the hypothesis that in ribohomopolymer synthesis GTP has a greater affinity than ITP for the initiation and/or elongation sites of core A. vinelandii RNA polymerase is shown in Table VII a. It can be noted that GTP inhibits incorporation of (^{3}H)IMP to a greater extent than ITP inhibits (^{3}H)GMP incorporation when both nucleoside triphosphates are present simultaneously. The data also indicates fidelity of transcription of poly dC for neither ATP, UTP nor CTP have an obvious effect on poly r(I) or poly r(G) synthesis at the concentrations utilized.

Poly d(I) may support synthesis of the ribohomopolymer, poly r(C), given the appropriate conditions. Poly r(C) synthesis in the absence of a primer is low since the initiation site has little affinity for a pyrimidine nucleoside triphosphate. However, the primer CpC may be used to bypass the problem of initiation with CTP. It was of interest to determine the effect of the presence of noncomplementary nucleotides on primed poly r(C) synthesis. Table VIII indicates that for CpC primed poly r(C) synthesis GTP and ITP inhibit 83 - 90% of poly r(C) synthesis. The inhibitory effect of GTP is greater than ITP, and poly r(G) or poly r(I) are synthesized as well as poly r(C). ATP and UTP inhibit only 47 - 55% of the (^{3}H)CMP incorporation. The effect of the purine nucleoside triphosphate is greater than that of the pyrimidine nucleoside triphosphate with neither synthesis of

TABLE VII

Effect of nucleoside triphosphates on poly r(I) and poly r(G) synthesis using a poly d(C) template

NTP's Added	nmoles (^{3}H)NMP Incorporated	% Synthesis
(^{3}H)GTP	1.98	100
(^{3}H)ITP	2.69	100
(^{3}H)GTP + CTP	2.13	107
(^{3}H)ITP + CTP	2.52	94
(^{3}H)GTP + ITP	0.86	43
(^{3}H)ITP + GTP	0.80	30
(^{3}H)GTP + ATP	1.87	94
(^{3}H)ITP + ATP	2.50	93

LEGEND TO TABLE VII

Effect of nucleoside triphosphates on poly r(I) and poly r(G) synthesis using a poly d(C) template

100 µl of Mn^{++} buffer as described in Figure 10, 10 nmoles poly d(C) and 5 µg. RNA polymerase were preincubated for 15 minutes at 37° C in a total volume of 110 µl. RNA synthesis was initiated by the addition of 50 nmoles (^{3}H)ITP (6824 cpm/nmole) or (^{3}H)GTP (4024 cpm/nmole) in the presence or absence of 50 nmoles of CTP, GTP, ITP or ATP. Synthesis was terminated after 90 seconds at 37° C and radioactivity incorporated into RNA determined. The nmoles of (^{3}H)GTP or (^{3}H)ITP incorporated in the absence of competing nucleoside triphosphates were used as the values for 100% synthesis for poly r(G) and poly r(I) synthesis respectively.

TABLE VIII

Effect of noncomplementary nucleoside triphosphates on CpC primed poly r(C) synthesis

NTP's added	nmoles (^{3}H)CMP incorporated	nmoles non-complementary (^{3}H)NMP incorporated	% Resistant (^{3}H)CMP Incorporation
CTP	3.36	0.00	100.0
CTP + ATP	1.53	0.00	45.4
CTP + GTP	0.35	0.55	10.5
CTP + ITP	0.56	0.46	16.6
CTP + UTP	1.79	0.00	53.3

LEGEND TO TABLE VIII

Effect of noncomplementary nucleoside triphosphates on CpC primed poly r(C) synthesis

100 µl Mn^{++} buffer as described in Figure 10, 40 nmoles CpC, 10 nmoles poly dI and 5 µg. RNA polymerase were preincubated for 15 minutes at 37° C in a total volume of 120 µl. RNA synthesis was initiated by the addition of 50 nmoles (^{3}H)CTP (3939 cpm/nmole) in the presence or absence of 50 nmoles of ATP, GTP, ITP or UTP. Synthesis was terminated after 30 minutes at 37° C and radioactivity incorporated into poly r(^{3}H)C determined.

To determine incorporation of radioactivity into poly r(A), poly r(U), poly r(I) or poly r(G), reactions were essentially performed as above with the addition of 50 nmoles unlabelled CTP in the presence of 50 nmoles (^{3}H)ATP (8121 cpm/nmole), (^{3}H)UTP (13,300 cpm/nmole), (^{3}H)GTP (4024 cpm/nmole) or (^{3}H)ITP (6824 cpm/nmole)

poly A nor poly U occurring. Therefore, the data of Table VIII suggests that the presence of noncomplementary purine or pyrimidine nucleoside triphosphates reduces the affinity of the CpC primer for the initiation site. The most significant effect in reduction of CpC affinity is noted when the rapidly initiating nucleoside triphosphate, GTP, is present. It is not apparent from the data whether synthesis of poly r(I) or poly r(G) occurs by an unprimed mechanism, by use of poly d(I)·r(C) as template or by a slippage mechanism using the CpC primer.

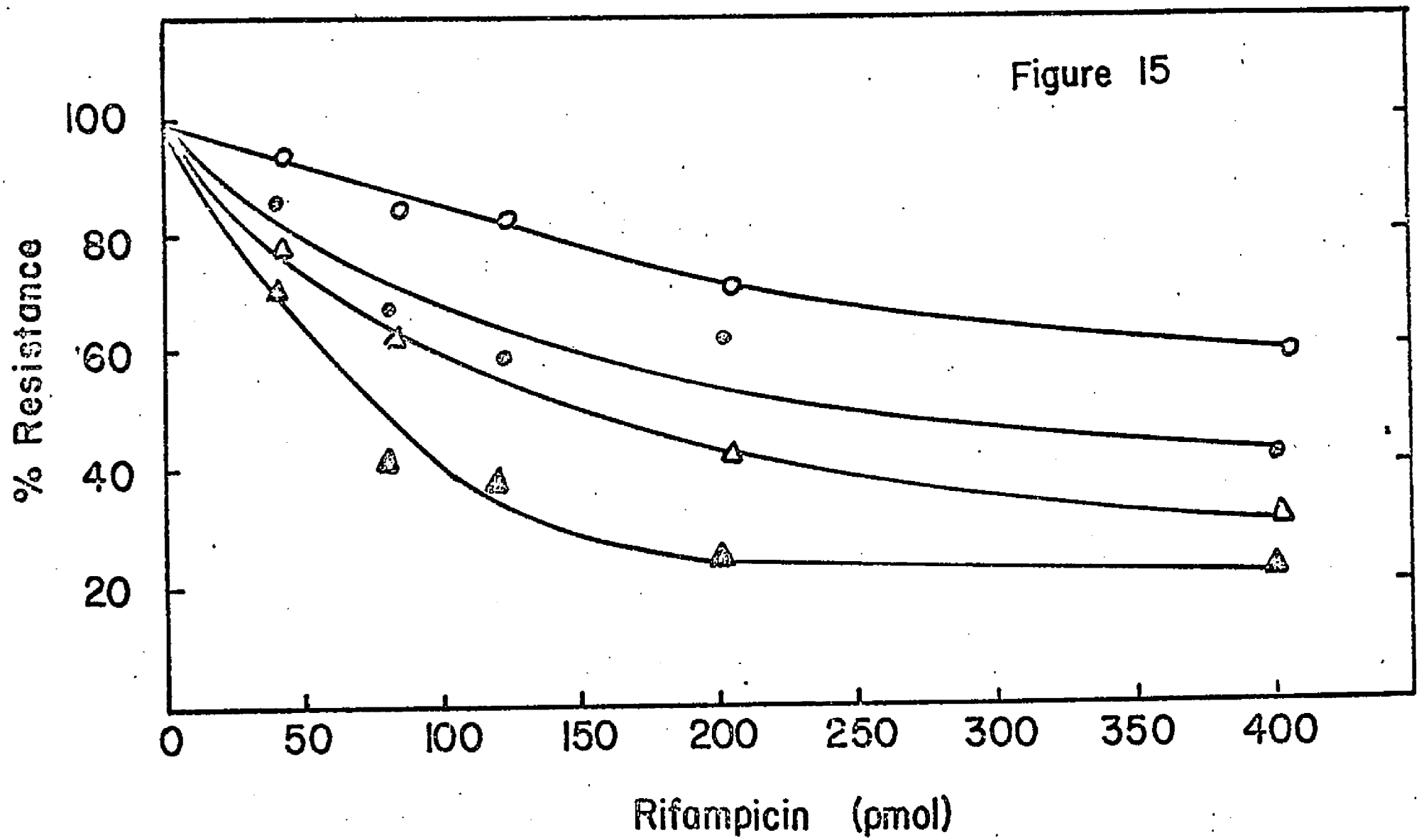
The RNA chain initiation reaction has been studied for poly r(I) or poly r(G) synthesis using rifampicin challenge experiments. In Figure 15 it can be seen that using the double helical poly d(I)·d(C) template, the rifampicin resistance of poly r(G) is significantly greater than that of poly r(I) synthesis in Mg^{++} or Mn^{++} buffers, and that Mn^{++} apparently enhances rifampicin 'resistance' of A. vinelandii RNA polymerase for poly r(G) or poly r(I) synthesis significantly as compared with the use of Mg^{++} as the divalent cation.

A comparison of poly r(G) and poly r(I) rifampicin resistance using the poly d(C) template (Figure 16) also demonstrates greater affinity of GTP for the initiation site of RNA polymerase.

The results of Figures 15 and 16 indicate that initiation is more efficient when poly d(I)·d(C) is the template than when poly d(C) is the template. Secondly, the role of Mn^{++} vs. Mg^{++} is not as clearly distinct in enhancing the initiation on the single stranded poly d(C) template as on the double helical poly d(I)·d(C).

The data from rifampicin challenge experiments for poly r(G)

Figure 15



LEGEND TO FIGURE 15

Rifampicin resistance of poly r(G) and poly r(I) synthesis:

Comparison of poly r(G) and poly r(I) synthesis during transcription of poly d(I)·d(C)

600 µl of Mn^{++} or Mg^{++} buffers as described in Figure 10, 120 nmoles $d(I)_n \cdot d(C)_n$ and 15 µg. RNA polymerase core enzyme were preincubated for 15 minutes at 37° C. Following preincubation 100 µl aliquots of the mixtures were added to 10 µl reaction mixtures containing 50 nmoles (^{3}H)GTP (4024 cpm/nmole) or 50 nmoles (^{3}H)ITP (5929 cpm/nmole) and 0 to 400 pmoles rifampicin. (The labelled GTP or ITP and rifampicin were preincubated at 37° C for 3 minutes.) Incubations were terminated after 90 seconds at 37° C and acid precipitable radioactivity determined.

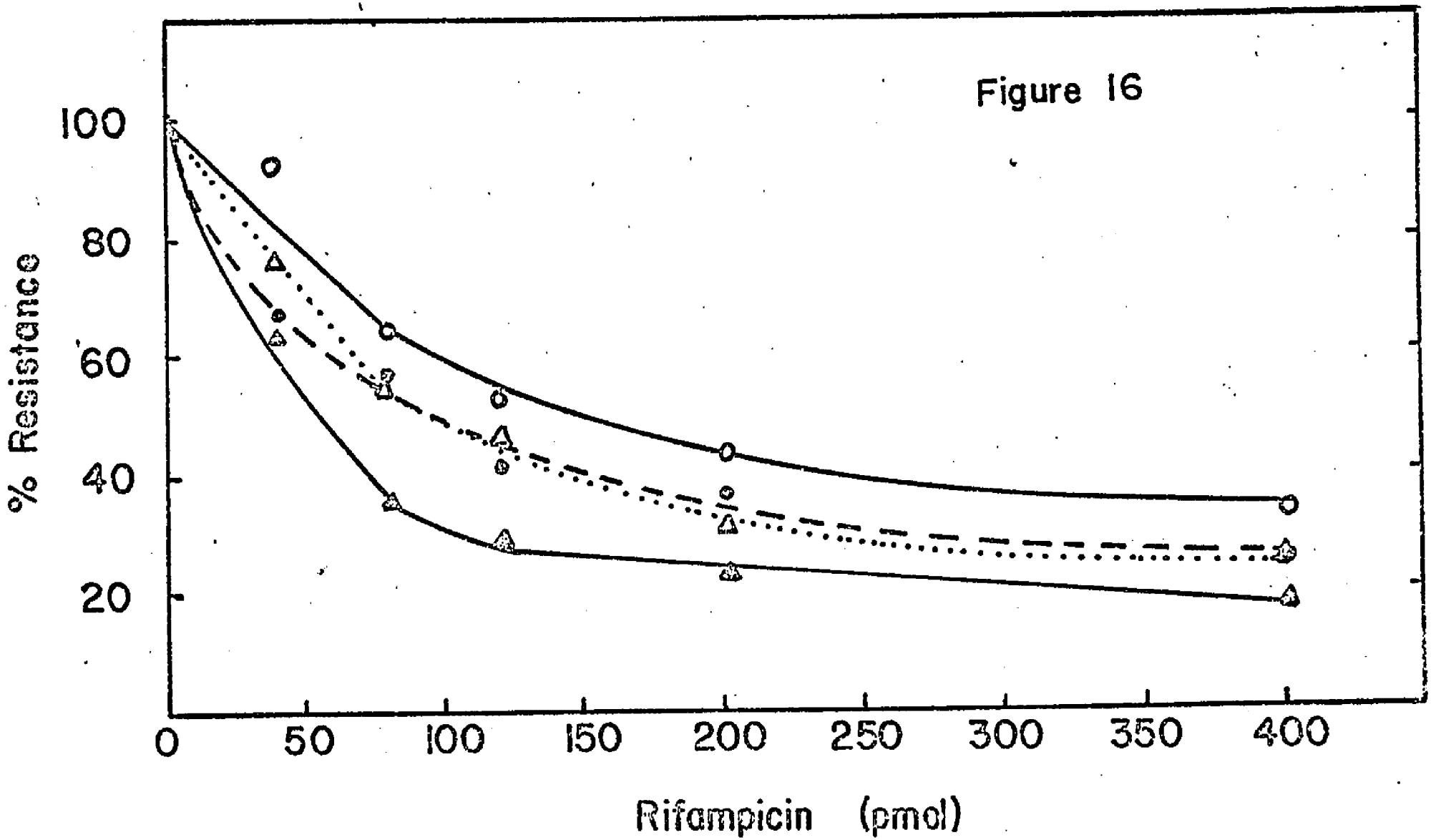
o - o Mn^{++}, (^{3}H)GTP 100% = 1.8 nmoles (^{3}H)GMP incorporated

● - ● Mn^{++}, (^{3}H)ITP 100% = 1.8 nmoles (^{3}H)IMP incorporated

Δ - Δ Mg^{++}, (^{3}H)GTP 100% = 2.4 nmoles (^{3}H)GMP incorporated

▲ - ▲ Mg^{++}, (^{3}H)ITP 100% = 1.7 nmoles (^{3}H)IMP incorporated

Figure 16



LEGEND TO FIGURE 16

Rifampicin resistance of poly r(G) and poly r(I) synthesis:

Comparison of poly r(G) and poly r(I) synthesis during transcription of poly d(C) template

600 µl of Mn^{++} or Mg^{++} buffer as described in Figure 10, 60 nmoles poly d(C) and 15 µg. RNA polymerase in a total volume of 690 µl were preincubated for 15 minutes at 37° C. Following preincubation, 100 µl aliquots of the mixture were added to reaction mixes containing 50 nmoles (^{3}H)GTP (4024 cpm/nmole) or (^{3}H)ITP (6824 cpm/nmole) and 0 to 400 pmoles rifampicin. Incubations were terminated after 90 seconds at 37° C and acid precipitable radioactivity determined.

o - o Mn^{++}, (^{3}H)GTP 100% = 2.6 nmoles (^{3}H)GMP incorporated

● - ● Mn^{++}, (^{3}H)ITP 100% = 3.4 nmoles (^{3}H)IMP incorporated

Δ - Δ Mg^{++}, (^{3}H)GTP 100% = 2.2 nmoles (^{3}H)GMP incorporated

▲ - ▲ Mg^{++}, (^{3}H)ITP 100% = 2.3 nmoles (^{3}H)IMP incorporated

synthesis in Figures 15 and 16 have been replotted in Figure 17. As can be seen the reciprocal of the rifampicin resistance of poly r(G) synthesis vs. the rifampicin concentration is linear regardless of buffer or template. Similar data have been obtained in the presence of a GpG primer for poly r(G) rifampicin resistance and for poly r(I) rifampicin resistance in the presence or absence of a primer.

The rate constant for initiation, k^*, and the half time for initiation, $t_{1/2}$, are dependent upon k_2, the relative rate of inactivation of the binary complex by rifampicin. It is possible that this rate of inactivation of the binary complex is dependent upon the stability of the binary complex and there is evidence from heparin dissociation of several binary E-DNA complexes (See below) that there is a wide range of stabilities dependent not only upon the nature of the DNA but also on the specific divalent cation present. However, it is possible in the absence of data for k_2 to determine the relative rate of initiation of GTP vs. ITP in the specific buffer systems used in the presence or absence of a primer and to compare these relative rates with those found using the $d(I\text{-}C)_n$ template as follows:

Equation 12 may be written as:

$$k^* = 0.01\ s^{-1}\ k_2 \qquad (13)$$

where s^{-1} is the reciprocal of the slope of a graph of % rifampicin resistance^{-1} vs. concentration of rifampicin. We may now define a relative initiation rate constant, i, as:

$$i = 0.01\ s^{-1} \qquad (14)$$

from which the relative half time for initiation, $t_{1/2}$, may be defined as:

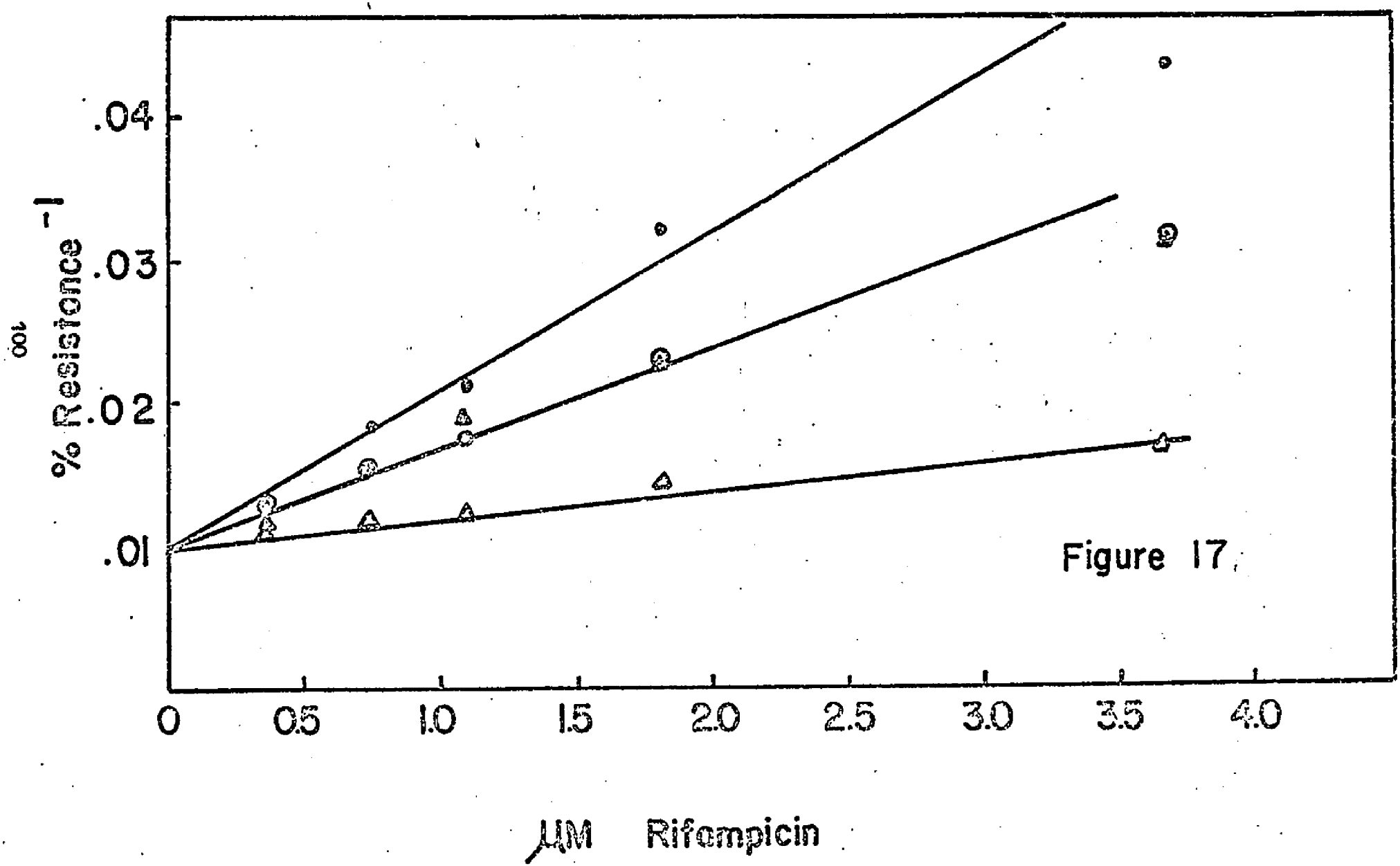


Figure 17.

LEGEND TO FIGURE 17

Application of equation 11 to rifampicin challenge experiments for poly r(G) synthesis

Data from Figures 15 and 16 are replotted according to equation 11.

● - ● from Figure 16 Δ - Δ

▲ - ▲ from Figure 16 O - O

O - O from Figure 15 O - O

Δ - Δ from Figure 15 Δ - Δ

$$t_{\frac{1}{2}} = \frac{0.69}{i} \qquad (15)$$

Equations 14 and 15 are valid when used for comparison of relative first order initiation rate constants and half times for initiation involving preformed binary complexes of similar stability.

Table IX lists relative initiation rate constants, i, and half times for initiation, $t_{\frac{1}{2}}$, for GTP and ITP during primed and unprimed ribohomopolymer synthesis. Table X compares ratios of half times for initiation for ITP and GTP, for ITP as a function of Mg^{++} vs. Mn^{++} buffers and for GTP as a function of Mg^{++} vs. Mn^{++} buffers. It is evident from the data that GTP initiates more efficiently than ITP regardless of template, divalent cation or presence of a primer and that a primer has a significant effect in reducing the half time for initiation during ribohomopolymer synthesis in both Mn^{++} and Mg^{++}-containing buffers. This is unlike the case of $r(G\text{-}C)_n$ and $r(I\text{-}C)_n$ synthesis in which significant reduction of the half time for initiation as a result of the presence of the primer is more related to the presence of Mg^{++} as the divalent cation.

Further, if one considers the relative incorporation of purine ribonucleoside triphosphates into poly r(G) and poly r(I) (See legend to Figures 15 and 16) it is possible that ITP has a greater affinity for the elongation site than GTP during ribohomopolymer synthesis. However, since structures of both polymers are not identical and since early termination due to the rigidity of poly r(G) may be a factor, it is not possible to form a firm hypothesis at this time without further experimentation to determine the apparent K_m's for elongation which are not within the scope of this paper.

TABLE IX

Values for relative rate constants, i, and relative half times for initiation, $t_{1/2}$, from rifampicin challenge experiments as a function of template, purine ribonucleoside triphosphate and divalent cation

Template	i x 10^7 M				$t_{1/2}$ x 10^{-4} sec^2 M			
	ITP Mn^{++}	ITP Mg^{++}	GTP Mn^{++}	GTP Mg^{++}	ITP Mn^{++}	ITP Mg^{++}	GTP Mn^{++}	GTP Mg^{++}
$d(I\text{-}C)_n$	17	6	86	23	41	111	8	30
$d(I\text{-}\overline{BrC})_n$	53	10	230	63	13	69	3	11
$d(I\text{-}\overline{IC})_n$	49	22	173	86	14	32	4	8
$d(I)_n \cdot d(C)_n$	20	6	53	15	35	110	13	45
$d(C)_n$	9	5	14	9	74	137	49	77
$d(I\text{-}C)_n$*	25	77	138	138	28	9	5	5
$d(I\text{-}C)_n$**	22	22	115	49	31	32	6	14
$d(C)_n$***	17	13	31	31	41	55	22	22
$d(I)_n \cdot d(C)_n$***	77	77	230	69	9	9	3	10
$d(I\text{-}\overline{BrC})_n$*	53	57	230	115	13	12	3	6
$d(I\text{-}\overline{IC})_n$	69	77	230	86	10	9	3	8

LEGEND TO TABLE IX

Values for relative rate constants, i, and relative half times for initiation, $t_{1/2}$, from rifampicin challenge experiments as a function of template, purine ribonucleoside triphosphate and divalent cation

Relative rate constants for initiation were calculated from data similar to that in Figure 17 using equation 14. Relative half times for initiation were calculated using equation 15.

* GpC primer present during preincubation of the binary complex

** CpG primer present during preincubation of the binary complex

*** GpG primer present during preincubation of the binary complex

TABLE X

Ratios of half times for initiation for ITP and GTP with various templates as a function of divalent cation, for synthesis in Mg^{++} vs. Mn^{++} buffers for ITP, and for synthesis in Mg^{++} vs. Mn^{++} buffers for GTP

Template	Primer	$t_{½}$ ratio for ITP/GTP as function of buffer		$t_{½}$ ratio for Mg^{++}/Mn^{++} buffer as function of	
		Mn^{++}	Mg^{++}	ITP	GTP
$d(I\text{-}C)_n$	none	5.1	3.7	2.7	3.8
$d(I\text{-}\overline{BrC})_n$	none	4.3	6.3	5.3	3.7
$d(I\text{-}\overline{IC})_n$	none	3.5	4.0	2.3	2.0
$d(I)_n \cdot d(C)_n$	none	2.7	2.5	3.1	3.5
$d(C)_n$	none	1.5	1.8	1.9	1.6
$d(I\text{-}C)_n$	GpC	5.6	1.8	0.3	1.0
$d(I\text{-}C)_n$	CpG	5.2	2.3	1.0	2.3
$d(C)_n$	GpG	1.9	2.5	1.3	1.0
$d(I)_n \cdot d(C)_n$	GpG	3.0	0.9	1.0	3.3
$d(I\text{-}\overline{BrC})_n$	GpC	4.3	2.0	0.9	2.0
$d(I\text{-}\overline{IC})_n$	GpC	3.3	1.1	0.9	2.7

LEGEND TO TABLE X

Ratios of half times for initiation for ITP and GTP with various templates as a function of divalent cation, for synthesis in Mg^{++} vs. Mn^{++} buffers for ITP, and for synthesis in Mg^{++} vs. Mn^{++} buffers for GTP

Ratios of half times for initiation were calculated from the data in Table IX.

Comparison of K_m values for poly r(G) vs. poly r(I) synthesis during transcription of a poly $d(G)_n \cdot d(C)_n$ template indicates a similar pattern to that exhibited for transcription of $d(I)_n \cdot d(C)_n$ (Table VIa). However, the problem of scatter was great using this template making further experimentation impractical at this time. Should the problem of scatter be minimized (e.g. by use of a template of more homogeneous length), it is most likely that data obtained would parallel that found for the $d(I)_n \cdot d(C)_n$ template with a possible lower half time for initiation by GTP as indicated by the lower values for K_m for GTP on this template.

Role of template with respect to K_m and rifampicin resistance

It is apparent that K_m and the first order rate constant for initiation are dependent upon the nature of the template (single stranded vs. double stranded, alternating ribocopolymer vs. ribohomopolymer - cf. Tables VIa and IX) and the nature of the two ribonucleoside triphosphates involved in phosphodiester bond formation. (Compare K_m values for GTP and ITP for $r(G-C)_n$ vs. $r(G)_n$ and $r(I-C)_n$ vs. $r(I)_n$ synthesis respectively in Tables IV and VIa as well as relative half times for initiation for GTP and ITP in Table IX.)

What is the role of template stability in $r(I-C)_n$ and $r(G-C)_n$ synthesis? Heparin (8) has been shown to interfere with template binding of $(^3H)d(I-C)_n$ to A. vinelandii RNA polymerase, the dissociation of the enzyme-template complex being enhanced by the presence of a divalent cation in a manner analogous to the effect of poly glutamyl tyrosine copolymer on A. vinelandii RNA polymerase (106). The action of heparin is less rapid than that of rifampicin. Thus, enzyme -

template complexes can be preincubated with heparin (in the absence of divalent cations) and the relative binary complex stability measured on the basis of % resistance to heparin attack.

The reaction of the binary complex with heparin may be represented by:

$$\underset{\text{active}}{\text{E-DNA}} + \text{heparin} \longrightarrow \underset{\text{inactive}}{\text{E-heparin}} + \text{DNA} \qquad (16)$$

Since E-heparin is an inactive complex, it is assumed that the total active binary complex concentration may be determined from the % resistance to heparin attack determined by the synthesizing ability of the active E-DNA binary complex:

$$\%\ \text{heparin resistance} = \frac{\text{nmoles } (^3\text{H})\text{NMP incorporated in presence of heparin}}{\text{nmoles } (^3\text{H})\text{NMP incorporated in absence of heparin}} \times 100 \qquad (17)$$

RNA synthesis has thus been used as a probe for the % active binary complex remaining after two minutes preincubation with heparin, in the absence of divalent cations: synthesis being initiated by the simultaneous addition of nucleoside triphosphates plus the divalent cation. This probe is useful though not quantitative, since heparin, which can abolish 100% of E-$(^3\text{H})\text{d(I-C)}_n$ binding to nitrocellulose filters upon 2 minutes preincubation of a 6:1 ratio of heparin to enzyme-$(^3\text{H})\text{d(I-C)}_n$ binary complex in the absence of ribonucleoside triphosphates, reduces the synthesizing ability of the binary complex by only 33% when added simultaneously with nucleoside triphosphates plus divalent cation and incubated for 2.5 minutes.

The use of heparin as a probe for the measurement of binary

complex stability is subject to the following assumptions:

a. Heparin can dissociate a binary complex, but cannot dissociate a stable elongation ternary complex (8).

b. The rate of formation of the phosphodiester bond necessary for the formation of a stable elongation ternary complex is equal to or directly related to the rate of initiation. Since ITP initiates less rapidly than GTP, it would be expected, for example, that the use of $r(I\text{-}C)_n$ synthesis as a probe for heparin stability should reflect lower heparin resistance than when $r(G\text{-}C)_n$ synthesis is used as a probe, for the lower initiation rate by ITP would necessitate a longer period of time in which heparin could be in contact with the binary complex.

c. It has been shown that the A. vinelandii RNA polymerase holoenzyme-$d(A\text{-}T)_n$ binary complex is less stable to poly glutamyl tyrosine copolymer in the presence than in the absence of Mg^{++} as a divalent cation (106). Heparin, like poly glutamyl tyrosine, is a polyanionic inhibitor of E-DNA binary complex stability and is subject to interaction with divalent cations such as Mg^{++} and Mn^{++}. Since the E-DNA binary complex can be shown to be stable to heparin attack in the event of simultaneous addition of heparin, Mn^{++} or Mg^{++} plus ribonucleoside triphosphates under the same conditions in which it is unstable to heparin attack in the absence of divalent cations or ribonucleoside triphosphates, it will be assumed that the relative differences in heparin sulfate-Mn^{++} vs. heparin sulfate-Mg^{++} interactions are unimportant in interpretation of the heparin resistance data.

d. Heparin resistance is not a probe of dissociation of the

binary complex alone. Since heparin resistance is subject to initiation rate and divalent cation interactions, it will not be considered a quantitative tool, but rather a qualitative probe to be used in conjunction with rifampicin challenge data and K_m data.

Figure 18 indicates that the % heparin resistance can reflect binary complex stability (when RNA synthesis is used as a probe for stable binary complexes) when the heparin/*A. vinelandii* RNA polymerase ratio is 3/1. (A molecular weight of 1.5×10^4 was assumed for heparin.) Binary complexes should appear more stable when a rapidly initiating nucleoside triphosphate is used as a probe for heparin resistance than when more slowly initiating nucleoside triphosphates are used. Figure 18 shows that this is the case. The greater ability to initiate in Mn^{++}- containing buffers is also apparently reflected.

Table XI contains a comparison of the heparin resistance of several E-DNA binary complexes as a function of divalent cation and initiating nucleoside triphosphate (GTP or ITP). The data shows a wide variation in resistance to heparin dissociation. Nevertheless, the pattern remains in which there is greater heparin resistance when GTP rather than ITP is the initiating purine ribonucleoside triphosphate and when Mn^{++} rather than Mg^{++} is the divalent cation cofactor used.

Comparison of heparin resistance in Table XI with the relative half times for initiation by nucleoside triphosphates in Table IX indicates that there is no linear correlation between % heparin resistance and relative half times for initiation. However, increased rate of initiation is always reflected by greater resistance to heparin attack. Since it is assumed that heparin resistance is a measure of

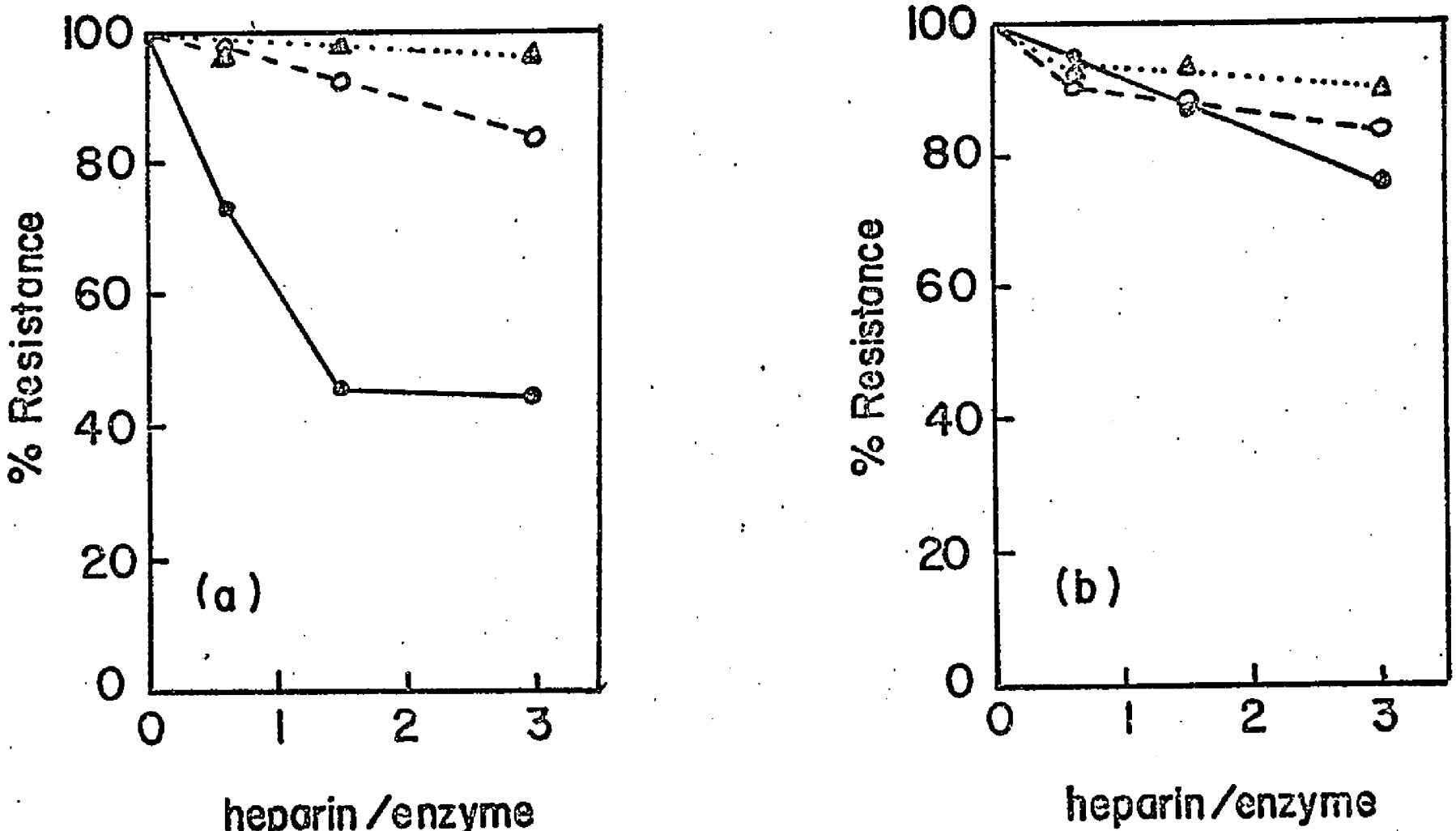
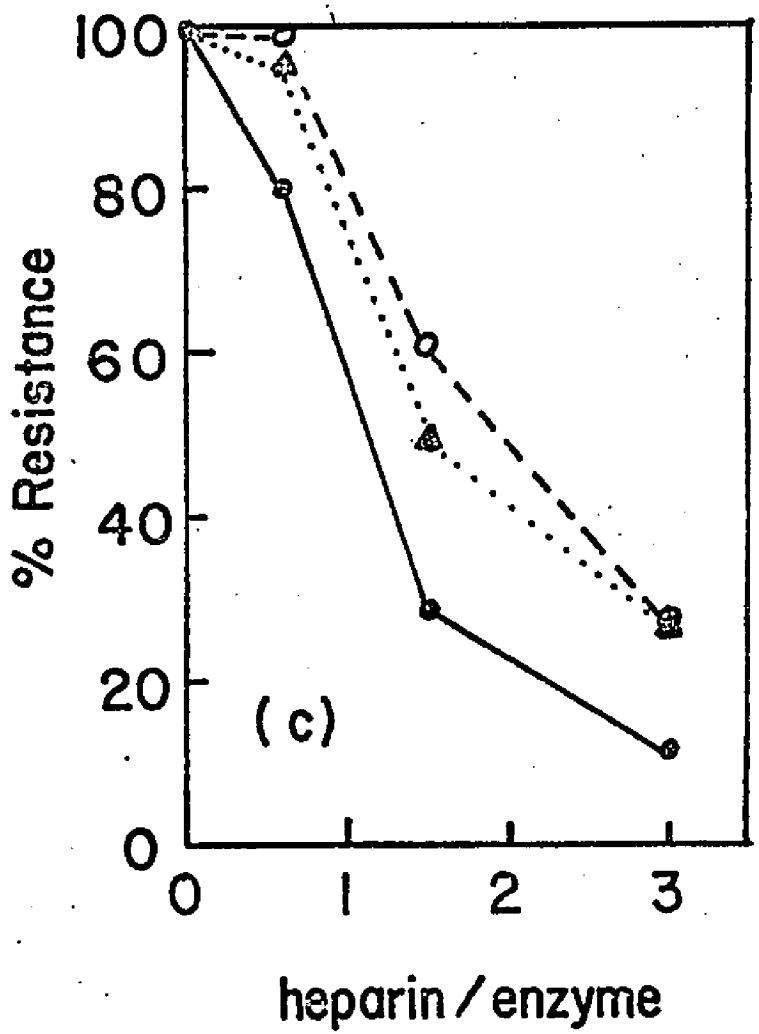


Figure 18



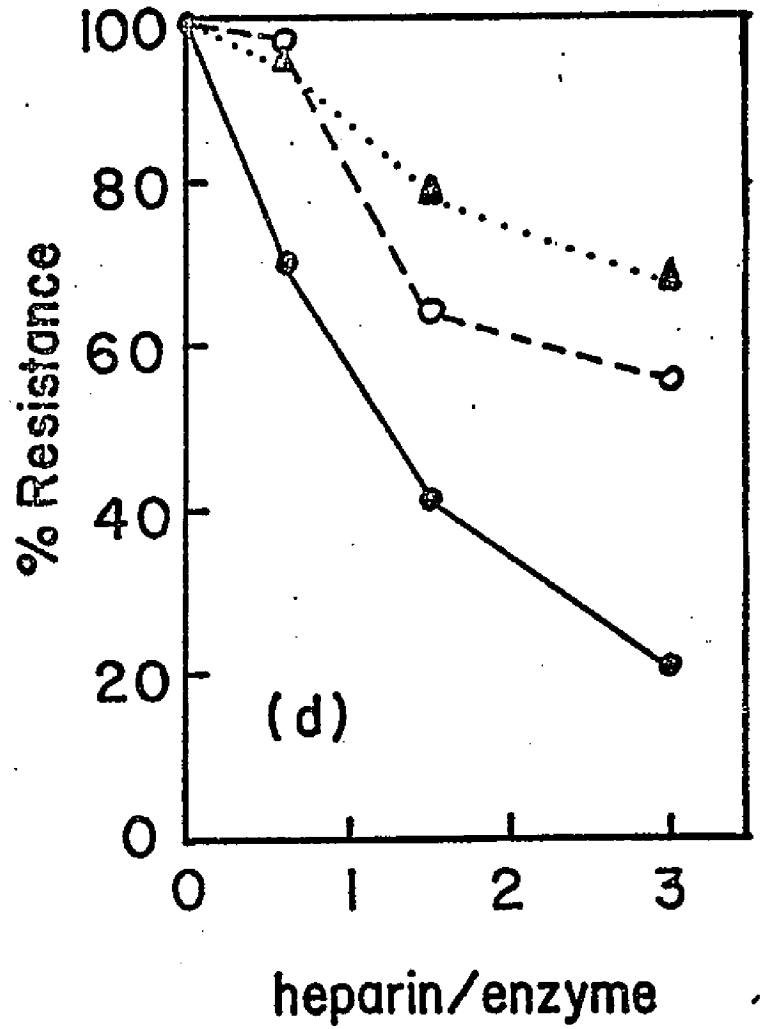


Figure 18

LEGEND TO FIGURE 18

Effect of heparin on dissociation of the E-d(I-C)$_n$, E-d(I-$\overline{BrC}$)$_n$ and E-d(I-$\overline{IC}$)$_n$ binary complexes

110 µl of a reaction mix containing 50 mM Tris pH 8.0, 50 mM ME, 10 nmoles d(I-C)$_n$, d(I-$\overline{BrC}$)$_n$ or d(I-$\overline{IC}$)$_n$ and 5 µg. RNA polymerase were preincubated for 10 minutes at 37° C; a 5 µl aliquot containing 0 to 0.5 µg. heparin (0 to 3 pmoles heparin/pmole enzyme) was preincubated for 2 minutes at 37° C with the preformed binary complex. Following preincubation, 15 µl of a reaction mix containing 50 nmoles (^{3}H)GTP (58.4 cpm/pmole) and 50 nmoles GTP or ITP and 0.5 µmoles $MgCl_2$ or 0.1 µmole $MnSO_4$ + 7.5 µmoles KCl was added to the preincubation mixture to initiate RNA synthesis. Reactions were terminated after 2.5 minutes at 37° C and acid precipitable radioactivity determined. Addition of 0 pmoles heparin represents 100% heparin resistance.

a. ▲..▲ d(I-$\overline{BrC}$)$_n$, r(I-C)$_n$, Mn^{++}
100% = 1070 pmoles (^{3}H)CMP incorporated

o--o d(I-$\overline{IC}$)$_n$, r(I-C)$_n$, Mn^{++}
100% = 1004 pmoles (^{3}H)CMP incorporated

●–● d(I-C)$_n$, r(I-C)$_n$, Mn^{++}
100% = 565 pmoles (^{3}H)CMP incorporated

b. ▲..▲ d(I-$\overline{BrC}$)$_n$, r(G-C), Mn^{++}
100% = 1342 pmoles (^{3}H)CMP incorporated

o--o d(I-$\overline{IC}$)$_n$, r(G-C)$_n$, Mn^{++}
100% = 1024 pmoles (^{3}H)CMP incorporated

LEGEND TO FIGURE 18 CONTINUED

● - ● $d(I\text{-}C)_n$, $r(G\text{-}C)_n$, Mn^{++}

100% = 737 pmoles (^{3}H)CMP incorporated

c. ▲ .. ▲ $d(I\text{-}\overline{BrC})_n$, $r(I\text{-}C)_n$, Mg^{++}

100% = 1067 pmoles (^{3}H)CMP incorporated

o -- o $d(I\text{-}\overline{IC})_n$, $r(I\text{-}C)_n$, Mg^{++}

100% = 963 pmoles (^{3}H)CMP incorporated

● — ● $d(I\text{-}C)_n$, $r(I\text{-}C)_n$, Mg^{++}

100% = 290 pmoles (^{3}H)CMP incorporated

d. ▲ .. ▲ $d(I\text{-}\overline{BrC})_n$, $r(G\text{-}C)_n$, Mg^{++}

100% = 802 pmoles (^{3}H)CMP incorporated

o -- o $d(I\text{-}\overline{IC})_n$, $r(G\text{-}C)_n$, Mg^{++}

100% = 716 pmoles (^{3}H)CMP incorporated

● - ● $d(I\text{-}C)_n$, $r(G\text{-}C)_n$, Mg^{++}

100% = 720 pmoles (^{3}H)CMP incorporated

TABLE XI

Heparin resistance of binary E-DNA complexes as a function of divalent cation and initiating nucleoside triphosphate

Binary Complex	ITP Initiation % Resistance		GTP Initiation % Resistance	
	Mg^{++}	Mn^{++}	Mg^{++}	Mn^{++}
E-d(I-C)$_n$	9.8	44.3	21.1	75.9
E-d(I-$\overline{BrC}$)$_n$	26.3	96.9	68.2	90.0
E-d(I-$\overline{IC}$)$_n$	27.3	83.8	55.7	84.6
E-d(I)$_n$•d(C)$_n$	40.8	50.9	41.6	79.8
E-d(C)$_n$	17.8	58.6	26.8	53.9

LEGEND TO TABLE XI

Heparin resistance of binary E-DNA complexes as a function of divalent cation and initiating nucleoside triphosphate

Reaction conditions as in Figure 18 except for the following: 10 nmoles $d(I\text{-}C)_n$, $d(I\text{-}\overline{BrC})_n$, $d(I\text{-}\overline{IC})_n$, $d(C)_n$ or $d(I)_n \cdot d(C)_n$ are preincubated with 0 to 3 pmoles heparin/pmole RNA polymerase at 37° C for 10 minutes. % heparin resistance is based on the ratio of synthesis when 3 pmoles heparin/pmole of enzyme or 0 pmoles heparin/pmole enzyme are preincubated with the preformed binary complex with incubation conditions as in Figure 18.

binary complex stability, the heparin resistance data implies that the more stable the binary complex formed the more rapid the rate of initiation. This hypothesis is supported by the fact that binary complexes between RNA polymerase and promotor regions of T_7 DNA are more stable than binary complexes between RNA polymerase and nonpromotor regions of T_7 DNA (10 - 14) and that enzyme bound to promotor regions of T_7 DNA supports more rapid initiation of RNA transcription than enzyme bound to non-promotor regions of T_7 DNA (13, 14).

Heparin resistance data thus indicate that formation of a binary complex which is stable enhances rapid rate of initiation. Correlation of this fact with the data of Table XII suggests that binary complex stability also is reflected in low K_m values. Since K_m is related to the relative affinities of the nucleoside triphosphates for the enzyme, it is apparent that there is a role of the template in the determination of the relative affinity of the initiation site for the nucleoside triphosphates GTP and ITP.

However, these data do not indicate whether the mechanism of transcription is processive or non-processive. The low heparin resistance and rifampicin resistance of the E-d$(C)_n$ binary complex as well as the associated high K_m values for both GTP and ITP gives some emphasis to the hypothesis that the mechanism for the transcription of single stranded templates may be nonprocessive due to the inability to form a nondissociable elongation ternary complex. However, all the evidence supports the hypothesis that transcription is processive in the case of double stranded templates (24, 53, 54).

The most stable binary complex is formed with the E-d$(I\text{-}\overline{BrC})_n$

TABLE XII

Comparison of apparent K_m values for GTP, ITP and CTP with respect to template and divalent cation

Template	Primer	Cation	$r(I\text{-}C)_n$ Synthesis				$r(G\text{-}C)_n$ Synthesis			
			K_I	K_C	V_I	V_C	K_G	K_C	V_G	V_C
$d(I\text{-}\overline{BrC})_n$	none	Mn^{++}	13	20	11	7	23	17	11	11
$d(I\text{-}\overline{BrC})_n$	none	Mg^{++}	33	83	10	8	33	25	6	6
$d(I\text{-}\overline{IC})_n$	none	Mn^{++}	100	33	12	33	25	13	8	10
$d(I\text{-}\overline{IC})_n$	none	Mg^{++}	25	50	11	6	50	29	6	6
$d(I\text{-}C)_n$	none	Mn^{++}	25	25	14	20	25	25	16	13
$d(I\text{-}C)_n$	none	Mg^{++}	200	200	22	33	200	50	13	19
$d(I\text{-}\overline{BrC})_n$	GpC	Mn^{++}	8	8	7	7	25	25	12	10
$d(I\text{-}\overline{BrC})_n$	GpC	Mg^{++}	25	33	9	10	20	20	7	7
$d(I\text{-}\overline{IC})_n$	GpC	Mn^{++}	14	13	8	7	20	11	10	7
$d(I\text{-}\overline{IC})_n$	GpC	Mg^{++}	17	40	10	8	25	8	5	6
$d(I\text{-}C)_n$	GpC	Mn^{++}	25	25	14	20	25	25	16	13
$d(I\text{-}C)_n$	GpC	Mg^{++}	50	50	27	27	50	50	19	13

LEGEND TO TABLE XII

Comparison of apparent K_m values for GTP, ITP and CTP with respect to template and divalent cation

K_m's and V_{max} values were determined from Lineweaver-Burk plots using conditions similar to those described in Figure 4.

Values for K_m are expressed in μM.

Values for V_{max} are expressed as pmoles/sec/5 μg. RNA polymerase

and E-d(I-$\overline{IC}$)$_n$ templates irrespective of initiating purine ribonucleoside triphosphate when Mn^{++} is the divalent cation cofactor during synthesis. There is great similarity in the heparin resistance of E-d(I-$\overline{BrC}$)$_n$ and E-d(I-$\overline{IC}$)$_n$ with respect to each other but not with respect to E-d(I-C)$_n$ indicating significantly greater stability of the binary complexes formed in the case of the analog templates.

In general, the values of K_m are lower when the binary complex is formed with the analog templates than with the d(I-C)$_n$ template (Table XII). The presence of a dinucleoside monophosphate primer during formation of the binary complex, leading to a ternary complex, E^{DNA}_{GpC}, causes a further lowering of the K_m in all cases. Secondly, the relative half times for initiation determined using the analog templates are significantly lower (Table IX) than those for d(I-C)$_n$. The effect of the GpC primer is less significant in the case of the analog templates than for d(I-C)$_n$ for both r(I-C)$_n$ and r(G-C)$_n$ synthesis. This is a further indication of rapid initiation resulting from the formation of a stable binary complex.

There is little effect of the primer on the maximum velocity of r(G-C)$_n$ or r(I-C)$_n$ synthesis regardless of template. It can also be noted, that for all templates in a primed system, the value for K_G, K_I and K_C when Mg^{++} is the divalent cation varies between 10 and 50 μM which approximates the value for the binding constant of GTP, CTP and ITP to the elongation site of E. coli RNA polymerase (35). This suggests that the values of K_m determined in the primed systems reflect the affinities of GTP, ITP and CTP for the elongation site of A. vinelandii RNA polymerase.

Thus, although there is a marked difference in affinity of GTP, ITP and CTP for the initiation site, GTP $>$ ITP $\gg$ CTP, it is apparent that the affinity of all three nucleoside triphosphates for the elongation site is approximately equal.

DISCUSSION

From the data presented in this paper it is clear that GTP has a greater affinity for the initiation site of Azotobacter vinelandii RNA polymerase core enzyme than ITP. This was the conclusion drawn for the A. vinelandii holoenzyme using pyrophosphate exchange experiments (37). However, pyrophosphate exchange cannot adequately probe initiation site affinity differences for the core enzyme (95). Using data from apparent K_m determinations and rifampicin challenge experiments (47), it is evident that the relative affinity for the initiation site may be described as: GTP $\rangle$ ITP $\gg$ CTP. This relative affinity relationship exists when the template is single stranded or double stranded and when the RNA synthesized is a ribohomopolymer or alternating ribocopolymer (Tables IV, VIa, IX, XII). It is also evident that Mn^{++} affects initiation differently than does Mg^{++} (Table IX, X). It is also evident that binary complex stability affects the rate of transcription, but not the relative affinities of the nucleoside triphosphates for the enzyme.

The synthesis of $r(G\text{-}C)_n$, $r(I\text{-}C)_n$, $r(G)_n$ and $r(I)_n$ proceeds linearly for the first 10 minutes after which a plateau region is evident (Figure 1a, b) presumably due to product inhibition (61). As previously reported by Fuchs et al. (67) for transcription of T_4 DNA and by Mangel and Chamberlin (73) for transcription of T_7DNA, chain elongation also appears to be optimum for both $r(I\text{-}C)_n$ and $r(G\text{-}C)_n$ synthesis using a $d(I\text{-}C)_n$ template between ionic strength 0.2 to 0.3 regardless of whether Mg^{++} or Mn^{++} is the divalent cation required for elongation (Figure 3c). The optimum for chain initiation is at a

somewhat lower ionic strength (73). This is indicated by the fact that increasing the ionic strength from 0.08 to 0.33 stimulates chain elongation (Figure 3d) but causes curvature in Lineweaver-Burk plots (Figure 6a, b). That this curvature is not due to product inhibition (61) is indicated since reactions were terminated in the linear portion of the reaction (Figure 3d). Secondly, the degree of synthesis at low nucleoside triphosphate concentrations is significantly lower at high ionic strength than at an ionic strength of 0.1. Figures 3a and 3d demonstrate that less effective binary complex formation at high ionic strength results in less effective ability to initiate transcription.

There is evidence for a conformational change occurring when the enzyme binds DNA (107, 108). Measurements of helix-coil transition enthalpy and T_m's indicate that the stability of DNA increases with increasing ionic strength (109); this effect is related to the G + C content of the DNA as well as to the presence of divalent cations (79, 80). RNA polymerase binding is proposed to open about 6 to 8 base pairs of DNA (10). Agents such as glycerol, ethylene glycol, dimethylsulfoxide, sucrose and 1,3 propanediol which lower the T_m of $\lambda_{\underline{gal}}$ DNA also activate transcription of total RNA and gal RNA (111). Thus, the instability of the E-d$(I-C)_n$ binary complex at an ionic strength of 0.3 may be related not only to electrostatic interactions with salt which would affect binding, but also the inability of the enzyme to melt the base pairs of the template for the formation of a stable binary complex.

The stability of the binary complex is an important factor in

determining the rate of initiation. Although there is no obvious linear correlation between % resistance of the binary complex to dissociation by heparin (Table XI), the relative half times for initiation (Table IX) and the K_m values for GTP, CTP and ITP (Tables VIa, XII), it is evident that when resistance to heparin is maximum, there is a lower K_m and a shorter half time for initiation. The data indicate that the most stable binary complexes, those formed in the presence of Mn^{++}, reflect the more rapid rate of formation of the first phosphodiester bond. Further, single stranded DNA's form less stable binary complexes than double stranded DNA's (24). Table IX shows that this lack of stability leads to significantly longer half times for initiation. This increase in half time for initiation may be related to a non-processive mechanism for transcription of single stranded templates due to formation of an easily dissociable ternary complex with the newly synthesized RNA. In this case, a single initiation event per RNA chain would not be measured, but rather a complex series of events. This is not the case for double stranded templates in which the mechanism is apparently processive (Table IX, XI, XII).

The difference in the response to Mn^{++} and Mg^{++} is exhibited by stimulation of the rate of synthesis of $r(I\text{-}C)_n$ and $r(G\text{-}C)_n$ (Figure 1) and the lowering of the apparent K_m values for GTP, ITP and CTP (Table VIa) regardless of whether the template is single stranded or double stranded in unprimed reactions. The effect of Mn^{++} in reducing the half time for initiation becomes less significant, however, in primed reactions, regardless of whether the template is single stranded or double stranded. This data confirms the results of Krakow (81) in a

study of the subunit interaction of the RNA polymerase core enzyme which implied that one effect of Mn^{++} is on the enzyme itself.

A divalent cation is not necessary for the formation of a binary E-DNA complex (Figure 2) and Mg^{++} does not stabilize the binary complex to dissociation by ionic strengths of 0.4 (Figure 2). Resistance to dissociation by salt and to attack by heparin are indicative of more stable binary complexes (24, 67, 71). The stimulatory effect of Mn^{++}, existent even for the less stable binary complexes of E-d$(I-C)_n$, suggests that the effect of Mn^{++} on binary complex stability is related to a conformational change on the enzyme enabling the enzyme to form a tighter complex with the template.

That this is not the only role of Mn^{++} is evidenced by the fact that K_m values in the presence of Mn^{++} are not so significantly lowered by the presence of a primer as those of Mg^{++} (Table XII). Since low K_m values reflect more rapid initiation, and since more rapid initiation suggests more rapid phosphodiester bond formation, one important role of the divalent cation may be in providing the steric conformation necessary for the cleavage of the P_α - O bond necessary for the release of pyrophosphate.

Mg^{++} has been shown to bind to the β phosphate of GTP and CTP (112). It is known that Mn^{++} binds not only to the β and γ phosphate groups but also to the N-7 of purine ribonucleoside triphosphates (77, 78, 113), a binding which may provide strain on the P_α - O bond. The Mg^{++}- NTP or Mn^{++}- NTP conformation before binding to the enzyme is not, however, of the greatest significance. Rather, the conformation of the RNA polymerase - Mg^{++}- NTP and RNA polymerase - Mn^{++}-NTP ternary

complex apparently plays a crucial role. Evidence for this hypothesis is that Mn^{++} has been shown to confer an unusual puckered conformation on the DNA polymerase I • Mn^{++} • dTTP ternary complex (114) which may enhance the ease of nucleophilic attack on the P_{α} - O bond necessary for the release of pyrophosphate. This may also be the case for the RNA polymerase - Mn^{++}- GTP ternary complex or the RNA polymerase - Mn^{++}- ITP ternary complex.

More rapid initiation in the case of Mn^{++} vs. Mg^{++} buffers is perhaps attributed to the difference not only in the effect of Mn^{++} vs. Mg^{++} on the template but also on the conformation of the nucleoside triphosphates prior to formation of the phosphodiester bond.

Pyrophosphate release is also necessary for elongation. Since elongation does not occur in the absence of a divalent cation, it is apparent that provision of the appropriate conformation for P_{α} - O cleavage which preceeds PP_i release is an additional role for Mg^{++} and Mn^{++}.

While Mg^{++} and Mn^{++} are essential for initiation of transcription and for elongation, they do not have a direct effect on the relative affinities of GTP, ITP and CTP for the initiation and elongation sites of A. vinelandii RNA polymerase. The data in this study shows a 4 to 6 times greater ability of the core enzyme to initiate with GTP than with ITP in the absence of a primer, regardless of divalent cation in alternating ribocopolymer synthesis, and a 2 to 3 times greater enhancement of GTP over ITP for ribohomopolymer synthesis (Table X).

The maximum velocity of poly r(I) synthesis is greater than that for poly r(G) synthesis under the same conditions (Table VIb) indica-

ting that the elongation site does not exhibit the differential preference regarding the nucleoside triphosphates as does the initiation site, again confirming the implications of previous studies (34 - 36, 48, 57, 58, 75, 76).

The presence of a dinucleoside monophosphate primer inhibits binding of the purine ribonucleoside triphosphate to the initiation site (48 - 50) and bypasses the binding of the initiating purine ribonucleoside triphosphate by formation of the 5' end of the RNA chain to be synthesized (51). The primers GpC, CpG and GpG have been shown to increase rifampicin resistance, especially when ITP rather than GTP is present in the system (Figures 11, 12, Table XI). This is a further support for the hypothesis that the initiation site exhibits greater selectivity with respect to the 5' terminus of the nascent RNA chain than does the elongation site for the nucleoside triphosphate proximal to the 3' OH terminus - i.e., in the presence of a primer, the major role of the core enzyme is elongation.

Although the primers increase rifampicin resistance as determined by rifampicin challenge experiments, they do not cause the half time for initiation to be reduced to zero as would be the case if the primer converted the binary complex to a 100% effective rapid starting initiation complex. Table III implies that the conformational change on the enzyme upon binding of the primer is not so significant unless GTP or ITP is bound. CTP does not confer the proper conformation on the enzyme for a stable ternary complex. These data support the hypothesis of Johnston and McClure (104) that a rifampicin resistant ternary elongation complex exists only following formation of the

second phosphodiester bond. However, it is also evident from these data that the 5' - 3' sequence of a primer can determine its degree of effectiveness in conferring rifampicin resistance (Figure 11) and lowering of K_m (Table IV) which are the indications of the formation of a stable ternary elongation complex.

The synthesis of $r(G-C)_n$ or $r(I-C)_n$ confers greater resistance of the $E-d(I-C)_n$ binary complex to salt dissociation. Since it is known that during chain elongation, approximately 10 to 12 residues of the nascent RNA chain proximal to the 3' OH terminus are protected from nuclease digestion (56), it is possible that primers of lengths approximating 10 to 12 nucleotides would cause the formation of 100% stable ternary complexes. It is known that longer polynucleotides are more efficient for priming RNA synthesis than dinucleoside monophosphates (115). Further, short T_7 RNA segments synthesized as preinitiated complexes (stable ternary complexes) were 100% stable to rifampicin (47). It is possible that binding of a dinucleoside monophosphate primer cannot truly simulate a 100% effective rapid starting initiation complex with the core enzyme in the absence of synthesis, but that once elongation proceeds beyond the formation of a dinucleoside tetraphosphate, the ternary complex may be rendered totally rifampicin resistant (104).

Thus, a second conformational change may be required of the enzyme for elongation to occur after the formation of the initiating dinucleoside tetraphosphate - a change which will not occur if the product terminus site is destabilized by the presence of rifampicin (116), if the dinucleoside tetraphosphate is released before the formation of the next phosphodiester bond, or if trans-

location is hindered.

The half time for initiation for GTP determined by rifampicin challenge experiments is less than that for ITP (Table IX) regardless of template, the half time for initiation being 0.6 sec. in Mg^{++}-containing buffers when $d(I-C)_n$ is the template. This is three times longer than the half time for initiation predicted for the holoenzyme (47, 116) irrespective of template. It is evident that Mn^{++} simulates the conformational change on the enzyme conferred by the presence of the sigma subunit, for in this system, the half time for initiation by GTP is identical to that determined for the holoenzyme (47, 116).

While ITP meets the structural requirements (117) for the availability of 2' and 3' OH groups of the ribose moiety and the anti rotomer conformation, it is not a suitable initiating nucleoside triphosphate (Table IV, VI, IX, XII) when compared with GTP (118) regardless of the divalent cation present. That the inefficiency of ITP is most directly a function of binding to the enzyme is evident from Tables III and XI.

A second difference existent between GTP and ITP is that GTP has three hydrogen bonds per base pair whereas ITP has only two hydrogen bonds per base pair (119). Substrate specificity is template determined (Table VII, VIII), but the fact that ATP initiates RNA synthesis with holoenzyme with the same half time for initiation as GTP in a Mn^{++} buffer for core enzyme (47, 116) suggests that hydrogen bonding differences are not likely a major factor here. Further, it has been shown for several purine nucleoside triphosphate analogs that if the purine residue is altered in the ring in such a way that

base pairing interactions are not affected, chain initiation may, nevertheless be greatly reduced (120 - 121). Examples of chain initiation inhibiting nucleoside triphosphate analogs with altered ring residues but normal Watson - Crick base pairing are tubercidin triphosphate (120, 121), 6 azaguanine triphosphate (75) and deazanebularin triphosphate (76). However, alterations of the substituents on the ring without altering the ring residues, as in the case of 7 methyl guanine triphosphate (124), 6 methyl amino purine triphosphate (125), and formycin triphosphate (120) also impair initiation. Thus, it is evident that the initiation site exhibits a rigid selectivity for ATP and GTP, not determined by hydrogen bonding characteristics alone but also related to the unique position of substituents on the purine ring residues of ATP and GTP.

The similarity of behavior of GTP and ITP in elongation is evident from this study. From studies of inhibition of continued RNA chain elongation with nucleoside triphosphates in the syn rotomer conformation, such as 8 bromo ATP and 8 amino purine (120), it is postulated that the anti rotomer conformation is a requirement either for translocation to or function of the product terminus site.

On the other hand, initiation inhibiting analogs in the anti rotomer conformation such as tubercidin triphosphate and formycin triphosphate (120) do not significantly inhibit chain elongation. This is further confirmed by the fact that the elongation site also accepts complementary pyrimidine nucleoside triphosphates in the anti conformation. It is thus apparent that the selectivity of the elongation site for specific ribonucleoside triphosphates is not so rigid as that of

the initiation site.

The role of the template in determining the affinity of binding to the initiation and/or elongation site remains unclear. Nevertheless, that the differences in affinity for the initiation site of core enzyme are determined primarily by the enzyme itself is implied by the above data.

The major effect of a stable binary complex in RNA synthesis may be demonstrated by a comparison of K_m values for GTP and ITP in unprimed reactions using the $d(I\text{-}C)_n$ vs. $d(I\text{-}\overline{BrC})_n$ and $d(I\text{-}\overline{IC})_n$ templates. In the case of the analog templates used in this study, it is known that substitution of halogens on the C-5 of pyrimidines affects base stacking interactions of nucleic acids with resultant increase in the thermal stabilities of the DNA (125). These interactions can cause changes in the affinity of DNA binding proteins (89). It is shown in Tables IX and XII as well as in Figures 18a, b, c and d that the analog templates can form more stable E-DNA binary complexes than $d(I\text{-}C)_n$. This is most evident in the increased heparin resistance of the E-$d(I\text{-}\overline{BrC})_n$ and E-$d(I\text{-}\overline{IC})_n$ binary complexes compared with that of E-$d(I\text{-}C)_n$ binary complex. However, it is not clear what relationship exists between base stacking interactions and helix unwinding from this study. It is evident that greater thermal stability of DNA does not of necessity imply lower ability of the enzyme to unwind that DNA for the formation of a stable binary complex.

Stable binary complex formation is essential for initiation, i.e. formation of the first phosphodiester bond. Binary complex stability is reflected in heparin resistance (Table XI), low values of K_m

(Table XII) and rapid initiation rates.

However, binary complex stability does not determine the relative affinity of GTP, ITP and CTP for the initiation and elongation sites of A. vinelandii RNA polymerase. It is apparent from this study that the affinity differences of GTP, ITP and CTP with respect to the initiation site, GTP ITP CTP are a property of the enzyme itself as are the affinity relationships for the elongation site, GTP $\not\approx$ ITP $\not\approx$ CTP. The role of the template is primarily in directing a complementary nucleoside triphosphate to the initiation site and to the elongation site. The stability of the enzyme-template complex formed will then determine the rate of initiation and elongation. This initiation and elongation rate are also dependent upon the presence of a divalent, Mg^{++} or Mn^{++} which acts as a cofactor for the binding of complementary purine ribonucleoside triphosphates to the initiation site, for binding of complementary ribonucleoside triphosphates to the elongation site and for provision of the appropriate steric conformation of ribonucleoside triphosphates necessary for cleavage of the phosphodiester bond.

SUMMARY

Differences in affinity of GTP and ITP for the initiation and elongation sites of A. vinelandii RNA polymerase core enzyme have been studied. Apparent K_m measurements made in Mg^{++}- containing buffers indicate that the apparent K_C for $r(G-C)_n$ synthesis is 50 μM when no primer is present, but 200 μM for $r(I-C)_n$ synthesis under identical conditions. Further, $K_G = K_I = 200$ μM. These data suggest that the lower apparent K_C during $r(G-C)_n$ synthesis reflects a greater affinity of GTP for the initiation site than that of ITP. This hypothesis is confirmed by the fact that the presence of a primer such as GpC which inhibits purine ribonucleoside triphosphate binding to the initiation site causes $K_G = K_I = K_C = 50$ μM in Mg^{++}- containing buffers. These data suggest that GTP, ITP and CTP have similar affinities for the elongation site as has been implied by earlier studies (34, 36, 48, 57, 58, 75, 76).

The greater affinity of GTP than ITP for the initiation site is also indicated by the fact that the presence of GTP renders the binary E-$d(I-C)_n$ complex more resistant to dissociation by salt solutions of ionic strength 0.4 in Mg^{++}- containing buffers (Table III) and suggests that the binding of GTP induces a conformational change on the enzyme in the binary complex conferring greater stability for the newly formed ternary complex than that conferred by ITP binding. Similar stabilizing effects of GTP binding are also noted in Mg^{++}- containing buffers when the binary complex is allowed to undergo rifampicin challenge (47) or heparin inhibition (8, 24, 26, 27) studies. Since rifampicin is known to inhibit RNA polymerase near the initiation

site (39) and heparin is believed to dissociate binary E-DNA complexes prior to initiation (8, 24, 26, 27), these studies suggest strongly that:

1. GTP has a greater affinity for the initiation site than ITP.

2. The half time for initiation with GTP is lower than that for ITP (Table V, Table X).

3. The greater affinity of GTP vs. ITP for RNA polymerase is a property of the core enzyme - i.e. affinity is not conferred by the presence of the sigma subunit.

The affinity of both GTP and ITP for the initiation site may be affected by the appropriate choice of divalent cation cofactor. In nearly all cases, apparent K_m values in Mn^{++}- containing buffers are lower (Table IV, Table XII) than those in Mg^{++}- containing buffers, resistance to dissociation by high salt is higher (Table III), and resistance to inhibition by rifampicin (Figures 10, 12, 15, 16) or heparin (Figure 18, Table XI) is greater. A possible implication of these data is that Mn^{++} may confer a conformational change on the enzyme similar to that conferred by the sigma subunit leading to enhancement of the initiation rate. That Mn^{++} enhances initiation rate is suggested since the apparent K_G, K_I and K_C are equal to 25 μM for both $r(G\text{-}C)_n$ and $r(I\text{-}C)_n$ synthesis regardless of whether a primer is present.

The enhanced rate of initiation of GTP compared with ITP is not an exclusive property of alternating ribocopolymer synthesis. Although apparent K_m values are neither the same nor predictable for different templates, it is apparent that the affinity of GTP for the initiation

site is greater than that of ITP regardless of whether the template is an alternating deoxyribocopolymer, a single stranded deoxyribohomopolymer, or double stranded deoxyribohomopolymer (Table XII). Thus, the role of the template in transcription is in binding to the polymerase for the purpose of directing a complementary ribonucleoside triphosphate to the initiation site and to the elongation sites.

In conclusion, it may be said that:

1. With respect to the initiation site, the affinities of the three ribonucleoside triphosphates studied are: GTP $\rangle$ ITP $\gg$ CTP regardless of template used.

2. With respect to the elongation site, the affinities of the three ribonucleoside triphosphates studied are: GTP $\eqsim$ ITP $\eqsim$ CTP.

3. With respect to ability to stimulate the rate of initiation: Mn^{++} - containing buffers Mg^{++} - containing buffers.

4. The above properties are not dependent upon the presence of the sigma subunit, but are a property of the core enzyme itself.

APPENDIX A

Preparation of Azotobacter vinelandii RNA polymerase

250 g. of frozen A. vinelandii cells were suspended in 500 ml 0.01 M Tris pH 8.0 and the pH adjusted to 8.0 with Tris base. The cell suspension was allowed to come to room temperature and 12 ml of 0.5 M EDTA pH 7.6 and 10 ml of 1% lysozyme in 0.01 M Tris pH 8.0 were added with constant stirring. 1.0 ml of 1 M ME was added to the lysate which was then chilled to 10° C. To the chilled lysate was added 25 ml of 5% Brij, 25 ml of 1 M $MgCl_2$ and 25 ml of 1 M Tris pH 7.8 with stirring for 15 to 20 minutes at 10° C.

The mixture was treated with 80 ml of 5% polymin P, pH 7.8 added at a rate of 2.5 ml/min. After stirring for 5 minutes with a non-aerating stirrer, the polymin treated lysate was centrifuged in the cold at 4° C at 12,000 x g for 20 minutes. The pellet was suspended in 800 ml of 0.05 M Tris pH 7.8, 0.01 M $MgCl_2$, 0.3 M NH_4Cl and 1 mM ME. The suspension was stirred for 20 minutes with a nonaerating stirrer and centrifuged at 12,000 x g for 20 minutes. The resulting pellet was suspended in 800 ml of 0.05 M Tris pH 7.8, 0.01 M $MgCl_2$, 0.45 M $(NH_4)_2SO_4$ and 1 mM ME. The suspension was stirred for 20 minutes with a nonaerating stirrer and centrifuged at 12,000 x g for 20 minutes. Ammonium sulfate was added to the supernatant to 30% saturation and the resultant suspension centrifuged for 20 minutes at 12,000 x g. The 30% ammonium sulfate supernatant was brought to 57% ammonium sulfate saturation and spun at 12,000 x g for 20 minutes. The resultant pellet was suspended in 400 ml of 0.02 M Tris pH 8.0, 0.01 M ME and 5% glycerol. The suspension was stirred with a nonaerating

stirrer for 20 minutes and centrifuged at 29,000 x g for 90 minutes at 4° C. The supernatant was diluted with 300 ml cold 10% glycerol in 0.01 M ME and loaded onto a DEAE cellulose column previously equilibrated with 0.04 M potassium phosphate pH 6.8, 10% glycerol and 0.01 M ME. The column was eluted with a linear gradient: 500 ml of 0.04 M potassium phosphate pH 6.5, 0.01 M ME and 10% glycerol to 500 ml of 0.04 M potassium phosphate pH 8.3, 0.01 M ME, 10% glycerol and 0.67 M KCl. Fractions were assayed for RNA polymerase activity and pooled fractions were brought to 60% saturation with ammonium sulfate and centrifuged at 12,000 x g for 20 minutes.

The ammonium sulfate pellet was dissolved in 10 to 20 ml of 0.02 M Tris pH 7.6, 0.01 M ME and 50% glycerol. The enzyme mixture was dialyzed against 0.01 M Tris pH 7.9, 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol and 1 M KCl, and chromatographed on an 8% agarose (Bio Gel A - 1.5 m) column equilibrated with the same buffer. Fractions were assayed for enzyme activity and active fractions with $A_{280/260} > 1.4$ were pooled, diluted 1/3 with the above buffer minus KCl, and brought to 60% ammonium sulfate saturation. The suspension was centrifuged at 12,000 x g for 20 minutes and diluted to 200 ml in 0.05 M Tris pH 8.0, 0.1 mM EDTA, 2 mM ME and 10% glycerol, and loaded on a phosphocellulose column equilibrated with 0.05 M Tris pH 8.0, 0.015 M KCl, 0.1 mM EDTA, 2 mM ME and 10% glycerol. The column was washed with the equilibration buffer and eluted with a linear gradient: 150 ml of 0.05 M Tris pH 8.0, 0.015 M KCl, 0.1 mM EDTA, 2 mM ME and 10% glycerol to 150 ml of 0.05 M Tris pH 8.0, 0.5 M KCl, 0.1 mM EDTA, 2 mM ME and 10% glycerol. Fractions were assayed

for RNA polymerase activity. Two pools of enzyme activity, holoenzyme and core enzyme, were isolated from the phosphocellulose column. After precipitating with 60% ammonium sulfate, these fractions were dissolved in 0.02 M potassium phosphate pH 6.8, containing 1 mM EDTA and 50% glycerol and stored at -15° C. Fractions were assayed for the presence or absence of sigma by SDS polyacrylamide gel electrophoresis in Tris-glycine buffer (101).

Assay of RNA polymerase activity

RNA synthesis was followed by determining the incorporation of (^{3}H)UTP into acid insoluble material retained on glass fiber filters. The assay mixture contained: 20 µl of 0.67 M Tris pH 7.6, 10 µl of 0.5 M $MgCl_2$, 10 µl of 0.01 M (^{3}H)UTP (2000 cpm/nmole), 10 µl of 0.01 M ATP, 10 µl of 1 M mercaptoethylamine, 20 µl of $d(A\text{-}T)_n$ (O.D. 3.0), 20 µl enzyme fraction and 150 µl water. The mixture was incubated for 10 minutes at 37° C and acid precipitable radioactivity determined.

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