KINETIC STUDIES OF DNA-DEPENDENT RNA POLYMERASE FROM E. COLI

by

PETER JOHN ROACH, B.Sc.

Presented for the degree of Doctor of Philosophy

Institute of Biochemistry

University of Glasgow

July 1972

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

TO ANNE

LIST OF CONTENTS

ACKNOWLEDGEMENTS

I should like to express my thanks to all who have helped me in my postgraduate work, and in particular

Professor J.N. Davidson and Professor R.M.S. Smellie for their support and for the provision of facilities for this work

Dr. A.F.H. Anderson with whom I have had an active and stimulating association during the past three years

Mr. E.W. Robertson with whom I collaborated for some more recent experiments and Mr. J.J. Greene for technical assistance on various occasions

Dr. Fiona Williams for help in setting up a computer program to run on the ERCC IBM 360/50

Mrs. Eileen Kemp for typing some of this thesis and Mr. A. Gibb for photographic work.

Anne for her encouragement and help.

The work described here was carried out while I was in receipt of a Science Research Council studentship.

LIST OF FIGURES

ABBREVIATIONS AND CONVENTIONS

The abbreviations, conventions and symbols used follow the recommendations of the Biochemical Journal (Biochem. J. (1972) 126, 1-19). Additional abbreviations are :

BSA	bovine serum albumin
FI	fluorescent intensity
NTP	unspecified nucleoside triphosphate, or in some instances, an equimolar mixture of ATP, CTP, GTP and UTP.
PuTP	purine nucleoside triphosphate
PyTP	pyrimidine nucleoside triphosphate
SDS	sodium dodecyl sulphate

SUMMARY

DNA-dependent RNA polymerase was isolated in a highly purified form from *E. coli* MRE 600 by the method previously established in this laboratory. The polypeptide composition of enzyme samples was routinely analysed by SDS-polyacrylamide gel electrophoresis. This technique gave not only an analysis of the purity of RNA polymerase samples but could also yield, in some instances, estimates for mol.wt.'s of polypeptides present.

RNA polymerase prepared in this laboratory had always contained a polypeptide, θ , in relatively high concentration. The separation of θ from RNA polymerase, by glycerol gradient centrifugation, is reported here. It was found also that θ existed as an aggregate of the 58 000 mol.wt. monomer observed by SDS-polyacrylamide gel electrophoresis ,during glycerol gradient centrifugation at high ionic strength. The properties of θ resemble those of a protein with ATPase activity reported very recently by Paetkau & Coy (1972). By use of the SDS-polyacrylamide gel electrophoresis technique, the precise polypeptide composition of RNA polymerase preparations was found, in general, to be dependent on the purification procedure used.

A fluorimetric assay for DNA directed RNA polymerase activity was developed. This method is based on the fluorescence properties of ethidium bromide-nucleic acid complexes and had been described originally for measuring umprimed RNA polymerase activity. A variation of the fluorimetric assay was employed to measure polynucleotide phosphorylase activity, which was present in some RNA polymerase samples.

The dependence of RNA polymerase activity on ionic conditions was investigated, in order to establish conditions for subsequent kinetic experiments. The time course of RNA synthesis by RNA polymerase was studied and, in particular, the RNA product was considered as an inhibitor of the reaction. A complex inhibition pattern was observed, which did,however, conform approximately to non-competitive inhibition at high substrate concentration.

Kinetic experiments were carried out using T7 DNA and calf thymus DNA templates. The kinetic behaviour of RNA polymerase was dependent on various factors : the nature of the template, the presence of the initiation factor, σ , and whether purine or pyrimidine nucleotides were varied. In order to use a better defined enzyme-template system, the kinetic properties of RNA polymerase core enzyme (apparently the simplest active form of the enzyme) with a poly(dA-dT) template were investigated. With this system, the steady state rate equation for a two substrate ping pong mechanism, with substrate inhibition by ATP, was found to be applicable over a certain range of substrate concentrations. At higher substrate concentrations, deviations from this rate law were observed. Model rate equations, of the general form of steady state rate laws, were analysed, by the least squares method, for their ability to fit the experimental data. The form of the equation that best described the experimental results could be explained in several ways ; one of these was the existence of alternative reaction pathways. This eventuality was investigated further by analysing the poly(A-U) product in various ways : nearest neighbour analysis, determination of the 5'-terminal, and the adjacent, base residues, and measurement of the gross base stoichiometry.

The results of these experiments provided some evidence for the operation of alternative reaction pathways at the stage of the initiation of poly(A-U) synthesis.

SECTION 1

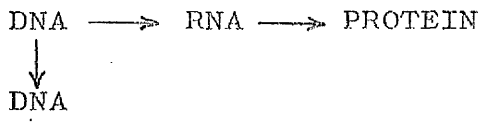
INTRODUCTION

Life, although eluding rigorous definition, is unquestionably a dynamic, non-equilibrium process. Schrodinger stated this tersely when he said that 'living matter evades the decay to equilibrium' (Schrodinger, 1944). The coordinated processes taking place within an organism are continually engaged in protecting that organism from the decline to equilibrium, which is death and disorder. Thus, there must be an adequate supply of free energy, as food, which can be utilised by the organism in question to overcome the continual production of entropy.

The organism is, then, an open system and the effective utilisation of food inputs, both as sources of materials and energy, requires the integrated performance of many chemical reactions. These reactions are catalyzed by enzymes which are, to quote Pauling, 'the molecules that we must understand to know how organisms carry on their metabolic activities' (Foreword to Webb, 1963). Enzymes not only act as catalysts, but also provide means by which the rates of chemical reactions may be controlled.

Another feature of living systems is that they perpetuate their kind by reproduction. This requires that each organism carries a plan of itself, which it passes on to its progeny. In fact, this information, stored in the base sequence of the DNA molecule, resides in nearly every cell. The expression of this information as specific cellular components has commanded much attention in recent years, and the path of information flow has been summarized in the so-called 'central

dogma of biology'.



This much seems clearly established. It remains to elucidate in detail the mechanisms whereby the above process is brought about and controlled, temporally and spatially. Some progress has been made, especially for simpler organisms like bacteria and viruses.

Like most cellular reactions, the processes of transcription, translation and replication are catalyzed by enzymes (or enzyme systems). These enzymes (or enzyme systems) not only control the rate of information transfer but also select which information is transferred, and so have unique roles in cellular life, bridging as they do the dynamic and informational aspects of living systems. A bacterial DNA-dependent RNA polymerase (E.C. 2.7.7.6) which catalyzes the transfer of information from DNA to RNA is the subject of this investigation. For bacteria especially, the rate and selectivity of RNA synthesis has an important influence on cell growth and reproduction.

As described more fully in the following sections, RNA polymerase is of complex structure and the reaction it catalyzes, a complicated one by comparison with many enzyme catalyzed reactions. In this study, some kinetic properties of RNA polymerase were investigated, with a view to assessing how far the enzyme's behaviour could be described by the conventional formulations of enzyme kinetics, in particular, steady state kinetics. Also, an operational description of the enzyme's properties provides a basis for characterizing RNA polymerase samples kinetically.

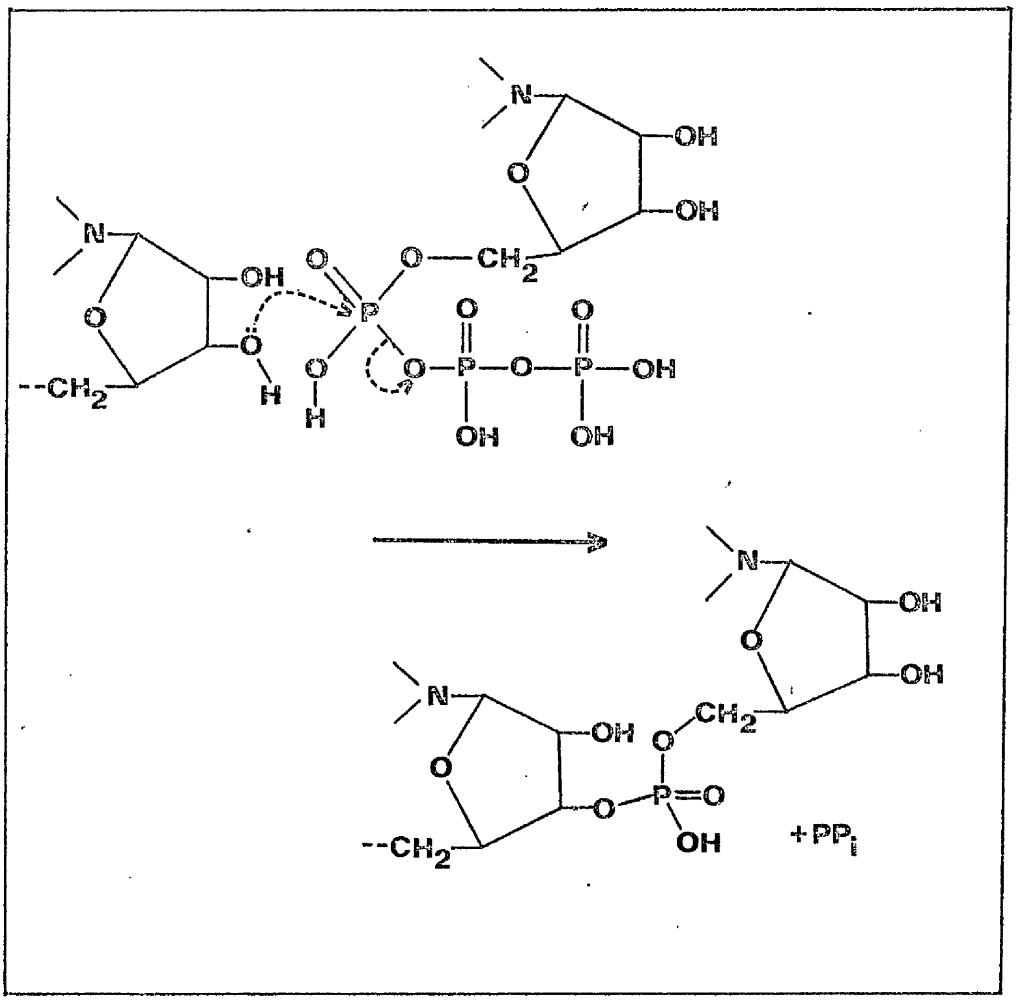


Fig.1 Possible mechanism for RNA synthesis by RNA polymerase

1A SYNTHESIS OF RNA BY RNA POLYMERASE

1A1 BASIC REACTION

A general equation for the reaction catalyzed by RNA polymerase is

$$DNA + NTP \xrightarrow[\text{divalent cation}]{\text{enzyme}} DNA + RNA + PP_i$$

The reaction is a programmed condensation polymerization of the four nucleoside triphosphates, ATP, CTP, GTP and UTP, leading to the production of RNA and PP_i. In terms of covalent bonds, the condensation of one nucleoside triphosphate involves the formation of a 3'-5' phosphodiester linkage between the ribose moieties of two nucleoside triphosphates (Fig. 1). This could result from nucleophilic attack by the oxygen of the 3'-hydroxyl of the terminal sugar moiety on the α-P of the incoming nucleotide, with PP_i as the leaving group (Florentiev & Ivanov, 1970). Nucleotides add to the 3'-end of the growing RNA molecule, both *in vitro* (Shigeura & Boxer, 1964; Bremer *et al.*, 1965) and *in vivo* (Goldstein *et al.*, 1965). The RNA product retains its 5'-terminal triphosphate (Bremer *et al.*, 1965). The synthesis of an RNA molecule of high molecular weight will evidently require the formation of a large number of 3'-5' phosphodiester linkages.

The fidelity of information transfer from DNA to RNA is established in the control by the template of the selection of the incoming nucleotide. Only a ribonucleoside triphosphate complementary, as dictated by Watson-Crick base pairing, to the prevailing base on the DNA, can be added to the growing RNA chain.

This implies an overall complementarity of the RNA product and the DNA template, as has been observed experimentally by hybridization studies (Bautz & Hall, 1962; Hayashi *et al.*, 1963).

As with any polymerization, the reaction may be conveniently divided into an initiation phase, an elongation phase and a termination phase. Control of initiation and termination offers the possibility of regulating precisely the regions of DNA which are transcribed. Several means of implementing such control have, in fact, been discovered and will be discussed in Section 1A6. Meantime, the main components of the reaction will be considered - the enzyme, the template, the substrates and the products.

1A2 RNA POLYMERASE

Enzymes capable of catalyzing RNA synthesis *in vitro* were isolated from both higher organisms (Weiss & Gladstone, 1959) and bacteria (Stevens, 1960; Hurwitz *et al.*, 1961; Ochoa et al., 1961; Chamberlin & Berg, 1962) as long ago as the early 1960's. That RNA polymerase has been well-studied since that time is apparent from the number of recent reviews of this enzyme and related topics (Richardson, 1969; Geiduschek & Haselkorn, 1969; Chamberlin, 1970; Travers, 1971; Burgess, 1971; Bautz, 1972). The enzyme purified from *Escherichia coli* has been the subject of the most extensive investigation and reference to the RNA polymerase from *E. coli* is implied in this dissertation unless otherwise specified.

1A2.1 Structure of RNA polymerase

Most RNA polymerase preparations contain four main subunits, designated β, β', α, and σ, in the stoichiometry $\alpha_2\beta\beta'\sigma$. Enzyme of this composition has been called 'holoenzyme'. It is now evident that most laboratories observe less than this stoichiometric amount of σ (Zillig et al., 1970; Berg et al., 1970). Holoenzyme may be separated into a second enzyme form, called core enzyme, and the subunit σ, by chromatography on a phosphocellulose column (Burgess et al., 1969; Berg et al., 1969). Core enzyme has stoichiometry $\alpha_2\beta\beta'$. Functionally, these two enzyme forms differ in their RNA synthesizing capabilities. Core enzyme can catalyze RNA synthesis from templates such as calf thymus DNA or poly (dA-dT), but by comparison has very low activity on intact DNA molecules like T7 or T4 DNA. σ stimulates RNA production by core enzyme manyfold with these phage DNA's and also imparts specificity on the transcription process. In the presence of σ, RNA chains are initiated at specific locations, promoter sites, on the DNA template (Bautz et al., 1969; Hall et al., 1969; Summers & Siegel, 1969).

1A2.2 Molecular Weights of RNA Polymerase Subunits

The molecular weights of the subunits, as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, are quoted by Burgess (1971) and Chamberlin (1970) as β', 150-165 000; β, 145-155 000; σ, 85-90 000; α, 39-41 000. The values for the mol. wt.'s of σ, β, and β', must be viewed as being the least certain since the method used in their determination is least accurate for high mol.wt.'s.

In this laboratory, lower mol.wt.'s for σ , β , and β (145 000 for the average of β and β' ; 78 000 for σ) have been measured using the gel electrophoresis technique (Lochhead, 1972) and, indeed, lower mol.wt.'s (130-137 000) were obtained for β and β' by sedimentation equilibrium (Burgess, 1969b).

1A2.3 Molecular weight of RNA polymerase

Taking the mol.wt.'s for the subunits quoted above, core enzyme would have a mol.wt. of about 400 000 and enzyme of structure $\alpha_2\beta\beta'\sigma$, a mol.wt. of about 490 000. Most estimates from sedimentation equilibrium experiments have, however, been somewhat lower (Richardson, 1966a ; Maitra & Hurwitz, 1967 ; King & Nicholson, 1971 ; Priess & Zillig, 1966 ; Berg & Chamberlin, 1970). These figures are for the monomer form of the enzyme which can, in fact, aggregate at low ionic strength (Richardson, 1966a ; Stevens et al., 1966 ; Berg & Chamberlin, 1970).

1A2.4 Individual roles of the subunits of RNA polymerase

Attempts to ascribe functions to the main subunits of RNA polymerase have not been entirely successful. σ appears to be clearly implicated in the initiation of RNA synthesis at specific sites on the DNA template (Section 1A6). Studies of RNA polymerase isolated from mutants resistant to the drugs rifampicin and streptolydigin have shown the β subunit to be modified. These drugs inhibit initiation and elongation of RNA chains respectively, suggesting that β may be the catalytic subunit (Zillig et al., 1970 ; Heil & Zillig, 1970).

poly-peptide	mol.wt.	source	reference
τ	120-130 000	E. coli	Burgess et al.,(1969) Davison et al.,(1970)
ω	10 000	E. coli	Burgess et al.,(1969) Berg & Chamberlin,(1970)
β	60 000	E. coli	Zillig et al.,(1970a)
ν	15 000		
θ	58 000	E. coli	Lochhead,(1972)
?	65-70 000	E. coli	Davison, cited in Paetkau & Coy,(1972)
?	65-70 000	E. coli	Stonington & Pettijohn,(1971)
ATPase	65-70 000	E. coli	Paetkau & Coy,(1972)
?	62 000	E. coli	Hirschbein et al.,(1969)
ε	65-70 000	A. vine-landii	Krakow & von der Helm,(1970)

Table 1.1 Some polypeptide species observed in RNA polymerase preparations

Investigations of the binding of subunits to DNA have indicated that β' is responsible for the binding of the enzyme to the template (Sethi, cited in Burgess, 1971). So far, it has not been possible to allocate a function to α and it is fair to say that much remains to be learned of the roles of the subunits of RNA polymerase in general.

1A2.5 Other components of RNA polymerase preparations

The subunits α, β, β', and σ appear to be clearly established as components of RNA polymerase. However, various laboratories have detected other polypeptide species when relatively pure RNA polymerase preparations were analysed by polyacrylamide gel electrophoresis (Table 1.1). These components are generally less consistent in their stoichiometry, certainly than α, β, and β', and little progress has been made in deciding whether they are true parts of the transcription machinery. Lacking any knowledge of possible functions, it is difficult to devise experiments that differentiate between co-purifying contaminants and true auxiliary factors to the basic RNA polymerase enzyme. Any system as complex as that required for transcription will be subject to this general problem of definition of composition.

Besides the polypeptides mentioned above, whose detection was the result of structural analysis of enzyme preparations, certain enzyme activities have been observed in purified RNA polymerase : RNase, DNase, polynucleotide phosphorylase, polynucleotide kinase (Burgess, 1971) and polyA polymerase (Terzi et al., 1970). Such activities are usually considered as impurities . This is perhaps correct but their lack of implication in RNA polymerase function should not be taken for granted, since the definition of the complete RNA synthesizing apparatus is not totally resolved at this time.

1A3. DNA TEMPLATE

RNA polymerase from E.coli is active in transcribing a number of DNA's (for example, Hurwitz et al., 1962). The rate and specificity of transcription depends upon the template and the enzyme composition (eg. σ content).

1A3.1 DNA structure

The DNA molecule is thought to have a double helical structure, with hydrogen bonding between complementary bases on opposite strands of the DNA molecule (Watson & Crick, 1953). At neutral pH in solution, the individual chains of the DNA would be forced apart by electrostatic repulsion, were the negative charges of the ionised phosphate groups not neutralized. Thus, double helical DNA in solution has an associated counterion and although various ions can act in this capacity the effectiveness varies from ion to ion (von Hippel & Scleich, 1969). Monovalent cations such as Na^+ or K^+ are much less effective than divalent ones like Co^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} or Mg^{2+}. Mg^{2+} is bound almost stoichiometrically by DNA (Dove & Davidson, 1962).

Many physical properties of DNA are sensitive both to ionic strength and to the nature of the ions present (Dove & Davidson, 1962 ; Marmur & Doty, 1962 ; Ts'o et al., 1963 ; Walker, 1964 ; von Hippel & Schleich, 1969 ; Wang, 1969). The T_m, for example, is very sensitive to ionic conditions (Dove & Davidson, 1962 ; Marmur & Doty, 1962). In a general way, then, it is reasonable to suppose that any effect on DNA properties might also influence transcription by RNA polymerase. In particular, the postulate that local unwinding of DNA occurs during the course of RNA polymerase action (Fuchs et al., 1967) implies an important influence

of the melting properties of DNA on the enzymic reaction. Different regions of the DNA may have different melting properties, responding differently to ionic conditions. This might have special relevance at the initiation of RNA synthesis.

1A3.2 Poly(dA-dT)

Poly(dA-dT) is an effective template for RNA polymerase (for example, Hurwitz et al., 1962). Although X-ray diffraction studies have shown that the helical structure of poly(dA-dT) resembles that of natural DNA's (Davies & Baldwin, 1963), the poly(dA-dT) structure has some features not associated with natural DNA's. In solution, double helical structure is observed but only at high polymer concentration does this arise from the participation of two separate poly(dA-dT) chains. Ordinarily, the double helix is due to the formation of a hairpin helix, i.e. a single strand folding back, and base pairing with itself (Schachman et al., 1960 ; Inman & Baldwin, 1962b). A further unusual property of poly(dA-dT) is its tendency to branching (Inman & Baldwin, 1962a). This phenomenon is sensitive to ionic strength, more branching occurring at high ionic strength. Transcription of poly(dA-dT) by RNA polymerase must be viewed in the light of the differences between the structure of synthetic poly(dA-dT) and that of natural DNA's, although a DNA resembling poly(dA-dT) has been isolated from Crabs (Laskowski, 1972).

1A4 SUBSTRATES

The complete complement of substrates for RNA polymerase comprises the four ribonucleoside triphosphates, ATP, CTP, GTP and UTP. The base moieties, adenine, cytosine, guanine and uracil are capable of lactam-lactim and/or enamine-ketimine

tautomerism. Also, the purine and pyrimidine rings exhibit the following ionizations;

base	position	pK
adenine	NH_3^+	4.2
cytosine	NH_3^+	4.5
guanine	NH_3^+	3.2
	N-1,C-6 OH	9.5
uracil	N-1	9.5

reference: Mahler & Cordes, 1967

In addition, ionizations of the triphosphate group occur with pK's of about 1.0 and 6.0 (Mahler & Cordes, 1967).

A property of these nucleotides is their ability to form complexes with metal ions. Complexes with a number of divalent cations, Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Sr^{2+}, and Ba^{2+} have been reported (Walaas, 1958 ; Nanninga, 1961 ; Burton, 1959; Phillips et al., 1966) as well as the weaker complexes formed with Na^+ and K^+ (Melchior, 1954 ; Smith & Alberty, 1956). Many enzymic reactions require the presence of metal ions and in those reactions involving nucleotides, a nucleotide-metal ion complex is, in some cases, the actual substrate. Although complexes between a number of different nucleotides and metal ions have been described, the complex MgATP has been most fully investigated (Burton, 1959 ; Hammes et al., 1961 ; Cohn & Hughes, 1962 ; Phillips et al., 1966 ; Norby, 1970). ATP^{4-} can form a 1:1 complex with Mg^{2+} (Martell & Schwartzenbach, 1956 ; Walaas, 1958). Evidence from infra-red and n.m.r. spectroscopy suggests that the Mg^{2+} is bonded only to the β and γ phosphate groups (Hammes et al., 1961 ; Cohn & Hughes, 1962 ; Brintzinger, 1963) with no bonding to a nitrogenous group of the adenine. The formation constants for Mg^{2+} complexes with ATP, CTP, GTP, and UTP are similar

involvement in complex formation. However, the association of Mg^{2+} with ATP probably causes an alteration in conformation of the triphosphate group (Phillips et al., 1966) and this has implications for the mechanism of ATP involvement in many biological reactions.

The quantitative measurement of these binding constants has been reported several times in the references cited above and, in particular, many estimates of the formation constant of $MgATP^{2-}$ have appeared. Phillips et al., (1966) present a compilation of such estimates. The formation constant, however, is dependent on ionic strength and temperature as well as the presence of other ions that can enter into complex formation (notably, Na^{+} and K^{+}). Since measurements of the formation constant have arisen from various different experimental procedures and conditions, comparison of results is not straightforward. Under a variety of conditions, values ranging from 3000 (Smith & Alberty, 1956) to 10 000 mol-1 (O'Sullivan & Perrin, 1961) have been quoted.

The synthesis of RNA is sensitive to the concentration of divalent cations (Fuchs et al., 1967 ; Bremer, 1970 ; Richardson, 1970a) and fairly complex responses to Mg^{2+} and Mn^{2+} concentration have been noted for the transcription of synthetic templates by the RNA polymerase from Micrococcus luteus (Straat & Ts'o, 1969 ; Steck et al., 1968). Optimal Mg^{2+} concentrations for RNA synthesis are considerably greater than the total nucleotide concentration (Fuchs et al., 1967), implying that all but a very small amount of nucleoside triphosphates are present as Mg^{2+} complexes. However, there appears to be no explicit statement in the literature that $MgNTP^{2-}$ is the substrate for the RNA polymerase reaction.

Formally, the products of RNA polymerase activity are PP_i and RNA.

1A5.1 Pyrophosphate

Pyrophosphate undergoes four ionizations with pK's of 0.85, 1.49, 5.77 and 8.22 (Barker, 1971) and at pH 8 pyrophosphate will be present as a mixture of $HP_2O_7^{3-}$ and $P_2O_7^{4-}$. Both of these species form complexes with Mg^{2+} (Lambert & Watters, 1957).

1A5.2 RNA

RNA polymerase catalyzes the synthesis of RNA molecules of high mol.wt. The essence of the reaction *in vivo* is that selected regions of the template only are transcribed. The true product is a set of RNA molecules of defined length and base sequence as dictated by the regulatory mechanisms of the cell. These RNA's are, for the cell, products in the same sense that glucose-6-phosphate, say, is the product of hexokinase activity. *In vitro*, the regulatory apparatus is usually incomplete and, in general, relatively unspecific transcription might be anticipated. Further, the quantity commonly determined in enzyme assays is total RNA synthesis, which is a measure of the total number of 3'-5' phosphodiester linkages formed, and takes no account of the size distribution or functional activity of the RNA synthesized. In experiments where RNA synthesis is so measured, one must recall the extrapolation from the measured quantity to the products of the enzyme in the strictly controlled environment of the cell. More detailed analysis of the product is possible, for instance, by physical studies of its mol.wt. distribution or by investigations of its base sequence. As these techniques

become more and more routine, this type of rigorous product analysis will, no doubt, become increasingly applied to RNA polymerase.

1A5.3 RNA structure

Anticipating a brief discussion of the thermodynamics of RNA synthesis, it is of interest to consider the factors that stabilize RNA structures in solution. An RNA molecule will in general consist of both single and double stranded regions.

Tinoco et al., (1971) have analysed the secondary structure of R17 viral RNA from the viewpoint of the formation base-paired double helical regions. For the RNA molecule, they compute ΔG's of the order of -4 to -10 kcal/mol., which works out as -0.08 to -0.2 kcal/mol. per base. These authors have not considered the contributions from single stranded stacking energy or the sequence dependent stacking between base pairs in double helical regions.

An approach to the energy of single stranded stacking can be made by considering dinucleotides since extrapolation to higher polymers appears to be vindicated (Cantor et al., 1966). Michelson (1968) quotes thermodynamic parameters for the transition from ordered to disordered states for a variety of dinucleotides and from these values, free energy changes at 20°C can be calculated for the transition. This yields free energy changes varying from +0.18 to -0.35 kcal/mol.

For an RNA molecule, both of the contributions above must be included as well as the sequence dependent stacking in order to account for stabilizations arising from the formation of secondary structure. A complete description would also include the effects of tertiary structure. A value for ΔG of 0 to -1 kcal/mol. per base to account for

the stabilization of secondary RNA structures relative to a random conformation seems reasonable on the basis of the above figures. This is of course only a gross approximation. As with DNA, RNA structure is sensitive to ionic conditions (for example, Spirin, 1963).

1A6 REGULATION OF RNA SYNTHESIS : SELECTIVE TRANSCRIPTION

Effective gene expression *in vivo* involves a high degree of selection at the level of transcription. For example, whilst mRNA is coded by greater than 95% of the bacterial genome, it represents only 1-2% of the cellular RNA at any given time (McQuillen, 1968). An important contribution to the understanding of the control of transcription in bacteria was that of Jacob & Monod (1961). In their theory of induction and repression the concept of a negative control element, the repressor, was introduced. In recent years, a number of positive regulatory factors have been reported (see reviews by Burgess, 1971 ; Bautz, 1972). In general, the control of RNA synthesis *in vivo* may be viewed as the imposition of various cellular regulatory mechanisms on the basic catalytic activity of RNA polymerase core enzyme. Indeed, core enzyme is still some four times larger than the RNA polymerase coded by T7 phage which is capable of highly specific transcription of T7 DNA (Chamberlin *et al.*, 1970). This difference in size probably reflects the much greater specificity of action demanded of the *E.coli* enzyme.

1A6.1 Sigma

The best studied positive control factor is σ which enables core enzyme to initiate RNA synthesis at specific locations on an intact DNA template. Several investigations of this effect have been reported (Bautz *et al.*, 1969 ; Hall *et al.*, 1969 ; Summers & Siegel, 1969) and the work of

Okamoto and co-workers has very clearly illustrated the effect of σ on the products of RNA polymerase activity (Okamoto et al., 1970 ; Sugiura et al., 1970 ; Takanami et al., 1970). In some instances, other control factors complement the effect of σ -i.e. σ is necessary but not always sufficient for correct initiation.

1A6.2 Psi

A protein factor designated Ψ has been described by Travers et al. (1970) which, in the presence of σ , stimulates the synthesis, by RNA polymerase, of rRNA from 0.2% of the total product RNA to 30-40%, when transcribing an E.coli DNA template. The function of Ψ is, however, not entirely clear, as yet (Haseltine, 1972).

1A6.3 Catabolite gene-activating protein (CAP or CRP)

Studies of the transcription of the lac operon have demonstrated the existence of a protein called CAP or CRP which has a specific role in this process (Pastan & Perlman, 1970). In vitro, RNA polymerase core enzyme will transcribe lac specific mRNA from a DNA containing the lactose operon only if σ , CAP, and cAMP are all present (Arditti et al., 1970).

This appears to be a well-documented example of the requirement of a positive control element in addition to σ , in order to effect specific cistron initiation.

1A6.4 M Factor

Davison et al. (1969) have isolated a protein, M factor, which stimulates RNA synthesis from λ or T7 DNA's, even in the presence of σ (Davison et al., 1970).

1A6.5 Termination

Specific termination of RNA synthesis is thought to result from various mechanisms. Specific DNA sequences can cause termination (Takanami et al., 1970 ; Maitra et al., 1970 Richardson, 1970a). Roberts (1969) has isolated a protein designated ρ factor which directs the specific termination of RNA synthesis on a number of templates. In addition, high salt concentration alone enhances specific termination (Richardson, 1970b).

Non-specific termination has been observed at low ionic strength (Fuchs et al., 1967 ; Bremer & Konrad, 1964) and also when RNA polymerase is transcribing single-stranded templates (Maitra & Hurwitz, 1967 ; Maitra & Barash, 1969).

The factors described in the previous section determine which sections of the genome are transcribed, in response to the prevailing environmental conditions. The 'environmental conditions' are basically established by the availability of energy sources, carbon sources and so on, in other words, the nutritional status of the medium supporting growth. One would anticipate, then, that the mechanisms regulating RNA synthesis would be capable of sensing the levels of small molecules. This idea is, of course, central to the induction-repression theory of Jacob and Monod (1961) in which gene expression is considered to be switched on or off by the presence of certain small molecules. A further instance is the lac operon where cAMP is a requisite for the synthesis of lac messenger RNA. The lac operon may indeed belong to a class of genes whose expression requires not only de-repression in the Jacob-Monod sense, but also a'go' signal from the cAMP pool. Another small molecule effect is that of ppGpp, a nucleoside tetra-phosphate that accumulates in stringent E. coli strains on the onset of amino acid starvation (Cashel, 1969 ; Cashel, 1970). This nucleotide may preferentially inhibit rRNA synthesis (Travers et al., 1970).

As with any enzyme catalyzed reaction, the substrate concentrations will have an effect on the rate of RNA synthesis by RNA polymerase, irrespective of which DNA regions are being transcribed. With RNA polymerase, however, it has been estimated that the enzyme spends most of its time starting and stopping RNA chain synthesis rather than in the elongation reactions (Chamberlin, 1970). Indeed, σ , by altering the rate of initiation, may not only enable specific initiation but effectively control the overall rate of RNA synthesis in vivo. Also, the apparent K_m's reported for both

initiation and elongation (for example, Anthony et al., 1969) are well below most estimates of in vivo nucleoside triphosphate concentrations (Neuhard & Munch-Peterson, 1966 ; Gallant & Harada, 1969). Substrate influences on the rate of RNA synthesis in vivo have been sought. In the course of various nutrient and inhibitor induced shift-downs of E. coli, no such effects were observed (Nazar & Wong, 1972) but on recovery from dinitrophenol inhibition or entry into phosphate starvation substrate control was operative (Nazar et al., 1972) The most significant effects of nucleoside triphosphates may be indirect, for example, via conversion to other nucleotides such as cAMP or ppGpp but nonetheless changes in triphosphate concentrations can alter the rate of RNA synthesis. The relative concentrations of ATP, CTP, GTP, and UTP may, in fact, be the most important parameter in this respect.

1B THE KINETICS OF ENZYME CATALYZED REACTIONS

1B1 KINETICS OF SINGLE SUBSTRATE REACTIONS

1B1.1 Historical Background

The quantitation of the kinetics of enzyme catalyzed reactions goes back to Henri (1901 ; 1902), Brown (1902), and Michaelis and Menten (1913), out of whose work emerged the now familiar hyperbolic rate equation and the concept of enzyme-substrate complexes. A simple uni-substrate reaction was described by the following scheme :

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_3} E + P \qquad \text{eqn. 1.1}$$

where E is enzyme, A substrate, P product and EA the enzyme-substrate complex. Originally, the enzyme-substrate complex was considered to be in equilibrium with free enzyme and free substrate. Application of the Law of Mass Action gives the equation :

$$v = \frac{V.a}{K_a + a} \qquad \text{eqn. 1.2}$$

where K_a is the dissociation constant for EA and equals k_2/k_1. The analysis assumes that the magnitude of k_3 does not affect the equilibrium of E, A, and EA. The equation may be written

$$1/v = 1/V \ (K_a/a + 1) \qquad \text{eqn. 1.3}$$

which enables simple graphical estimation of K_a and V by plotting 1/v against 1/a (Lineweaver & Burk, 1934). Other similar rearrangements of the equation are possible (Hofstee, 1952). Eqn. 1.2 represents a hyperbola and the scheme depicted in eqn. 1.1 can be characterized by two parameters, K_a and V, which have a clear physical

interpretation. K_a is determined by the affinity of the enzyme for the substrate and V represents the maximum velocity of the reaction, which v approaches asymptotically as a tends to infinity. The form of eqn. 1.2 resembles that of the Langmuir isotherm (Langmuir, 1916) and illustrates the basic similarity between enzymic and surface catalysis (Laidler, 1965).

An algebraically equivalent rate equation to eqn. 1.2 was derived by Briggs and Haldane (1925) without invoking the original equilibrium assumption. Instead, they applied the more convincing concept of a steady state in which, after a brief initial period, d(EA)/dt was assumed equal to zero. The rate equation so derived is

$$v = \frac{V.a}{K_m + a} \qquad \text{eqn. 1.4}$$

where the only difference from eqn. 1.2 is the replacement of K_a by K_m. Km equals $(k_2 + k_3)/k_1$. K_m is a function of all the individual rate constants and, in general, does not represent the affinity of the enzyme for the substrate. This is an often quoted but often ignored point (Dalziel, 1962).

1B1.2 Steady state

The steady state rate equation for a single substrate is important both in its frequent applicability in ostensibly more complex situations and because the steady state assumption is fundamental to a large body of enzyme kinetic theory. The justification of the steady state assumption requires that -

(1) The time taken for the attainment of the steady state concentration of enzyme-substrate complex is small on the time scale of product formation.

(2) After reaching the steady state level of intermediate, d(EA)/dt must in fact equal zero.

Laidler (1955) has investigated the conditions under which these requirements are met, and in addition to the more common criterion of $s_o \gg e_o$ (where the subscript refers to zero time), the steady state assumption is also valid for

1) $e_o \gg s_o$

2) $k_2 + k_3 \gg k_1 e_o$

3) $k_2 + k_3 \gg k_1 s_o$

A more recent assessment of the steady state assumption has been reported by Heineken et al. (1967) who go so far as to say that it is 'somewhat scandalous' from a mathematician's point of view. This is, however, a rigorous treatment and the application of steady state kinetics in practice has been both successful and useful. Nonetheless, the restrictions imposed by the steady state assumption should not be forgotten.

1B1.3 Reversible Reaction.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P \qquad \text{eqn. 1.5}$$

The above mechanism can be analysed to give a steady state rate equation of the form

$$v = \frac{K_m^b . V^f . a - K_m^f . V^b . p}{K_m^f . K_m^b + K_m^b . a + K_m^f . p} \qquad \text{eqn. 1.6}$$

where p is the product concentration and the superscripts refer to the forward (f) and back (b) reaction. At t=0, p=0 and eqn. 1.6 reduces to eqn. 1.4. The use of initial velocities is experimentally common as it obviates the need among other complexities to account for the effect of the back reaction (unless product is deliberately added to the system for product inhibition studies). When v = 0 in

equation 1.6, the reaction is at equilibrium and

$$K_m^b . V^f . a - K_m^f . V^b . p = 0$$

from which it can be seen that

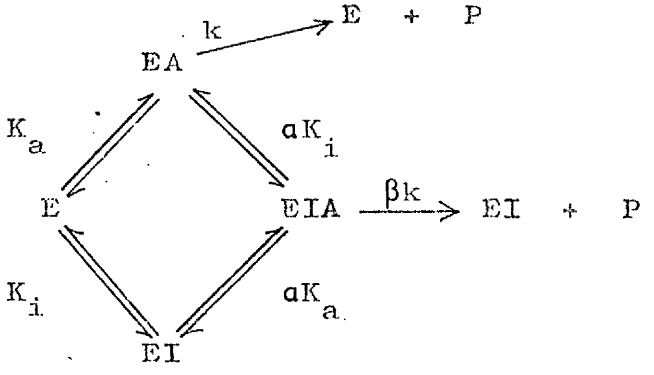
$$\frac{K_m^b . V^f}{K_m^f . V^b} = \frac{p_{eq}}{a_{eq}} = K_{eq} \qquad \text{eqn. 1.7}$$

K_{eq} is the equilibrium constant and eqn. 1.7 is the simplest of the so-called Haldane relationships.

Integrated kinetics have been described (Foster & Niemann 1953 ; Alberty & Koerber, 1957 ; Schwert, 1969a) but their practical application has been rather limited (for an example, Schwert, 1969b).

1B1.4 Inhibition of Enzymic Reactions

Enzymic reactions can be inhibited by substrates, products or other agents. Different types of inhibition can be recognized and several classifications have been described. Webb (1963) details a general reaction scheme which reduces to the more common inhibition types as the result of appropriate constraints. Although this is based on an equilibrium rather than a steady state treatment, it provides a basis for the discussion of inhibition in more complex systems.



The inhibitor, I, is assumed to affect either substrate binding (via the factor, α) or the breakdown of the enzyme-substrate complex (via the factor, β). The general

$$v_i = \frac{a.(\alpha K_i + \beta.i)}{a.i + \alpha(K_i.a + K_a.i + K_a.K_i)} \qquad \text{eqn. 1.8}$$

1) Completely competitive inhibition. $\alpha = \infty$ and $\beta = 1$

No EIA complex is formed as the inhibitor totally prevents substrate binding.

2) Completely non-competitive inhibition. $\alpha = 1$ and $\beta = 0$

The inhibitor does not affect substrate binding but abolishes the breakdown to products.

3) Uncompetitive inhibition or activation.

$\alpha < 1$ $\beta \geqslant 1$: activation

$\beta < 1$: possible inhibition

This behaviour is characterized by a set of parallel reciprocal plots (1/v against 1/a) with different inhibitor concentrations.

Webb lists further classes of inhibition arising from other constraints on the values of α and β. Other schemes for the classification of inhibition have been proposed, notably by Cleland (Cleland, 1970) who defines the types of inhibitors according to the effect on a double reciprocal plot. This operational definition is useful in more complex cases which do not readily conform to the general scheme of eqn. 1.8, and differs from Webb's mainly in that Cleland's classification does not distinguish between some of the types of categorized by Webb. In Cleland's scheme, inhibition is broadly classed as

competitive : effect on the slope but not the vertical intercept of a double reciprocal plot.

non-competitive : effect on slope and intercept.

uncompetitive : effect on intercept but not the slope.

Further distinction can be made by the nature of the variations of slope or intercept with inhibitor concentration, for example, S-linear, I-hyperbolic.

Inhibition by products and substrates can often be described by one of the classifications above, but most generally the actual effect on a model of any inhibitor action can be derived by a steady state analysis.

1B2. KINETICS OF MULTI-SUBSTRATE REACTIONS

1B2.1 Bisubstrate Reactions

Most enzyme catalysed reactions have more than one substrate and in going from single to multi-substrate reactions, an important complication in postulating reaction schemes is encountered; namely, there is not unique sequence. *A priori*, it is not known in what order the substrates bind (or the products are released), and indeed, there may be alternative reaction pathways. The characterization of a two substrate reaction requires not only the measurement of kinetic parameters, but also the identification of the actual reaction mechanism. Early contribution to the theory of bisubstrate reactions came from Alberty (1953 ; 1958) and Dalziel (1957 ; 1958) who initially showed that all linear (i.e. no alternative pathways) mechanisms could be described by a general equation of the form (with the parameters in the notation of Dalziel)

$$\frac{v}{e_o} = \frac{a.b}{\Phi_o.a.b + \Phi_1.a + \Phi_2.b + \Phi_{12}} \qquad \text{Eqn. 1.9}$$

Various particular mechanisms conforming to the above general equation can be distinguished.

The possibility of branched or alternative reaction mechanisms was explored by Dalziel (1958) and, for the two substrate case in particular, a fairly exhaustive treatment is given by Wong & Hanes (1962). Cleland (1963a, b, c) also developed the steady state analysis of multi-substrate reactions. The object of most steady state formulations has been to enable the differentiation between various feasible reaction mechanisms based on knowledge of the structure of the relevant rate equations. This required the derivation of steady state rate equations for a number of different reaction models, a task considerably simplified by the use

of the determinant technique of King & Altman (1956). Kistiakowsky & Shaw (1953) had originally used a determinant method in the derivation of rate equations and King & Altman developed a determinant-based schematic rule which obviated all algebraic manipulation. Their procedure effectively solves the set of linear simultaneous equations which define the steady state for all of the enzyme-containing intermediates of a given enzymic reaction. Volkenstein & Goldstein (1966) have worked out a method based on graph theory to solve these same equations, again free from algebraic juggling. These techniques can be applied to the solution of any model in steady state terms and much of the progress in multi-substrate kinetics has been the rationalization of the various rate equations so derived.

1B2. General Steady state rate equation

The general steady state rate equation with respect to a particular substrate, A , is given by Wong & Hanes (1962) as

$$v = \frac{p_o a^z + p_1 a^{z-1} + \ldots\ldots \quad \ldots\ldots p_{z-1} a + p_z}{q_o a^z + q_1 a^{z-1} + \ldots\ldots \quad \ldots\ldots q_{z-1} a + q_z} \qquad \text{eqn. 1.10}$$

where the terms of both the numerator and the denominator are subject to certain 'structural rules'. Different reaction Mechanisms differ in the value of z and in the composition of the coefficients p and q both as regards other substrate concentrations and the individual rate constants involved. The degree of the equation z is the degree with respect to A and in general equals the number of different enzyme-containing species to which the substrate A binds. The degree of the numerator can be less than or equal to that of the denominator.

The complete rate equation, with all substrates variable, can evidently be a complex function and its experimental confirmation requires the study of the variation of the coefficients, p and q, with the fixed substrates (i.e. substrates other than 'A'). The effects of inhibitors or activators can be incorporated by introducing putative modifier interactions in the initial reaction model.

1B2.3 Analysis of a linear bisubstrate reaction

To illustrate the common experimental rationale, the linear bisubstrate reaction will be considered.

ordered : $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \rightleftharpoons EPQ \underset{Q}{\rightleftharpoons} EP \underset{P}{\rightleftharpoons} E$

ping-pong : $E \overset{A}{\rightleftharpoons} EA \underset{P}{\rightleftharpoons} E' \overset{B}{\rightleftharpoons} E'B \rightleftharpoons EQ \underset{Q}{\rightleftharpoons} E$

The two main types of bisubstrate linear reactions are shown in the above schemes, and as mentioned in section 1B2.1 can be described generally by eqn. 1.9. The ping-pong mechanism is distinguished by the fact that $\Phi_{12} = 0$.

Experimentally, a is varied at a number of fixed concentrations of b, which is designated the 'changing fixed' substrate in this context (Cleland, 1970). The pattern of the double reciprocal plots of 1/a against 1/v at different b levels, is diagnostic. With an ordered mechanism, the double reciprocal plots meet to the left of the 1/v axis but are parallel with a ping-pong mechanism. For each concentration of b, a slope and a vertical intercept is obtained which can then be re-plotted against 1/b. This procedure allows the estimation of four parameters, two from each replot. Further specific linear bisubstrate mechanisms, arising from cases where the individual rate constants are constrained in a specific fashion, can be elucidated in a similar manner, and more complicated

reactions can be approached by extension of the same technique. It should be noted, however, that even in the two substrate situation, complete resolution of the reaction is not always possible. It is not possible, for instance, to determine which substrate of an ordered mechanism adds first, and this general problem increases with more complex reactions. Indeed, conformity to a kinetic rate law does not unequivocably prove the veracity of the reaction model from which the rate law was derived.

1B2.4 Competitive Substrate Inhibition

$$E \overset{A}{\rightleftharpoons} \underset{EAA}{\overset{A}{EA}} \overset{P}{\rightleftharpoons} E' \overset{B}{\rightleftharpoons} E'B \overset{Q}{\rightleftharpoons} E$$

The scheme above shows the diversion of enzyme into a fruitless complex with A, cometimes called a 'dead end' complex. This can be recognized as competitive inhibition. With a ping-pong bisubstrate reaction, the following eqn. 1.11 would be obtained from steady state analysis

$$\frac{v}{e_o} = \frac{a.b}{\Phi_o a.b. + \Phi_1.a + \Phi_2.b + \Phi_i.a^2}$$

Eqn. 1.11

In general, this situation leads to the introduction of an extra term in a^2 into the denominator of the rate equation for the uninhibited reaction (Wong & Hanes, 1962). If a is the variable substrate, this type of inhibition shows as a minimum in the plot of 1/v against 1/a, which asymptotically approaches the vertical axis as a tends to infinity. If a is the changing fixed substrate, the behaviour in response to the variation of b is hyperbolic, but the slopes of the double reciprocal plots increase with increasing a, and the plots intersect to the right of the vertical axis.

1B3 NON-HYPERBOLIC KINETICS

From the general steady state equation (eqn. 1.10), it is apparent that hyperbolic kinetics ($z = 1$) are a special case, although, in fact, conformity to the simple Michaelis-Menten law is common. Deviation from this rate law may be termed 'non-hyperbolic' behaviour and may result from a number of causes. The following classification is based on that of Wong & Endrenyi (1971).

1B3.1 Inapplicability of steady state rate equation

Interaction between enzyme molecules will cause deviation from the steady state rate law of eqn. 1.4, for example, if enzyme polymerization occurs (Frieden, 1967; Nichols *et al.*, 1967). Such behaviour is characterized by a non-linear dependence of v on enzyme concentration, even when $s_o \gg e_o$. Other failures of the steady state equation will occur if the assumptions of the steady state are violated, as would result from the inapplicability of the inequalities defined by Laidler (Section 1B1.2).

1B3.2 Branching (Alternative) Reaction Pathways

For an enzyme with a single binding site for a substrate, A, non-hyperbolic behaviour obtains if, via binding with other substrates or modifiers, a number of different enzyme-containing intermediates are capable of binding A. Saturation by the other substrates or modifiers can effectively reduce the number of intermediates that can bind with A so that the degree of the dependence (z) on A may be reduced. Such a response is diagnostic of this general mechanism, as is also the fact that the equation describing the binding of A to the enzyme is of degree 1, since the enzyme has a single A binding site.

1B3.3 Cooperative or Allosteric Behaviour

Where an enzyme has a number of substrate binding sites, (as, for example, with an oligomeric enzyme), binding of substrate at one site may affect the affinity of the other sites for substrates (for example, Koshland, 1970). If there is no interaction, no cooperativity is observed either in binding experiments or kinetic studies. Interactions may be on binding of substrate or the breakdown to product, as indeed, with simple modifiers, but in this case, the geometric arrangement of the sites is also important. Postulations concerning the nature of the allosteric interactions leads to various models of cooperative behaviour (Koshland *et al.*, 1966 ; Pauling, 1935 ; Monod *et al.*, 1965). Allosteric enzymes may be described by eqn. 1.10 in general and the mathematical implications of different models are seen in the different relationships amongst the coefficients of the polynomials.

The most common analytical procedure for cooperative behaviour is the Hill plot (Hill, 1952), in which log $v/(V-v)$ is plotted against log a. The maximum slope of the Hill plot ideally defines the number of binding sites for the substrate. Another diagnostic plot is that of log v against log a, the maximum slope of which again is an estimate of the number of sites. In practice, the maximum slope in both cases is an underestimate of the true number of binding sites unless the cooperativity is very high.

1B3.4 Substrate Involved Twice in Reaction Sequence

In a linear mechanism, where a substrate combines more than once with different enzyme-containing species, the rate function can be of degree more than one. The substrate binding points in the reaction scheme must be connected by a reversible sequence.

1B3.4 Substrate inhibition

Substrate inhibition is a special case of a branching pathway and was described in Section 1B3.2.

The above instances of non-hyperbolic behaviour are theoretical and in practical situations, there may be other causes, which although of less theoretical interest, may be important.

1B3.5 Impure enzyme

The presence of two enzymes catalysing the same reaction will give, even when both conform to hyperbolic rate equations, an overall rate law of the form

$$v = v_1 + v_2$$

$$= \frac{a.V_1(K_2 + a) + a.V_2(K_1 + a)}{(K_1 + a) \cdot (K_2 + a)} \qquad \text{(Cleland, 1970).}$$

1B3.6 Inhibitors or Activators as impurities of substrate or enzyme.

The presence of inhibitors or activators in either enzyme preparations or substrate solutions may impose deviations from an otherwise hyperbolic rate equation.

1B3.7 Error in Velocity Measurements

Systematic error in the determination of initial velocities may yield an erroneous departure from hyperbolic kinetics. For example, if v is systematically underestimated by an amount δv, then for a true hyperbolic rate law, the double reciprocal plot of 1/v against 1/a will be concave up when δv becomes a significant proportion of v.

The analysis of enzyme kinetic rate laws has been facilitated in many instances by the frequent conformity of enzymic reactions to hyperbolic rate laws, with respect to any particular substrate. This form of equation allows easy graphical analysis as described earlier, but the evaluation of kinetic constants in this way has been criticised (Hearon et al., 1959 ; Cleland, 1967). Statistical procedures have thus been devised (Wilkinson, 1961 ; Johansen & Lumry, 1961 ; Cleland, 1967) and it is now common to fit experimental values of v and a directly to the equation $v = V.a/(K_m + a)$ using the least squares method. This has the advantages of providing better estimates of the parameters and giving an objective analysis of experimental results.

In this instance, statistical methods yield a refinement in the estimation of kinetic parameters but in no way alter the validity of the underlying kinetic analysis. The interpretation of many enzymic reactions involving hyperbolic rate equations has been adequately founded on the judgement of the experimentor in drawing a straight line through a set of data points. When the degree of the rate equation is greater than 1 (z in eqn. 1.10), however, visual examination of the data can give little more than qualitative assessment of deviations from hyperbolic behaviour. If a more rigorous analysis is desired, then more sophisticated techniques are required and this usually involves fitting the data to higher degree rate equations, by statistical methods. This is no panacea since the least squares analysis of functions non-linear in parameters is not a trivial problem in numerical analysis.

1B4.1 Statistical Considerations in the Evaluation of Rate Equations.

The general steady state rate equation is given in eqn. 1.10 and any model equation is defined by the parameters, p and q. Statistical analysis serves two purposes. If the appropriate rate equation is known, the defining parameters may be estimated statistically. Lacking knowledge of the rate equation, experimental data can be fitted to different model functions, and the best fitting model selected as the most likely. Again, this does not alone furnish positive proof of a mechanism, but some feasible reaction schemes can be excluded with certainty.

Least Squares Analysis

Given a set of n data points (x_i, y_i), the problem is to evaluate the parameters a,b,c,..... that describe the best fit of the data to a putative model equation

$$y = f(x ; a,b,c,.....).$$

The uncertainty in x is considered negligible compared to the uncertainty in y. The sum of the squares, S, is defined by

$$S = \sum_{i=1}^{n} (y_i - f(x_i))^2$$

In the least squares method, the best estimates of the parameters a,b,c,... are those which minimise S,

$$\left(\frac{\partial S}{\partial a}\right)_{b,c,..} = \left(\frac{\partial S}{\partial b}\right)_{a,c,..} = ... = 0$$

If the model is linear with respect to the parameters a,b,c,.., then this defines a set of linear simultaneous equations which can be solved explicitly for a,b,c..... If, as is generally the case for eqn. 1.10, the model is non-linear in the

parameters, no explicit solution is possible, and initial estimates of the parameters must be iteratively modified until a minimum in S is obtained. The parameter space is effectively searched for a minimum of S. The execution of this operation generally requires the aid of a computer and various numerical techniques can be employed to help and accelerate the minimization procedure (Bevington, 1969).

To account for the differing uncertainties in the experimental values, y_i, the statistic 'chi squared' may replace S.

$$\chi^2 = \sum_{i=1}^{n} (1/\sigma_i^2) \cdot (y_i - f(x_i))^2$$

where σ_i is the variance of the i^{th} data value of y. The minimum of S or chi squared is obviously to some extent an indication of the goodness of fit of the data to the model equation. Commonly, the 'reduced chi squared' is used in this context.

$$\chi_\nu^2 = \chi^2/\nu$$

where ν = degrees of freedom.

This statistic, in fact, is the ratio of the observed variance with respect to the fitted model and the mean variance in the data, y_i, so that if $\chi_\nu^2 = 1$, the model accounts for all of the observed variance. In general, lack of applicability of the model is seen as a high value of χ_ν^2.

1C REACTION CATALYSED BY RNA POLYMERASE : KINETICS AND MECHANISM

1C1 THERMODYNAMICS OF RNA SYNTHESIS

Owing to the difficulty in defining the products of the reaction catalysed by RNA polymerase, the addition of a single nucleotide residue to a growing RNA chain will be considered.

$$RNA_{n-1} + NTP = RNA_n + PP_i$$

where n and n-1 refer to the number of base residues in the RNA chain. The free energy change associated with the forward reaction may be ascribed to -

1) The condensation of the incoming nucleotide, ΔG_E^o

2) The change in conformation of the product RNA, ΔG_R^o

ΔG_E^o may be estimated by consideration of simple phosphate ester formation. The free energy for the hydrolysis of a phosphodiester is about - 6.0 kcal/mol. (Mahler & Cordes, 1967). The reverse reaction, the formation of a phosphodiester is then endergonic to the extent of + 6.0 kcal/mol. This, however, would represent the following equation when adapted to the synthesis of RNA

$$RNA_{n-1} + NMP = RNA_n + H_2O \quad ; \quad \Delta G^o = 6.0 \text{ kcal/mol.}$$

For RNA polymerase, the hydrolysis of the nucleoside triphosphate substrate would formally favour the forward reaction. For ATP, Alberty (1969) gives a value $\Delta G^o = -8$ kcal/mol. for hydrolysis to AMP and PP_i at common reaction levels of Mg^{2+} and pH. If the same value is taken for the CTP, GTP and UTP, then

$$RNA_{n-1} + NTP = RNA_n + PP_i \quad ; \quad \Delta G_E^o = -2 \text{ kcal/mol.}$$

The contributions of secondary structure to RNA stability were discussed in Section 1A5.2. ΔG^{o}_{R} will include such terms which may be of the order of 0 - 1 kcal/mol. In any case, ΔG^{o}_{R} is presumably negative since RNA does adopt secondary and tertiary structures in solution. This is a simple and approximate analysis but indicates that one might expect RNA synthesis to be exergonic by at least about 2 kcal/mol.

An additional point is that the enzyme pyrophosphorylase will be active *in vivo*, catalysing

$$PP_i + H_2O = 2P_i \quad ; \quad \Delta G^{o} = -6 \text{ kcal/mol.} \qquad \text{eqn. 1.12}$$

The value of ΔG^{o} is from Alberty (1969). Coupled to the RNA polymerase reaction and because of the high H_2O concentration, this would make RNA synthesis considerably more exergonic than the estimate above.

1C2 BINDING OR RNA POLYMERASE TO DNA

The first event in RNA synthesis is the binding of RNA polymerase to the DNA template. This process is sensitive to ionic conditions (Richardson, 1966b). Core enzyme is capable of randomly binding to DNA (Hinkle & Chamberlin, 1970 ; Darlix *et al.*, 1969). At certain specific locations on the template, however, the affinity of the enzyme for DNA is thought to be greatly enhanced (Hinkle & Chamberlin, 1970), allowing the formation of complexes resistant to the inhibitors rifampicin (Sippel & Hartmann, 1970 ; Bautz & Bautz, 1970) and heparin (Zillig *et al.*, 1970). The formation of such complexes requires the presence of σ and a temperature in excess of $20^{o}C$ although nucleoside triphosphates are not implicated in the reaction (Sippel & Hartmann, 1970 ; Bautz & Bautz, 1970).

With nicked DNA's, single stranded DNA's or a DNA with single stranded regions such as poly(dA-dT), there is no requirement for elevated temperature or σ factor in the formation of these stable complexes (Chamberlin, 1970) but the complexes are not formed at specific template sites. Local melting of DNA has been invoked in the RNA polymerase reaction (Fuchs et al., 1967 ; Hinkle & Chamberlin, 1970) and this might be important in the formation of stable enzyme-DNA complexes. The influence of ions on RNA polymerase action, both at initiation and elongation of RNA chains, may be due in part to the effect of ions on DNA melting (Section 1A3.1).

Details of the interactions involved in the binding of RNA polymerase to DNA are as yet unresolved. It is not known, for instance, whether σ or core enzyme or both determine the specificity of the initial binding although the binding of core enzyme appears to provide most of the binding energy (Zillig et al., 1970). There is evidence from analysis of 5'-terminal sequences of RNA products, that core enzyme preferentially starts at pyrimidine clusters (Okamoto et al., 1970 ; Sugiura et al., 1970).

1C3 INITIATION OF RNA SYNTHESIS

Following the binding of enzyme to DNA, the next clearly defined occurrence is the binding of the first substrate molecule but there is some evidence for changes in the properties of the enzyme-DNA complex between these two events and this is discussed by Burgess (1971). In a number of studies, the first nucleotide of the product RNA (i.e. at the 5'-end) has been a purine residue (Maitra & Hurwitz, 1965 ; Maitra et al., 1967 ; Okamoto et al., 1970 ; Sugiura et al., 1970) irrespective, in fact, of the initial presence of σ . The second substrate then binds and the

formation of the first 3'-5' phosphodiester linkage follows. At some early stage in the synthesis of the RNA chain, σ, if present, is released (Berg et al., 1969 ; Krakow & von der Helm, 1970).

Definition of Initiation

In polymerization reacions studied in chemical laboratories, initiation is generally taken to mean the formation of the first covalent bond between two of the monomer reactants. In transcription, a number of events precede this occurrence. Thus, RNA synthesis may be committed to starting at a particular location on the DNA before any covalent bonds have been formed and the selection of the site of enzyme binding on the template may have been irreversible. To avoid problems with loose usage of the word 'initiation', the term 'covalent initiation' is used here to describe the actual formation of the first phosphodiester linkage. Otherwise 'initiation' is used to include all the events involved in the effective commencement of RNA synthesis and includes both the binding of enzyme to DNA (specifically or nonspecifically) and the formation of the first covalent bond.

I.C4 ELONGATION OF RNA CHAINS AND MODELS OF RNA POLYMERASE ACTION.

It has been suggested on the basis of kinetic studies (Anthony et al., 1969), equilibrium dialysis (Wu & Goldthwait, 1969a) and fluorescence quenching (Wu & Goldthwait, 1969b) that RNA polymerase has two sites capable of binding nucleoside triphosphates. At one site, designated the initiation site, purine triphosphates bind preferably, with no requirement for Mg^{2+}. This binding is inhibited by rifampicin. At the

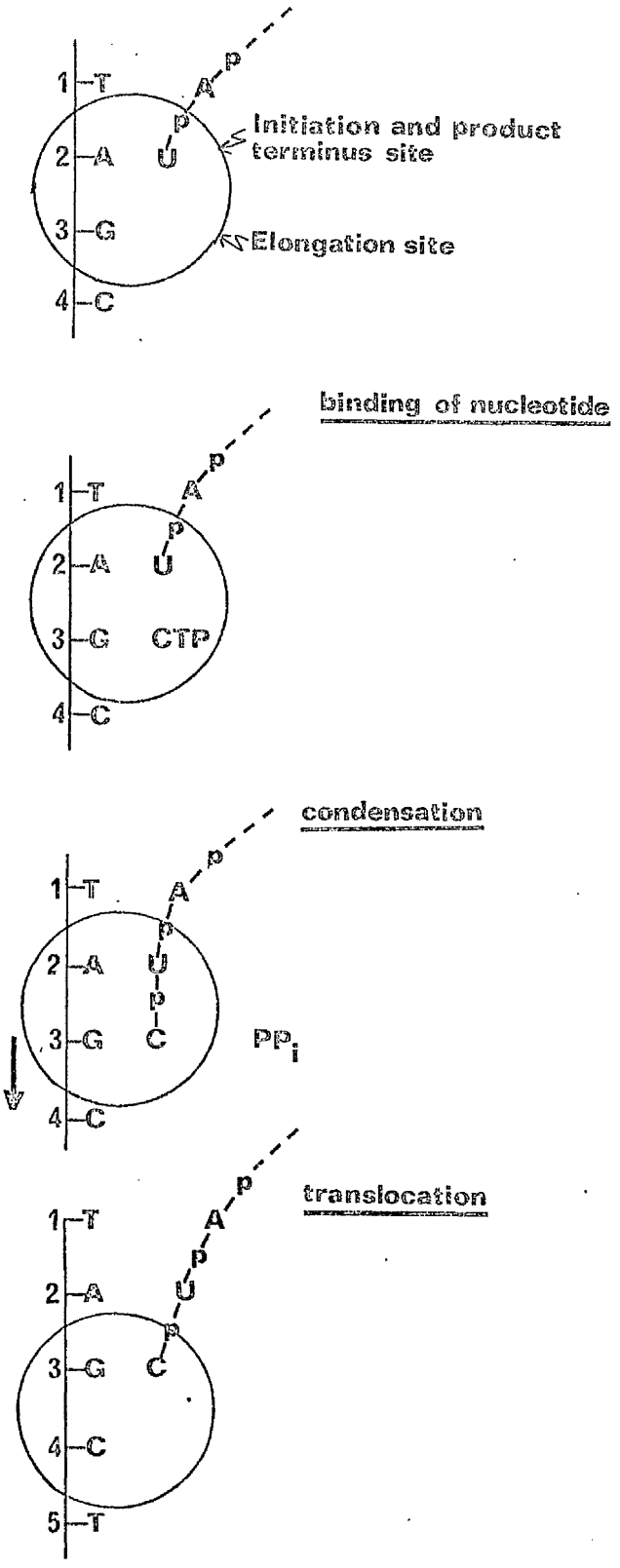


Fig. 2 Two site model of RNA polymerase action

second site, a polymerization site, all four nucleoside triphosphates bind with approximately equal affinities and this requires Mg^{2+}. Krakow has also proposed a model for the action of the enzyme from Azotobacter vinelandii with a poly(dA-dT) template (Krakow & Fronk, 1969 ; Krakow & von der Helm, 1970), based on pyrophosphate exchange experiments that is in basic accord with the proposals of Anthony, Wu & Goldthwait (Fig. 2). At the stage of covalent initiation, the initiation site first binds a purine triphosphate, followed by the template directed binding of the next nucleoside triphosphate at the adjacent polymerization site. The catalytic process involves the formation of a 3'-5' phosphodiester linkage from the incoming nucleotide to the 3' end of the product chain (which is only one unit long before covalent initiation). Pyrophosphate is released. Before the formation of the next bond, the enzyme must move with respect to the template so that the polymerization site is vacated and is now in register with the next base of the template sequence. With this migration, the 3'-terminal residue of the product becomes located at the initiation or product-terminus site. Elongation is the repetition of such reactions.

Expressed as a turnover number, the observed activity of RNA polymerase in vitro has varied from about $2s^{-1}$ (Bremer & Konrad, 1964) up to about $25s^{-1}$ (Richardson, 1969), depending on enzyme preparations and reaction conditions. The upper value approaches estimates of the rate of RNA chain elongation, in vivo, of approximately 55 nucleotides/s (Bremer & Yuan, 1968). For a polymerization reaction, however, the quantities quoted above are not a simple measure of the catalytic function of a single catalytic site, but are determined by the rates of both the initiation and the

elongation phases of RNA synthesis.

An interesting implication of this model is that, since Mg^{2+} is required for nucleotide binding to a polymerizing enzyme, the nucleotides are presumably in the form of their Mg^{2+} complexes. On the other hand, free ATP or GTP can bind to the initiation site in the covalent initiation step. This might imply conflicting requirements of Mg^{2+} for initiation as compared to elongation.

The results of Ishihama & Hurwitz (1969) do not however appear to concur entirely with the above two site model. These workers observed the binding of nucleoside triphosphates at 8 or 9 sites per RNA polymerase molecule. These sites were identical in their binding capacity for each of the nucleotides, specificity for purines only being imparted in the presence of DNA. The reason for the disparity between these results and those of Anthony, Wu & Goldthwait is as yet unclear.

Pyrophosphate can exchange into the nucleoside triphosphate substrates (Ishihama & Hurwitz, 1969 ; Krakow & Fronk, 1969 ; Krakow & von der Helm, 1970) indicating reversibility of the condensation step. With a poly(dA-dT) template, greater exchange occurs into UTP than ATP (Krakow & Fronk, 1969 ; So and Downey, 1970). This can be explained by the two site model on the basis that the product-terminus or initiation site has a greater affinity for purine triphosphates. A 3'-terminal UMP residue would take longer to transfer from the polymerization site to the product-terminus site, thus spending more time susceptible to pyrophosphate attack.

Further investigations of the active site were carried out by Ishihama & Hurwitz (1969) who made chemical modifications of the enzyme. Reagents reacting with sulphydryl

groups prevented DNA binding, those reacting with amino acids generally, inhibited a later step whilst reagents specifically blocking histidine abolished the polymerization reaction but not pyrophosphate exchange.

1C5 EFFECT OF IONIC CONDITIONS ON THE RNA POLYMERASE REACTION

From the previous sections it is apparent that ionic strength and the particular ions present can affect the properties of RNA polymerase, DNA, RNA, PP_i, and the nucleoside triphosphates. It is to be expected, then, that RNA synthesis by RNA polymerase is strongly influenced by ionic conditions, and any effect may include contribution from the ionic influences on the individual participants in the reaction. Some ionic effects have been described and a brief compilation of these is,

1) High salt inhibits initiation to a greater extent than polymerization (Fuchs et al., 1967) and Richardson (1966b) states that, in general, the affinity of RNA polymerase for DNA decreases with increasing ionic strength. Optimal KCl concentration has been quoted as 0.2 M-KCl (So et al., 1967).

2) The enzyme associates at low ionic strength into dimers and possibly higher polymers (Richardson, 1966a ; Stevens et al., 1966 ; Berg & Chamberlin, 1970).

3) The time course of RNA synthesis is dependent on ionic conditions (Fuchs et al., 1967). Lags are observed at high ionic strength and high Mg^{2+} concentration. Elevated Mg^{2+} or the presence of Mn^{2+} of Co^{2+} prolongs the duration of the linear phase of RNA synthesis with time.

4) Release of RNA products from the enzyme is facilitated at high ionic strength (Richardson, 1970b) and the dependence of the RNA products on KCl concentration has been demonstrated by Qasba & Zillig (1969) and Bremer (1970).

5) A divalent cation is a specific requirement of the enzyme at a concentration an order of magnitude lower than the main salt e.g. KCl . Mg^{2+} has been most commonly used in experiments (optimum at 0.012 M-$MgCl_2$; So et al., 1967) but the reaction proceeds in the presence of Mn^{2+} (Furth et al., 1962 ; Geiduschek et al., 1961 ; Fuchs et al., 1967) or Co^{2+} (Fox & Weiss, 1964).

6) Mg^{2+} has differential effects on the binding of nucleotides to the two putative binding sites on the enzyme (Section 1C4).

1C7 KINETICS OF RNA POLYMERASE

RNA polymerase is a large enzyme which catalyzes a complex reaction, requiring four substrates, divalent cations and a DNA template. The reaction is a condensation polymerization and the precise composition of the product RNA is highly sensitive to the reaction conditions. The enzyme kinetic properties of RNA polymerase have not been studied as extensively as many other aspects of the enzyme's action. Part of the reason is almost certainly the problem of relating RNA synthesis to established theories of enzyme kinetics.

1C7.1 Effect of enzyme:template ratio on the rate of RNA synthesis

The rate of RNA synthesis by RNA polymerase depends on the relative concentrations of enzyme and template, as well as the nature of the template (Hurwitz *et al.*, 1962 ; Ishihama & Kameyama, 1967 ; So *et al.*, 1967 ; Zillig *et al.*, 1970). The rate of RNA synthesis increases approximately hyperbolically with DNA concentration. It has been suggested that the absolute concentration of enzyme affects the kinetics of RNA polymerase (Bremer, 1970 ; Downey & So, 1970). The latter authors do not, however, state whether this was under conditions of template excess (poly(dA-dT) in this case). Depending on ionic conditions, such concentration -dependent properties of the enzyme could arise from alterations in the equilibrium between enzyme monomer and its aggregates. Certainly the DNA:enzyme ratio influences the number of RNA chains synthesized and the average chain length (Maitra *et al.*, 1967 ; Downey & So, 1970). In general, many of the factors that affect the binding of the enzyme to DNA (Section 1C2) are likely to modify the variation of the reaction rate with DNA concentration ; for

instance, the presence of σ, ionic conditions, nature of the template.

1C7.2 Kinetics of the Reaction with respect to Substrates

The variation of RNA polymerase activity with substrate concentrations has been reported a number of times (See references in Table 1.2). Comparison of the various results is greatly complicated by the wide range of experimental conditions used, such as the enzyme source, purification and definition of subunit structure; template; ionic conditions; concentration of the fixed substrates; experimental design. Of particular interest is the fact that, in much of the work, enzyme of unspecified σ content was used, especially, but not only, in the earlier reports. σ strongly affects RNA polymerase kinetics (Sugiura et al., 1970). A number of different values for K_m have, thus, been quoted and both hyperbolic and non-hyperbolic kinetics have been observed (Table 1.2). It seems true to say that the kinetic behaviour of RNA polymerase has not been unequivocably established.

In many instances, a simple hyperbolic rate law appears to have been adequate to describe the behaviour of RNA polymerase. The interpretation of the derived parameters, K_m and V is of course, uncertain but they are presumably characteristic of the enzyme under given conditions. In other experiments, non-hyperbolic velocity-substrate curves were observed. With a number of synthetic single stranded polyribonucleotide templates, Niyogi & Stevens (1965 a,b) reported a non-hyperbolic response to substrate concentration, as did Straat & Ts'o (1969) with single-stranded synthetic templates and the enzyme from M.luteus. In the latter work, corresponding double-stranded templates gave a hyperbolic response. For all of these synthetic templates, apparent

K_m's were significantly higher than for natural DNA's.

In experiments using naturally occurring DNA's, (T4DNA, calf thymus DNA and M.luteus DNA), Anthony et al., (1969) also observed non-hyperbolic kinetics under certain conditions. Hyperbolic behaviour, however, was obtained either by pre-initiating RNA synthesis or by maintaining a high concentration of the substrate that was 5'-terminal in the product RNA. In general, these authors observed a differential response of the reaction rate to purine nucleotides, in particular the initiating nucleotide, as compared to pyrimidine nucleotides. In the case of an M. luteus DNA template, for which 85% of the transcribed RNA is 5'-terminal in guanine, the K_m with respect to GTP was an order of magnitude larger than for ATP, CTP, or UTP. Anthony et al., (1969) relate their findings, along with the results of equilibrium dialysis (Wu & Goldthwait, 1969a) and fluorescence quenching (Wu & Goldthwait, 1969b) to the two-site model for polymerase action already described in Section 1C4. The kinetics with respect to a nucleotide involved in initiation, which must be a purine, are different (sigmoid, high apparent K_m) than for a nucleotide involved only in polymerization (hyperbolic, low apparent K_m). Similar results were obtained with poly (dA-dT).

For the poly (dA-dT) directed reaction, Downey & So (1970) found no such differential kinetics for ATP compared with UTP, the apparent K_m's being equal, even when only initiation was measured. The dimer ApU appeared to allow the chemical initiation step to be bypassed, but no other dinucleotide had this property. The authors interpreted their results on the basis that the formation of the first phosphodiester linkage was rate limiting, and a bimolecular reaction. Elongation was considered a uni-molecular process.

The reason for the disparity between the results of Anthony et al., (1969) and Downey & So (1970) for poly(dA-dT) directed synthesis is not apparent. It may be significant that neither set of authors define the σ content of their enzyme preparations, which could strongly influence the observed kinetics (Sugiura et al., 1970). Non-hyperbolic behaviour could be explained if the enzyme had allosteric properties. Anthony et al., (1969) argue against this suggestion on several experimental grounds, the most compelling being the failure of ATP to stimulate poly C production with a polydI: poly dC template (Anthony et al., 1966). However, the possible existence of allosteric effects in RNA polymerase kinetics will require more careful experimentation.

Several theoretical treatments of biopolymerizations have appeared (Volkenstein et al., 1963 ; Zimmerman & Simha, 1966 ; MacDonald et al., 1968 ; Maniloff, 1969) but currently experimental data for the RNA polymerase reaction is inadequate for the rigorous testing of what are generally complex model equations. Most kinetic data has been analysed by the estimation of V and apparent K_m even where strict adherence to hyperbolic rate law was not observed. This is justifiable firstly as a characterization of the enzyme preparation and also in view of the difficulty in relating the RNA polymerase reaction to more conventional enzyme kinetic formulations. Bremer (1967), and Hyman & Davidson (1970) have quantitated RNA synthesis in terms of the sum of the times (step times) for the addition of each ribonucleotide. This effectively defines a steady state rate equation of the form

$$v = \frac{agcu}{gcu.K_1 + agu.K_2 + acu.K_3 + acg.K_4}$$

This equation cannot, however, explain any variations from hyperbolic behaviour when a single substrate is varied, but may be applicable under certain conditions.

variable substrate	fixed substrate	apparent K_m (mM)	template	ref.	comments
ATP UTP CTP GTP	other 3	0.005 0.008 0.012 0.012	Salmonella typhimurium DNA	1	
ATP UTP ATP ATP ATP	- - - - UTP	0.6 0.6 0.6 4.0 1.6	polyC polyA polyU polyAU polyAU	2	hyperbolic and non-hyperbolic
ATP UTP	- -	0.5-0.7 0.5-0.7	polyU polyA	3	
all 4	-	0.12	T4 DNA	4	hyperbolic
GTP ATP CTP UTP all 4 other 3	other 3 other 3 other 3 other 3 - GTP	0.15 0.016 0.015 0.013 0.17 0.082	M. luteus DNA	5	hyperbolic and non-hyperbolic
ATP CTP	other 3	0.015 0.015	calf thymus DNA		
ATP UTP both	UTP ATP -	0.03* 0.013 0.08*	poly (dA-dT)		
CTP GTP	other 3	0.014 0.011	T4 DNA	6	hyperbolic
ATP ATP UTP UTP ATP ATP UTP UTP	- - - - - - - -	0.7 1.0 1.1 1.2 0.4 0.1 0.9 0.3	rU rA.rU rA rA.rU dT dA.dT dA dA.dT	7	M. luteus enzyme. hyperbolic and non-hyperbolic

Table 1.2 Apparent K_m's for RNA polymerase - part one

variable substrate	fixed substrate	apparent K_m (mM)	template	ref.	comments
all 4	-	0.63 0.63	T4 DNA T7 DNA	8	hyperbolic
GTP CTP ATP UTP	other 3	0.03* 0.02* 0.03* 0.03*	T4 DNA	9	hyperbolic
ATP UTP	UTP ATP	0.025 0.025	poly (dA-dT)		hyperbolic and non-hyperbolic
ATP	UTP	0.03	poly (dA-dT)	10	initiation measured
both	-	0.02	poly (dA-dT)		high enzyme
GTP ATP CTP	other 3	0.011* 0.011* 0.007*	T7 DNA	11	hyperbolic
ATP ATP ATP ATP GTP GTP GTP GTP	other 3	0.17* 0.03* 0.06* 0.03* 0.17* 0.03* 0.05* 0.03*	T4 DNA phage fd RF 1 DNA T4 DNA phage fd RF 1 DNA	12	$-\sigma$ $+\sigma$ $-\sigma$ $+\sigma$ hyper- $-\sigma$ bolic $+\sigma$ $-\sigma$ $+\sigma$
all 4	-	0.07* 0.32*	E. coli DNA	13	direct filtering. acid precipitate

Table 1.2 Apparent K_m's for RNA polymerase - part two

* denotes values calculated from reported data.
In ref. 7, a different notation for polynucleotides is used for brevity. Enzyme from E. coli except where indicated.

References :

1. Stevens & Henry, (1964)

2. Niyogi & Stevens, (1965a)

3. Niyogi & Stevens, (1965b)
4. Bremer, (1967)
5. Anthony et al., (1969)
6. di Mauro et al., (1969)
7. Straat & Ts'o, (1969)
8. Bremer, (1970)
9. Cassani et al., (1970)
10. Downey & So, (1970)
11. Hyman & Davidson, (1970)
12. Sugiura et al., (1970)
13. Nazar et al., (1972)

SECTION 2

MATERIALS AND INSTRUMENTS

2A MATERIALS

2A1 CHEMICALS

In alphabetical order :

Acrylamide	BDH Chemicals Ltd. (Poole), England This chemical was recrystallized from benzene before use.
Amido black (Naphthalene black 10 B)	George T. Gurr, Searle Scientific Services, High Wycombe, England
Ammonium sulphate	BDH enzyme grade (especially low in heavy metals)
Bromophenol blue	BDH pH indicator
Dithiothreitol	Koch Light Laboratories Ltd., Colnbrook, England
2,5-Diphenyloxazole (PPO)	Koch Light
Ethidium bromide	Calbiochem, Los Angeles, California 90054
Hyamine hydroxide	Nuclear Enterprises (GB) Ltd., Sighthill, Edinburgh, Scotland
Mercaptoethanol	Koch Light
Nucleotides :	
ATP, ADP, CTP, GTP, UTP	P.L Biochemicals Inc. (Milwaukee)
2',3'-AMP, 2',3'-UMP, pppAp	Sigma London Chemical Co. Ltd., Kingston-upon-Thames, England
Radioactive nucleotides (^{3}H and ^{32}P)	The Radiochemical Centre (Amersham), England

NN'-Methylene-bisacrylamide	BDH (Analar)
NNN'N'-Tetramethylene diamine	BDH (Analar)
Phosphoenol pyruvate	Sigma London
Unisolve	Koch Light

Other chemicals were BDH (Analar) grade .

2A2 CHROMATOGRAPHIC MATERIALS

Biogel A1.5M and A5M	Bio Rad, Richmond, California
DEAE-cellulose DE 52 (Cat.No.24521)	Whatman Biochemicals Ltd., Maidstone, Kent, England
DEAE-Sephadex A25	Pharmacia Fine Chemicals, Uppsala, Sweden
Dowex AG 50W-X2 Hydrogen form	Bio Rad
Phosphocellulose (P11; Cat.No.21112)	Whatman

2A3 BIOLOGICAL MATERIALS (INCLUDING SYNTHETIC POLYNUCLEOTIDES)

BSA	Armour Pharmaceutical Co. Ltd., Eastbourne, Essex, England
DNA's	
1) Calf thymus DNA	Schwartz/Mann, Orangeburg, N.Y. 10962
2) T7 DNA	was a gift from D.J.Jolly
3) Poly(dA-dT)	Miles Laboratories Inc., Research Products Div., Elkhart, Indiana 46514 ; or Biopolymers Laboratory, General Biochemicals, Chagrin Falls, Ohio 44022
DNase (electrophoretically pure)	Sigma London

E. coli	*E. coli* MRE 600 were obtained from the Microbiological Research Establishment, Porton, Salisbury, England. The cells, grown in continuous culture at 37°C under conditions of carbon limitation, were harvested as described by Elsworth *et al.* (1968) and washed with a buffer of 0.01 M-$Mg(CH_3CO_2)_2$; 0.01 M-tris-HCl, pH 7.4. Cells were stored at -70°C.
Oligoadenylates	Miles Laboratories
Polynucleotide phosphorylase (*E. coli* and *M. luteus*)	P.L Biochemicals
Pyruvate kinase	Sigma London
Yeast tRNA	BDH

2A4 OTHER MATERIALS

Dialysis tubing (Visking)	The Scientific Instrument Centre Ltd., 1, Leeke St., London Before use, tubing was boiled successively in 50 g/l Na_2CO_3 (twice), 0.05 M-EDTA, pH 7 and distilled water.
Filters	
1) Membrane filters (0.45μ pore size)	Sartorius Membran filter GmbH, Gottingen, Germany
2) Glass filters	Whatman GF/C

2B INSTRUMENTS

2B1 SPECTROPHOTOFLUORIMETER

All fluorescence measurements were made on an Aminco-Bowman Spectrophotofluorimeter (American Instrument Co. Inc., Silver Spring, Maryland ; Cat. No. 4-8106B). During the course of this work, the original Photomultiplier Microphotometer was replaced by a solid-state version, with blank subtract facilities (Cat. No. 10-280). The fluorimeter was coupled to an X-Y recorder in order to record spectra (Advance Electronics, model HR-96). The fluorimeter is a single beam instrument, and usually absolute measurements were not made. The instrument was used mostly to measure RNA concentration, and to quantitate this determination, a standard RNA sample was used for reference.

Various components of the spectrophotofluorimeter have been changed during this work and there have also been adjustments to the settings of the instrument, particularly the slit arrangements.

1) Xenon lamps : lamp 1
lamp 2 These were both standard Xenon lamps as supplied by Aminco.

2) Photomultiplier tubes :
tube 1 IP 28
tube 2 R136 both supplied by Aminco.

3) Slit arrangements

The various slits, situated around the cell compartment of the fluorimeter, are shown in Fig. 2.1 . A further slit, slit 7, is located just in front of the photomultiplier tube.

The photomultiplier tube was always operated at a voltage of 700 v.

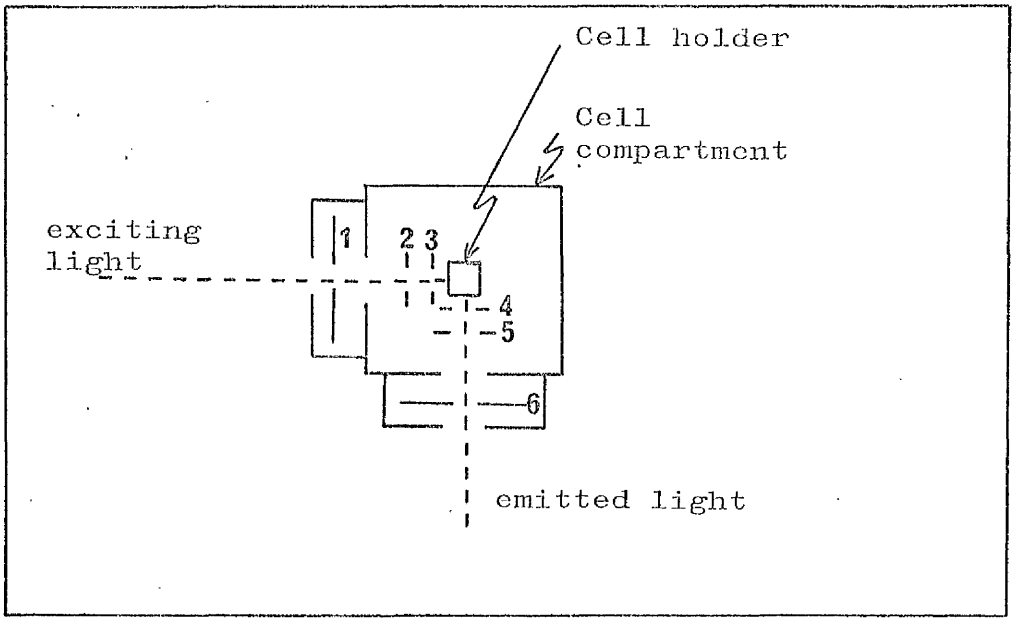


Fig. 2.1 Location of slits in Aminco-Bowman Spectrophotofluorimeter

2B2 OTHER INSTRUMENTS

Other instruments are described at various points in the text, with the exception of the following :

1) pH was measured on a Beckman Zeromatic pH meter.
2) u.v. and visible light absorption was measured on a Cary 15 recording spectrophotometer.
3) A PDP 8L (Digital Equipment Co. Ltd.) was used for numerous calculations that are not described in detail.

Nucleotide	Molar Extinction Coefficient (litre.mol^{-1}.cm^{-1})	Wavelength (nm)
ATP	15400	259
CTP	9000	271
GTP	13700	252
UTP	10000	262
ADP	15000	259

Table 3.1 Extinction coefficients of some nucleotides.

Reference : P.L. Biochemicals Inc. (Milwaukee) Reference Catalog 102 (1970).

Nucleic Acid	ε(P) (litre.mol^{-1}.cm^{-1})	Reference
T7 DNA	6550	Crothers & Zimm (1965)
Calf Thymus DNA	6600	Mahler & Cordes (1967)
Poly(dA-dT)	6650	Inman & Baldwin (1964)

Table 3.2 Exctinction coefficients (at 260 nm) of some nucleic acids.

SECTION 3

MEASUREMENTS AND ANALYSES

3A PROTEIN DETERMINATION

Protein concentrations were routinely estimated by the method of Lowry *et al.* (1951), using three times recrystallized bovine serum albumin as standard. On some occasions, the concentration of purified RNA polymerase solutions was determined by measuring the u.v. absorption at 280 nm. A specific extinction coefficient at 280 nm of 6.5 was used (Richardson, 1966b).

3B NUCLEOTIDE DETERMINATIONS

The concentration of nucleotide solutions was calculated from u.v. absorption. The molar extinction coefficients of Table 3.1 were used.

3C DNA DETERMINATION

DNA concentrations were invariably measured from u.v. absorption at 260 nm. The extinction coefficients of Table 3.2 were used.

3D RNA DETERMINATION

The measurement of RNA concentration is the basis of the assay for RNA polymerase activity described later in Section 5. The concentration of yeast tRNA, which was used as a reference standard, was determined by u.v. absorption, as described by Fleck & Munro (1962). A suitable dilution of the standard RNA was incubated at 37°C in 0.3 M-NaOH until the absorption at 260 nm was constant (approx. 5 h). The

molar extinction coefficient with respect to phosphorus was taken as $\varepsilon(P) = 9700\ l.mol^{-1}.cm^{-1}$ at 260 nm.

3E MAGNESIUM DETERMINATION

The method for Mg^{2+} determination was derived from that described by Schachter (1959). The change in fluorescence of 8-hydroxyquinoline is measured when it forms a complex with Mg^{2+} ions (Burton, 1959). The 8-hydroxyquinoline reagent was 2.5 g/l 8-hydroxyquinoline and 800 ml/l ethanol, buffered by 0.01 M-tris-HCl, pH 7.9. Samples to be estimated were diluted 1:500 with 8-hydroxyquinoline reagent and the fluorescent intensity was measured on an Aminco-Bowman spectrophotofluorimeter. The excitation wavelength was 415 nm and the emission wavelength 520 nm. $MgCl_2$, $MgSO_4$ and $Mg(CH_3COO)_2$ were used as reference standards. There was a linear relationship between fluorescent intensity and final Mg^{2+} concentration (i.e. after dilution with 8-hydroxyquinoline reagent) up to a Mg^{2+} concentration of approx. 0.04 mM. The Mg^{2+} concentration in RNA polymerase assay solutions was estimated by reference to a standard curve.

3F SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

The method was based on that described by Shapiro et al. (1967).

3F1 PREPARATION OF GELS

Polyacrylamide gels were prepared from the following solutions.

Solution A was buffered by 1.5 M-tris (adjusted to pH 8.9 with 1 M-HCl) and contained 2.3 ml/l NNN'N'-tetramethylene diamine.

Solution B contained 200 g/l acrylamide and 6 g/l NN'-methylenebisacrylamide, made up to volume with 0.1 M-sodium phosphate buffer, pH 7.1, which was also 1 g/l with respect to sodium dodecyl sulphate.

Solution C contained 1.4 g/l ammonium persulphate, made up to volume with 0.1 M-sodium phosphate buffer, pH 7.1, which was also 1 g/l with respect to sodium dodecyl sulphate.

Solutions A and B were stored in the dark at $4^{o}C$ and were renewed every 4 weeks. Solution C was prepared freshly before each experiment.

The final 'gel' solution was prepared immediately before use and consisted of Soluton A (1 volume), Solution B (1 volume) and Solution C (2 volumes). This gave a gel 5% (w/v) with respect to acrylamide. The gels were formed in clean, constant bore glass tubes of internal diameter 5 mm and length 100 mm. With the gel tube sealed at the bottom and in a vertical position, 1.5 ml of 'gel solution' were added to each tube and overlaid with about 0.2 ml of distilled water. Polymerization of the 'gel solution' occurred in 30-45 min

although in the most recent experiments, this time was decreased to 15 min by increasing the ammonium persulphate in Solution C to 2 g/l. The distilled water was removed from the top of the gel just before the application of the sample.

3F2 PREPARATION OF SAMPLES

Protein samples were incubated for approx. 3 h at 37°C in 0.01 M-sodium phosphate buffer, pH 7.1, which contained also 10 g/l mercaptoethanol. The samples were then dialysed for about 16 h at room temperature against 500 ml of 0.01 M-sodium phosphate buffer, pH 7.1, containing 1 g/l sodium dodecyl sulphate and 1 g/l mercaptoethanol.

A solution of Bromophenol blue and glycerol was prepared by mixing glycerol and a saturated aq. Bromophenol blue solution in the proportions 9:1 by vol. The Bromophenol blue solution was added to the dialysed protein samples (Bromophenol blue-sample, 1:3 by vol.) before application to the gel.

The volume of the sample applied to the gel depended on protein concentration and was preferably from 5-100µl, although up to 200 µl volumes have been successfully run on occasion. The amount of protein applied depended on the experimental aim. For good resolution, 10-20 µg were optimal but when for example looking for trace components in enzyme preparations, more protein was applied.

3F3 ELECTROPHORESIS

The electrode buffer was 0.1 M-sodium phosphate with 1 g/l sodium dodecyl sulphate. Electrophoresis was carried out at 8 mA per tube, with the positive electrode in the lower compartment and was continued until the Bromophenol blue was about 5 mm from the bottom of the tube.

3F4 FIXING AND STAINING

After removal from the glass tubes, the gels were fixed for approx. 16 h with 200 g/l sulphosalicylic acid solution. The gels were then stained for about 3 h in an Amido black solution (10 g/l Amido black, 70 ml/l glacial acetic acid) from which the insoluble material had been removed by filtration through filter paper. Destaining was carried out by immersion in 70 ml/l acetic acid solution and took about 12 h.

3F5 ANALYSIS OF GELS

Gels were successfully stored, in 70 ml/l acetic acid, in sealed test tubes for a period of months. In most experiments, the final gels were photographed, to provide a permanent record, soon after being destained. In some cases the photographic method of Oliver & Chalkley (1971) was used.

The main reason for polyacrylamide gel electrophoretic analysis in this study was to assess the subunit composition of RNA polymerase preparations and to monitor the presence of trace protein components in these preparations. Since the α, β, and β' chains of the RNA polymerase were commonly present in the samples for analysis, the stained bands corresponding to these polypeptides could be used as reference points for visual comparison of different gels. The reproduciblity of the method meant that this procedure could be used for visual assignment of other protein components which were present. However, by utilising the mathematical relationship between mol.wt. and electrophoretic mobility (Shapiro *et al.*, 1967 ; Shapiro & Maizel, 1969 ; Weber & Osborn, 1969), more precise information could be obtained.

Electrophoretic mobility is defined as

$$\text{mobility} = \frac{\text{distance of protein migration}}{\text{distance of marker dye migration}}$$

In the fixing and staining procedure, Bromophenol blue diffuses out of the gel. However, before this, the protein bands cannot be seen so that the marker dye and the stained protein bands are never visible simultaneously. It is usual then to measure the migration of the dye before fixing and staining, and the migration of the protein bands after destaining. A correction must then be applied for the change in total length of the gel that occurs in the acidic conditions used for staining and destaining. Then,

$$\text{mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

$$= \frac{d_i}{l_2} \times \frac{l_1}{s}$$

It was found (Shapiro et al., 1967 ; Weber & Osborn, 1969) for a certain mol.wt. range, that the mol.wt., M, is related to mobility, r, by the relationship

$$M = a.10^{-br} \qquad \text{eqn. 3.1}$$

where a and b are constants which depend on a number of factors of which the gel composition is important. With a given gel composition, there is a linear relationship between log M and r over the mol.wt. range for which eqn. 3.1 is applicable. Thus, the mol.wt.'s of unknown polypeptides may be determined either by means of a standard curve or by reference to standards run at the same time as the unknowns. Both procedures, however, depend on the availability of

reference proteins of known mol.wt.

Eqn. 3.1 is applicable only over a certain mol.wt. range and becomes concave up at high mol.wt. The linearity of the plot of log M against mobility is extended to higher mol.wt.'s as the acrylamide content of the gel is decreased. However, the estimation of high mol.wt.'s (greater than say 100 000) is fraught with more uncertainty than lower ones for several reasons. There are few reference polypeptides of mol.wt. greater than about 80 000 and also the linearity of the log M against r plot is more uncertain at high mol.wt. Further, the logarithmic relationship of eqn. 3.1 means that, at low mobility, a small uncertainty in the mobility yields an amplified uncertainty in the mol.wt. obtained from eqn. 3.1 . These problems should be considered for any analysis using the relationship of eqn. 3.1 .

If one gel contains a number of polypeptides, then the migration of the marker dye is the same for each polypeptide. Since $r = d_i/s$, then eqn. 3.1 becomes

$$M = a.10^{-d_i b/s}$$
$$= a.10^{-d_i . b'} \qquad \text{eqn. 3.2}$$

where a and b' are constants.

Thus, for a single gel, the mol.wt. of each polypeptide component can be related to its migration distance, d_i , rather than its mobility, r_i . The constant b', which replaces b in eqn. 3.1, refers to a particular gel and will, in general, vary from gel to gel. The constant a on the other hand should be independent of marker dye migration. If two or more of the polypeptide bands on the gel have known mol.wt., then the mol.wt.'s of any others can be estimated using eqn. 3.2 .

Thus, by taking values for the mol.wt.'s of the major components of RNA polymerase preparations (β', β , α , σ , and θ ; see Section 1A2.2), the mol.wt.'s of any other polypeptide species present can be interpolated as described above. The migration distance, d_i , of polypeptide bands after SDS-polyacrylamide gel electrophoresis was measured from photographs of the original gels using vernier callipers. Even with this relatively simple means of measurement, graphs of log M against d_i for β , β', α , σ , and θ were consistently linear (Fig. 3.1). As discussed in Section 6C, trace bands in enzyme preparations were only observed at certain recurring mol.wt.'s. Also, the constant a of eqn. 3.2 was essentially invariant for a number of different gels run under similar experimental conditions (a = 341 000 ; S.E.M. = 17 300 for 10 different gels) and this gives us confidence in the use of eqn. 3.2 .

The values of mol.wt.'s derived from the above treatment depend entirely upon the values of mol.wt.'s adopted for the reference polypeptides. There is some variation in the published values of the mol.wt.'s of RNA polymerase subunits (Section 1A2.2). The values previously found in this laboratory were therefore used (Lochhead, 1972) as being consistent with the bacterial source and isolation procedure used in this work.

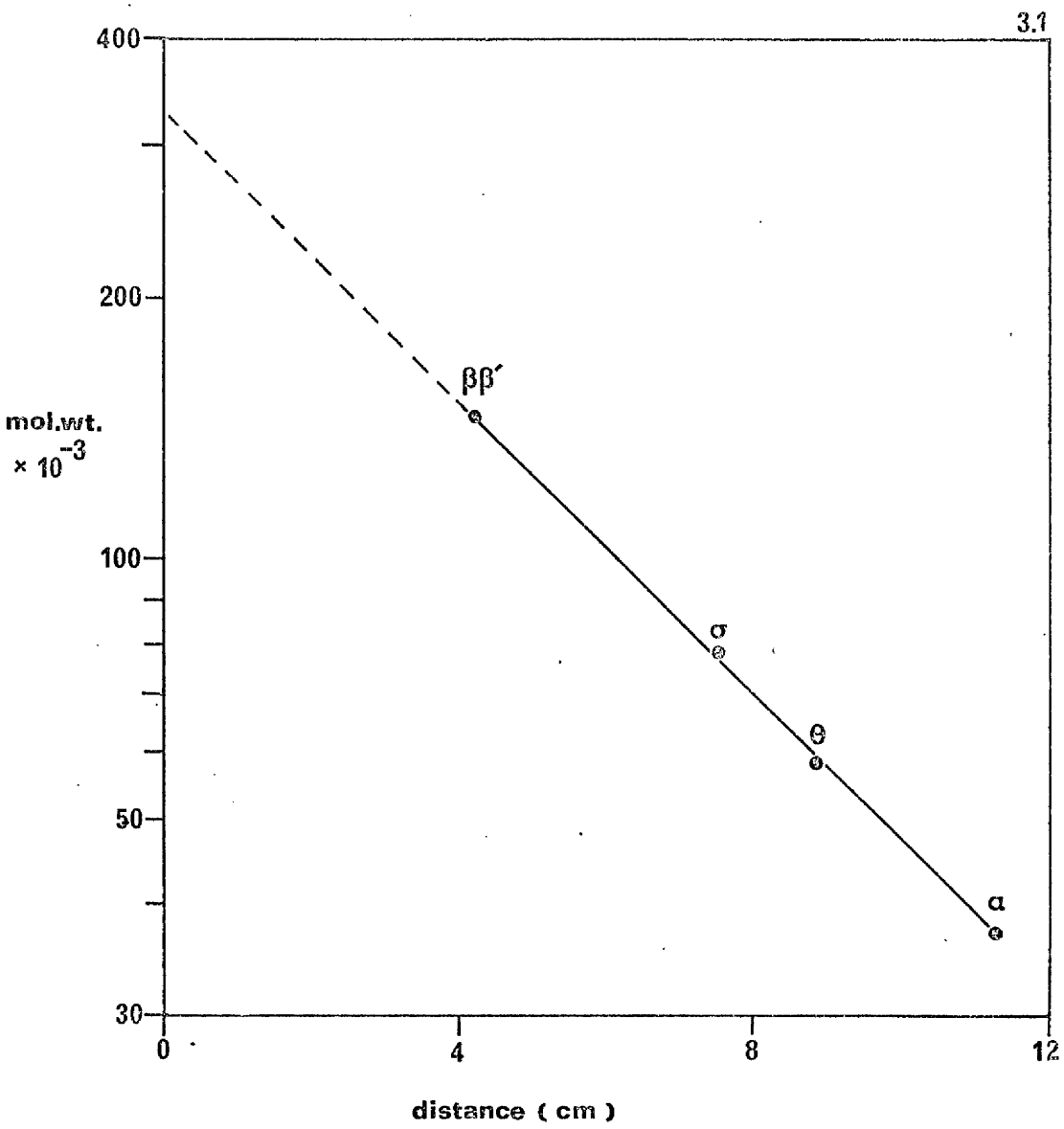
3G ANALYSIS OF NUCLEOTIDES BY PAPER CHROMATOGRAPHY

The method for the chromatographic separation of ribonucleotides was a modification of that described by Krebs & Hems (1953). The solvent for chromatography had the following composition : isobutyric acid (100 vol.), distilled water (55.8 vol.), concentrated NH_3 solution (4.2 vol.) and 0.1 M-EDTA (1.6 vol.). The solvent had a pH value of 4.6 .

Samples were applied to Whatman No. 3 paper and then subjected to descending chromatography with the solvent for 18 h at room temperature. The position of u.v. absorbing material was observed by spraying the chromatogram with a solution of fluorescein (20 mg/l fluorescein in methanol) after which nucleotides could be seen as dark spots under u.v. illumination.

Fig. 3.1 Relationship between mol.wt. of RNA polymerase subunits and their distance of migration after SDS-polyacrylamide gel electrophoresis.

The RNA polymerase sample was prepared by the routine purification procedure (Section 4A) and the electrophoresis was carried out as described in Section 3F. The mol.wt. axis of the graph is logarithmic so that the linear plot indicates the applicability of eqn. 3.2 . The values for the mol.wt.'s of the subunits were from Lochhead (1972) and $\beta\beta'$ represents the average of the mol.wt.'s of β and β' .


400
200
100
50
30
mol.wt.
× 10^-3
ββ´
σ
θ
α
0
4
8
12
distance (cm)

SECTION 4

PURIFICATION PROCEDURES

4A PURIFICATION OF RNA POLYMERASE

The procedure for the purification of RNA polymerase from E. coli was based on that of Burgess (1969a), with two agarose gel filtration steps replacing the final glycerol gradient centrifugations. The other difference from the Burgess method was the means used to disrupt the cells at the start of the purification. The following description is of the laboratory's routine enzyme isolation procedure (Lochhead, 1972)

4A1 BUFFER SOLUTIONS

The designation of the various buffers is that employed by Burgess (1969a) and the solutions were prepared from the following stocks.

1) 1 M-tris-HCl, pH 7.9 at 25°C
2) 1 M-tris-HCl, pH 7.5 at 4°C
3) 0.1 M-EDTA, pH 7.0
4) 1 M-$MgCl_2$
5) 0.1 M-dithiothreitol

These stock solutions were used to prepare the following buffers.

Buffer A : 0.01 M-tris-HCl pH 7.9 ; 0.01 M-$MgCl_2$; 0.1 mM-EDTA ; 0.1 mM-dithiothreitol and 50 ml/l glycerol.

Buffer C : 0.05 M-tris-HCl, pH 7.9 ; 0.1 mM-EDTA ; 0.1 mM-dithiothreitol and 50 ml/l glycerol.

Buffer G : 0.05 M-tris-HCl, pH 7.5 ; 0.01 M-$MgCl_2$; 0.1 mM-EDTA ; 0.2 M-KCl and 50 ml/l glycerol.

Buffers with added KCl are described as, for example, A + 1.0 M-KCl. This means 1 mol of KCl made up to 1 litre with buffer A.

4A2 CELL DISRUPTION

Frozen *E. coli* MRE 600 were suspended in 300 ml of Buffer G in an Automix blender. The cells were disrupted by shaking the bacterial suspension with glass beads (diameter 0.1 mm) for 2 min at 80 Hz in a vibrational homogeniser (Zillig & Holzel, 1958). Bacterial suspension (90 ml) was mixed with cold, acid-washed glass beads (135 ml) in the 200 ml stainless steel vessel of the homogeniser and the shaking was done at 4°C. The combined homogenates were diluted with 300 ml of Buffer G. To this, 2.5 mg of DNase in Buffer G were added and the homogenate left at 4°C for 30 min, with occasional stirring. The glass beads were then removed by filtering the homogenate through glass wool with gentle suction. The beads were re-extracted several times with Buffer G. Excessive foaming was avoided and the combined filtrates were fraction 1 (about 1 litre).

4A3 ULTRACENTRIFUGATION

Fraction 1 was centrifuged at 78 000 g in a Spinco 30 rotor for 4 h to remove cell debris and ribosomes in one step. The supernatant, about 800 ml, was fraction 2.

4A4 AMMONIUM SULPHATE FRACTIONATION

Ammonium sulphate (23.1 g per 100 ml) was added very slowly, with stirring, to fraction 2 to give a 33% saturated solution. The pH was prevented from dropping below 7 by the addition of 1 M-NaOH if necessary. After being stirred for 30 min, the solution was centrifuged in an MSE 6L centrifuge in 750 ml polypropylene bottles at 5200 g for 45 min at 4°C. The precipitate was discarded. Further ammonium sulphate (10.75 g per 100 ml) was added, as above, to give a 50% saturated solution, which was centrifuged under the conditions

described above. The precipitate, which contained the RNA polymerase was suspended in 260 ml of Buffer A, 42% (w/v) in ammonium sulphate, stirred for 30 min and centrifuged under the above conditions for 70 min. The resulting pellet contained the enzyme and was dissolved in Buffer A. More Buffer A was added until the specific conductivity of the solution was about 9.6 $mMho.cm^{-1}$, as measured by a Radiometer conductivity meter (type CDM 2e), to give fraction 3 (about 500 ml).

4A5 DEAE-CELLULOSE CHROMATOGRAPHY

Fraction 3 was applied to a DEAE-cellulose column (2.5 by 20 cm) which had been equilibrated with Buffer A. The column was eluted stepwise, first with 600 ml of Buffer A and then with about 300 ml of Buffer A + 0.13 M-KCl (Fig. 4.1). The fraction containing the RNA polymerase was finally eluted with Buffer C + 0.23 M-KCl. This was fraction 4.

4A6 LOW SALT AGAROSE GEL FILTRATION CHROMATOGRAPHY

The protein of fraction 4 was precipitated by the addition of 1.5 volumes of Buffer C saturated with ammonium sulphate, and the suspension centrifuged at 5200 g in the MSE 6L centrifuge for 70 min. The precipitate was taken up in 20 ml of Buffer C and dialysed for 5 h against Buffer C. This protein solution was applied to a column of Biogel A5M (5 by 95 cm) which had been equilibrated with Buffer C. The column was eluted with Buffer C at a flow rate of 60 ml/h (Fig. 4.2). The fractions containing the enzyme activity were pooled to give fraction 5.

4A7 HIGH SALT AGAROSE GEL FILTRATION CHROMATOGRAPHY

The protein in fraction 5 was precipitated and collected in the same way as fraction 4. The pellet after centrifugation was dissolved in 4 ml of Buffer A + 1.0 M-KCl and applied to a column of Biogel A1.5M (2.5 by 95 cm) which had been equilibrated with Buffer A + 1.0 M-KCl. The column was eluted with this same buffer at a flow rate of 24 ml/h (Fig. 4.3) and the tubes containing the enzyme activity pooled to give fraction 6.

4A8 STORAGE OF ENZYME AND GENERAL COMMENTS

Fraction 6 was usually concentrated by ammonium sulphate precipitation in the same way as that described for fractions 4 and 5. Centrifugation was at 11 000 g for 40 min in an MSE 18 centrifuge. The enzyme was stored in a buffer containing 0.01 M-tris-HCl, pH 7.9 ; 0.01 M-$MgCl_2$; 0.1 M-KCl ; 0.1 mM-dithiothreitol ; 0.1 mM-EDTA and 500 ml/l glycerol at a concentration of protein preferably greater than 5 mg/ml. At - 20 ^{o}C, the enzyme remained active for a period of months.

In the preceding preparative procedure, all manipulations were carried out at 4^{o}C where temperature is unspecified. In routine purifications, the reproducibility of the method meant that few enzyme assays were required.

4B PURIFICATION OF RNA POLYMERASE CORE ENZYME

RNA polymerase core enzyme was prepared from σ and θ containing enzyme by chromatography on phosphocellulose (Bautz et al., 1970).

The phosphocellulose was prepared as follows. After being stirred for 30 min in approx. 5 volumes of 0.5 M-NaOH, the phosphocellulose was washed with distilled water until the pH of the washings was 8. The phosphocellulose was then suspended in about 5 volumes of 0.5 M-HCl, stirred for 30 min and rinsed with distilled water until the pH of the washings was 6. About 4 four volumes of 0.05 M-tris-HCl, pH 7.9, were added to the washed phosphocellulose and the slurry stirred for 15 min after which the pH of the slurry was adjusted to pH 7.9 by the addition of 6 M-KOH.

The treated phosphocellulose was poured to form a column of dimensions 2.5 cm by 20 cm, which was equilibrated with Buffer C + 0.05 M-KCl until the pH and conductivity of the effluent from the column was identical with that of Buffer C + 0.05 M-KCl. The sample to be applied to the column (about 10 mg of RNA polymerase , fraction 6 ; see Section 4A7) was dialysed for 16 h at 4°C against Buffer C + 0.05 M-KCl. The dialysed enzyme was applied to the column slowly, followed by Buffer C + 0.05 M-KCl. The flow rate was 36 ml/h. After the wash-through peak of protein (peak A) had been eluted, the salt concentration of the eluting buffer was increased to 0.25 M-KCl. This buffer eluted another protein peak (peak B). The core enzyme (peak C) was released from the column by the passage of Buffer C + 0.4 M-KCl (Fig. 4.4). The RNA polymerase core enzyme was concentrated and stored under the same conditions as the routine enzyme preparations (Section 4A8)

4C GLYCEROL GRADIENT CENTRIFUGATION

Glycerol gradient centrifugation was used for investigating the effect of different preparative procedures on the composition of RNA polymerase preparations from *E. coli* MRE 600.

The glycerol gradients were run either at low or at high KCl concentration and were prepared as follows. Buffer solutions (Buffer A for low salt or Buffer A + 1.0 M-KCl for high salt) were made

a) 100 ml/l with respect to glycerol

b) 300 ml/l with respect to glycerol

by the addition of glycerol. Using a simple mixing device, linear gradients were formed from equal volumes of each glycerol concentration. The gradients were collected in cellulose nitrate test tubes that fitted the bucket of a Spinco SW 25.2 rotor. The protein sample, which had been dialysed for 2 h at 4°C against the appropriate buffer (Buffer A or Buffer A + 1.0 M-KCl), was layered carefully onto the gradient. Up to 60 mg of protein were applied to each tube. Centrifugation was at 25 000 rev./min and 4°C for either 24 h (low salt) or 36 h (high salt). The gradients were then analyzed by passage through the flow cell of a Gilford spectrophotometer set to record absorption at 280 nm. Fractions were collected manually.

4D PURIFICATION OF ATP AND UTP

ATP and UTP were purified by ion exchange chromatography, eluting with a gradient of the volatile triethylammonium carbonate buffer system (Brown & Reichard, 1969 ; Smith & Khorana, 1963).

4D1 PREPARATION OF TRIETHYLAMMONIUM CARBONATE BUFFER

A stock solution of 1 M-triethylammonium carbonate, pH 7.5, was prepared. For 1 litre, CO_2 was passed through a mixture of 500 ml of distilled water and 140 ml of freshly redistilled triethylamine, cooled in an ice bath. The passage of CO_2 was continued until the pH decreased to pH 7.5, a process that took up to 6 h. The pH 7.5 solution was diluted to 1 litre volume to give the stock buffer, from which buffers of other concentrations were prepared by dilution.

4D2 ION EXCHANGE CHROMATOGRAPHY

Nucleoside triphosphates, purchased from P.L. Biochemicals (Milwaukee) were applied to a column of DEAE-Sephadex A25 which had been equilibrated with the appropriate starting concentration of triethylammonium carbonate buffer. About 0.3 g of nucleotides were applied to a column 5 cm diameter by 20 cm. The column was eluted with a concentration gradient of the buffer.

4D2 .1 ATP

The elution gradient was formed by mixing 1 M-triethylammonium carbonate buffer, pH 7 5, with 400 ml of a 0.1 M solution of the same buffer at a flow rate of 54 ml/h. 9 ml fractions were collected. ATP was observed at an elution volume of about 700 ml and was preceded by traces of ADP and AMP.

4D2.2 UTP

In this instance, the elution gradient was formed by running 1 M-triethylammonium carbonate buffer pH 7.5, into 250 ml of a 0.3 M solution of the same buffer at a flow rate 34 ml/h. Fractions of 9 ml were collected. The elution volume for UTP was about 400 ml. With this nucleotide, an appreciable amount of UDP as well as traces of UMP and two unidentified

compounds which absorbed light at 260 nm were observed.

4D2.3 Analysis of purified nucleotides

For both ATP and UTP, the pooled fractions from the chromatographic separation were freeze dried and analyzed by paper chromatography as described in Section 3G. Under these conditions, both ATP and UTP appeared as single spots on the paper chromatograms. Evidence for lack of diphosphate contamination was further provided by the failure of the purified nucleoside triphosphates to act as substrates for putative polynucleotide phosphorylase activity in RNA polymerase preparations (Section 6C2)

Fig. 4.1 RNA polymerase purification : DEAE-cellulose chromatography.

RNA polymerase, fraction 3, was applied to a column of DEAE-cellulose. Elution was stepwise, using Buffer A, Buffer A +0.13 M-KCl and Buffer C + 0.23 M-KCl in that order. The solid line indicates the absorption of the effluent at 280 nm , measured as it passed through a 3 mm pathlength cell of an LKB Uvicord. The broken line is the RNA polymerase activity measured by the standard radioactive assay (Section 5C) with a calf thymus DNA template (150 μg/ml). The column dimensions were 2.5 cm by 20 cm. The sample was applied to the column at 54 ml/h and eluted at 90 ml/h. 15 ml fractions were collected.

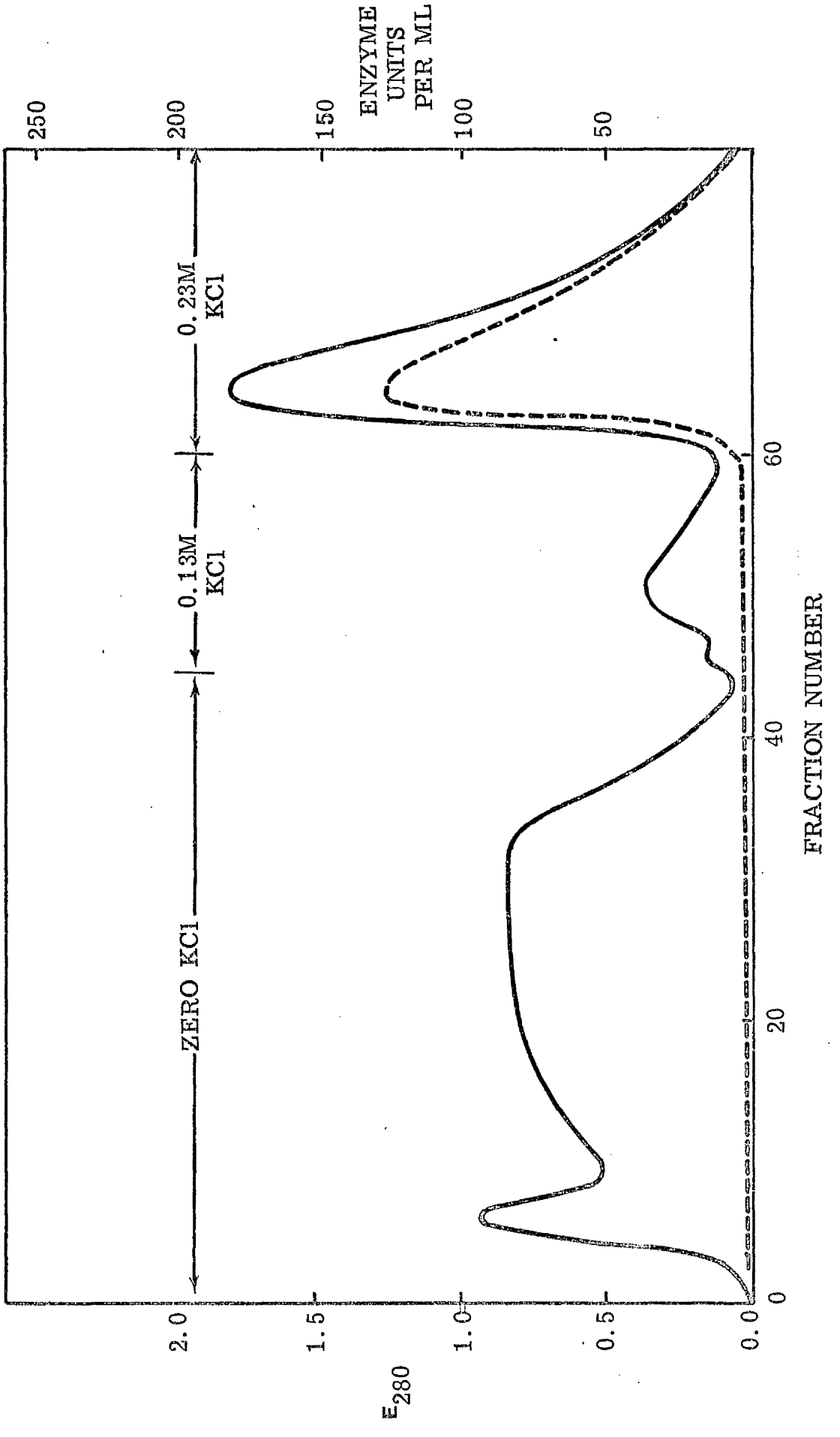

ENZYME UNITS PER ML
250
200
150
100
50
0.23M KCl
0.13M KCl
ZERO KCl
2.0
1.5
1.0
0.5
0.0
E_{280}
0
20
40
60
FRACTION NUMBER

Fig. 4.2 RNA polymerase purification : low salt agarose gel filtration chromatography.

RNA polymerase, fraction 4, was applied to a column of Biogel A5M. The column was eluted, at a flow rate of 60 ml/h, with Buffer C. The solid line indicates the u.v. absorption of the effluent from the column at 280 nm, measured as it passed through the 3 mm pathlength cell of an LKB Uvicord. The broken line is the RNA polymerase activity measured by the standard radioactive assay procedure (Section 5C) with a calf thymus DNA template (150 μg/ml). The column dimensions were 5 cm by 65 cm and the fraction size was 6 ml.

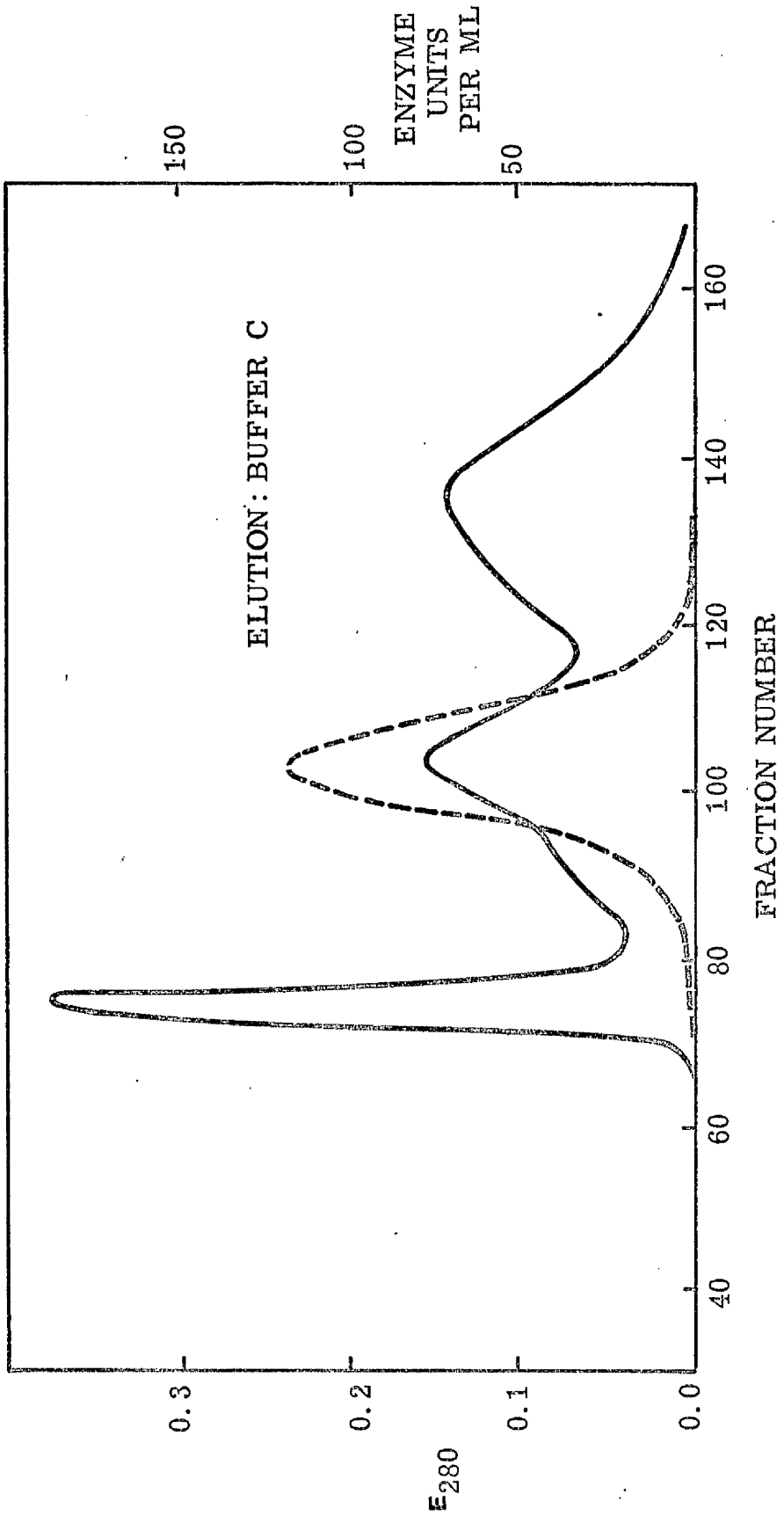

ELUTION: BUFFER C
ENZYME UNITS PER ML
150
100
50
E_{280}
0.3
0.2
0.1
0.0
40
60
80
100
120
140
160
FRACTION NUMBER

Fig. 4.3 RNA polymerase purification : high salt agarose gel filtration chromatography.

RNA polymerase, fraction 5, was applied to a column of Biogel A1.5M. The column was eluted, at a flow rate of 24 ml/h, with Buffer A + 1.0 M-KCl. The solid line indicates the u.v. absorption of the effluent from the column at 280 nm, measured as it passed through the 3 mm pathlength cell of an LKB Uvicord. The broken line is the RNA polymerase activity measured by the standard radioactive assay procedure (Section 5C) with a calf thymus DNA template (150 μg/ml). The column dimensions were 2.5 cm by 95 cm and the fraction size was 6 ml.

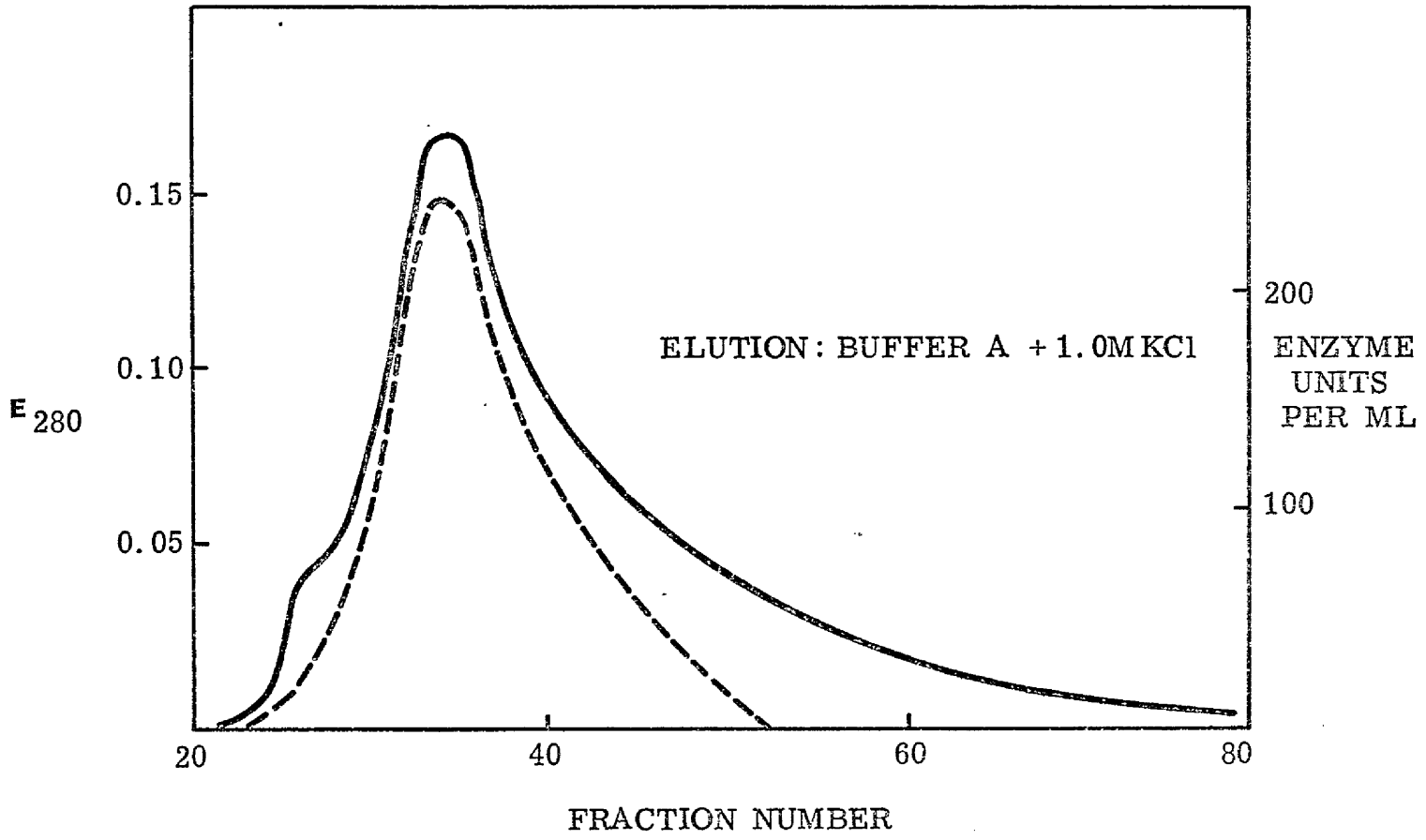
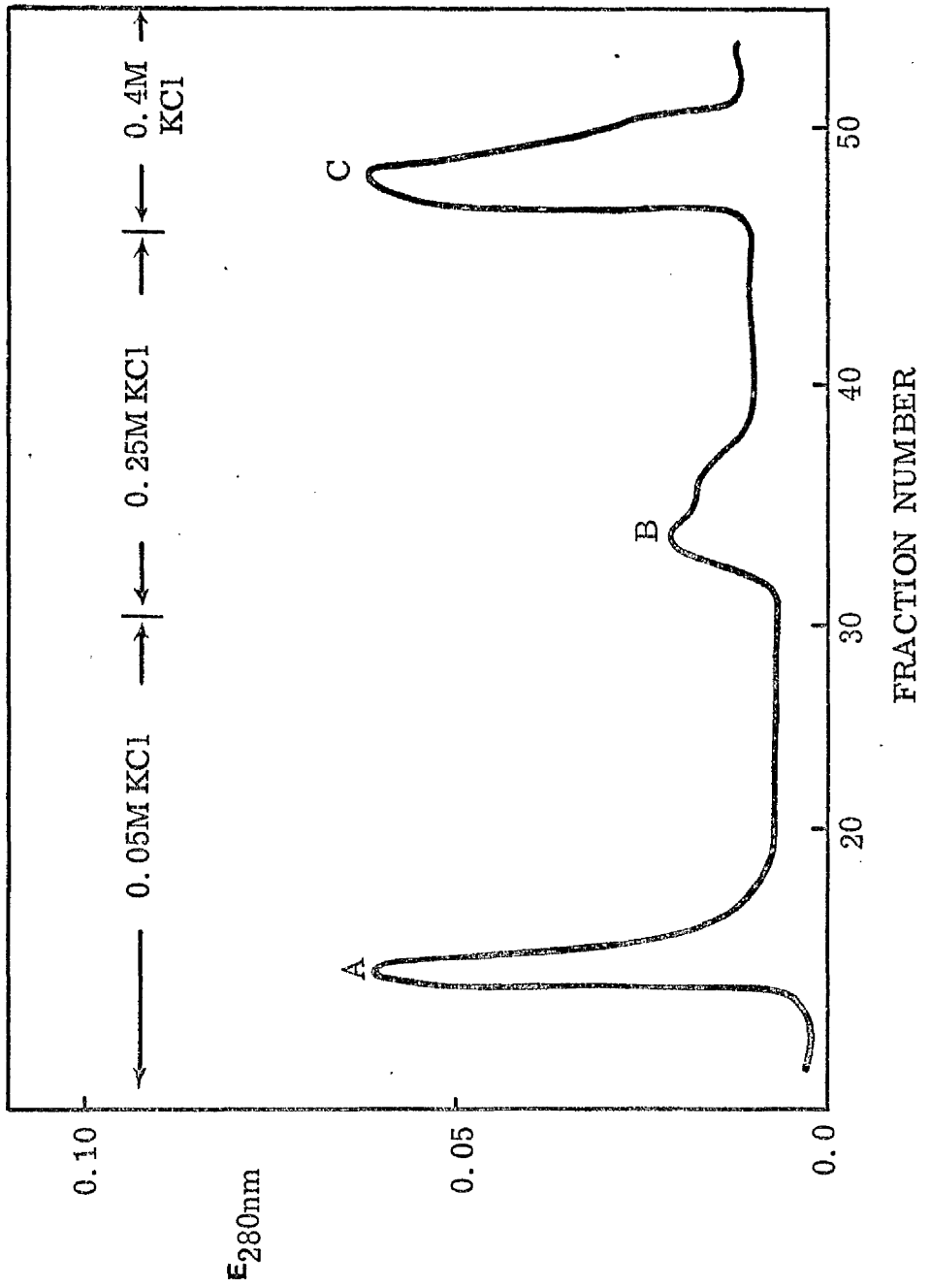

0.15
0.10
0.05
E 280
200
100
ENZYME UNITS PER ML
ELUTION: BUFFER A + 1.0M KCl
20
40
60
80
FRACTION NUMBER

Fig. 4.4 Chromatography of RNA polymerase on phosphocellulose.

RNA polymerase, prepared by the routine purification procedure (Section 4A), was subjected to chromatography on phosphocellulose as described in Section 4B. The vertical axis of the figure is the u.v. absorption at 280 nm of the effluent from the column, measured by passage through a 3 mm pathlength flow cell of an LKB Uvicord. The eluting buffer was Buffer C , plus the KCl concentration indicated on the figure. The column dimensions were 2.5 cm by 20 cm and the flow rate was 36 ml/h. 9 ml fractions were collected. RNA polymerase core enzyme was contained in peak C.


0.05M KCl
0.25M KCl
0.4M KCl
A
B
C
0.10
0.05
0.0
E_{280nm}
20
30
40
50
FRACTION NUMBER

SECTION 5

THE MEASUREMENT OF RNA POLYMERASE ACTIVITY

5A INTRODUCTION

Several methods have been described for measuring the activity of RNA polymerase.

5A1 INCORPORATION OF RADIOACTIVELY LABELLED NUCLEOSIDE TRIPHOSPHATES INTO RNA

This general method has been used many times in the past to assay for RNA polymerase and has varied mainly in the means used to separate the radioactive RNA product from the substrates. In the earlier procedures, acid-precipitable RNA was collected, with several washes, by centrifugation (for example, Furth et al., 1962). In later investigations, the acid-insoluble RNA product has been trapped on membrane filters (for example, Burgess 1969a). Sentenac et al. (1968) have also described a method in which the RNA is collected directly on membrane filters without previous precipitation by acid. The allows the retention of shorter RNA molecules which are not acid-precipitable.

Using radioactive nucleotides of high specific activity, these techniques can be very sensitive measures of RNA polymerase activity. The main disadvantage is the tedious work required to carry out a large number of such assays.

5A2 MEASUREMENT OF PYROPHOSPHATE

5A2.1 Enzymic pyrophosphate determination

Pyrophosphate can be determined enzymically by virtue of its participation in the reaction catalyzed by UDP-glucose pyrophosphorylase. A system based on this reaction has been successfully used to measure the pyrophosphate produced in the course of RNA synthesis by RNA polymerase (Johnson et al., 1971).

This gave a continuous recording assay of RNA polymerase. The disadvantages are, firstly, that the number of enzymes required greatly complicates the reaction conditions and secondly, that the method is, in practice, scarcely less tedious than those of Section 5A1. Nevertheless, this method is of importance in being virtually the only continuous recording one that has been described. Attempts were made to use this method in the present study, but it would seem to require further development.

5A2.2 Radioactive pyrophosphate determination

If nucleoside triphosphates are radioactively labelled at either the β or γ phosphate, then the pyrophosphate released after polymerization by RNA polymerase will be labelled. Maitra & Hurwitz (1967) have used this to measure RNA polymerase activity. The radioactivity, in a reaction mixture, that was not absorbed by activated charcoal was taken as a measure of the pyrophosphate released by the action of RNA polymerase.

5A3 PYROPHOSPHATE EXCHANGE

The exchange of pyrophosphate into nucleoside triphosphates during the course of RNA polymerase action has been studied (Maitra & Hurwitz, 1967 ; Krakow & Fronk, 1969) and this technique provides a useful way of studying the reaction mechanism. As an assay for RNA polymerase, however, its value is restricted by the interpretation of the measured quantity in terms of product formation or substrate disappearence.

5A4 FLUORIMETRIC MEASUREMENT OF RNA

The fluorimetric estimation of RNA in the presence of ethidium bromide has formed the basis for an assay of unprimed RNA polymerase activity (Krakow et al., 1969). This idea was extended in this investigation to template directed reactions and details are given later in this section .

5A5 5'-TERMINAL NUCLEOTIDE MEASUREMENT

Only the 5'-terminal residue of RNA synthesized by RNA polymerase retains its triphosphate group (Section 1A1). Thus, if a nucleotide substrate for the reaction is labelled at the β or γ phosphorus, the radioactivity of the RNA product will be a measure of the number of residues of that nucleotide that are 5'-terminal. Maitra *et al.* (1967) have used this technique, for example.

5A6 PENULTIMATE NUCLEOTIDE MEASUREMENT

Maitra *et al.* (1967) have also described a method for determining the nucleotide residue next to 5'-terminal in RNA. It is in effect a limited nearest neighbour analysis. RNA is synthesized by RNA polymerase, and one of the substrates is labelled at the α phosphorus. The RNA product is then hydrolysed by alkali, so that the α-phosphorus atom of the penultimate residue is then attached to the 2'- or 3'-carbon of the terminal residue. This can be detected because, after hydrolysis, only the 5'-terminal residue is present as the tetraphosphate. The fate of this labelled phosphorus is outlined in the scheme below .

$$pppN_1 + ppp^*N_2 + \ldots \longrightarrow pppN_1p^*N_2p\ldots$$

$$\xrightarrow{OH^-} pppN_1p^* + N_2p + \ldots.$$

5B REACTION CONDITIONS

The following conditions will be designated 'standard reaction conditions'.

1)	ATP,CTP,GTP and UTP	1.5 mM
2)	KCl	0.2 M
3)	$MgCl_2$	0.02 M
4)	Dithiothreitol	0.1 mM
5)	EDTA	0.1 mM
6)	Tris-HCl, pH 7.9 at 25°C	0.04 M

In the course of various experiments, some of these concentrations have varied, but the figures above relate to the routine assay system. Certain differences were :

1) With a poly (dA-dT) template, CTP and GTP were omitted.

2) With crude RNA polymerase samples (during the enzyme purification), an ATP regenerating system was included. This comprised phosphoenol pyruvate (0.01 M) and freshly prepared pyruvate kinase (0.02 mg/ml).

The reaction volume was normally 0.5 ml or 1 ml and the reaction temperature was 37°C. In most experiments, the reaction was started by the addition of enzyme.

5C MEASUREMENT OF RNA SYNTHESIS - RADIOACTIVE METHOD

This method follows the principles described in Section 5A1. The reaction, in a volume of 0.5 ml, was stopped by the addition of 1 ml of an ice cold BSA solution (200 µg/ml) or 1 ml of ice-cold water if the assay contained more than 200 µg of protein. 1 ml of cold trichloracetic acid reagent (125 g/l trichloracetic acid ; 10 g/l sodium pyrophosphate) was then added with mixing. The acidified reaction mixtures were left at 0°C for 15 min and then passed through Sartorius membrane filters (0.45µ pore size). The material trapped on the filters

was washed four times with 5 ml volumes of trichloracetic acid (50 g/l trichloracetic acid ; 10 g/l sodium pyrophosphate). The filters were removed, dried at 60°C, and counted in 8 ml of toluene/2,5-diphenyloxazole scintillator (5 g/l 2,5-diphenyloxazole in toluene) using a liquid scintillation counter (Nuclear Chicago 720 series liquid scintillation system).

The unit of enzyme activity used (EU) is defined as follows :

1 EU = 1 nmol. nucleotide incorporated/10 min

Assuming equal proportions of the four bases in the product RNA, the rate of incorporation of any nucleotide will equal a quarter of the rate of RNA synthesis. In the case of a poly(dA-dT) directed reaction, the rate of AMP or UMP incorporation will be one half of the rate of poly(A-U) synthesis.

ATP was usually the labelled nucleotide and the ^{3}H-isotope was preferred to ^{14}C because of the higher specific radioactivities of ^{3}H-nucleotides that are readily available. Radioactive nucleotides from The Radiochemical Centre (Amersham) were not further purified but an assessment of their radioactive purity was obtained by paper chromatography (Section 3G). The radioactive purities of one batch of ^{3}H-nucleotides was found to be : ATP 96% ; CTP 97% ; GTP 89% ; UTP 93% .

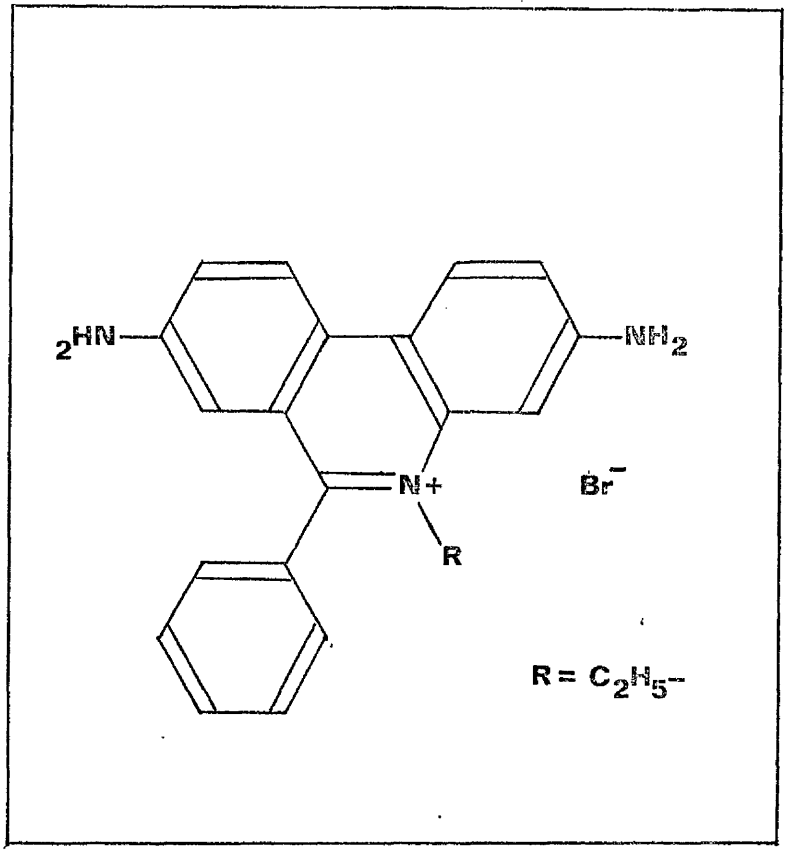


Fig. 5.1 Structure of ethidium bromide

5D FLUORESCENCE OF ETHIDIUM BROMIDE-NUCLEIC ACID COMPLEXES AND THE FLUORIMETRIC DETERMINATION OF NUCLEIC ACIDS

5D1 THE FLUORESCENCE OF ETHIDIUM BROMIDE AND ITS COMPLEXES WITH NUCLEIC ACIDS

Ethidium bromide (3,8-diamino-6-phenyl-ethylphenanthridinium bromide) has the structure shown in Fig. 5.1 . This dye has several biological effects (Dickinson et al., 1953 ; Henderson, 1963 ; Elliott, 1963 ; Waring, 1964) which appear to be the result of its property of binding to nucleic acids (LePecq et al., 1964 ; Waring, 1965). The complex formation between ethidium bromide and nucleic acids causes an alteration of the physical properties of both the nucleic acid and the dye (LePecq & Paoletti, 1967). Besides the physico-chemical interest in this complex formation, a number of techniques have been developed which utilise this binding phenomenon (for example, LePecq, 1971).

The fluorescent properties of the dye are altered in the presence of nucleic acid with a marked increase in the quantum efficiency of fluorescence of the bound dye. Following the increase in fluorescence of ethidium bromide therefore can give a quantitative estimation of nucleic acids (LePecq et al., 1964 ; LePecq & Paoletti, 1966). Krakow et al. (1969) described an RNA polymerase assay using this phenomenon to follow unprimed(non-template directed) RNA synthesis. The background fluorescence from a DNA template makes the detection of RNA synthesis more difficult. The investigations of the fluorescence of ethidium bromide reported here were carried out with a view to developing an assay for DNA-directed RNA polymerase activity, by measuring the RNA product fluorimetrically.

Were the object of this work to obtain accurate characterization of the fluorescence properties of ethidium bromide and its complexes with nucleic acids, then the problems associated with the use of a single beam spectrophotofluorimeter would have to be overcome. With a single beam instrument, for example, the properties of the photomultiplier tube, xenon lamp and monochromators are functions of wavelength. In fact, the fluorescence of ethidium bromide was characterized only sufficiently that RNA concentration could be related to fluorescent intensity. Reported spectra are uncorrected and instrumental slit settings were wide for maximum sensitivity rather than optimal resolution of wavelength. Nevertheless, maxima of excitation and emission still reflect conditions at which the observed signal is maximum.

5D1.1 Fluorescence of ethidium bromide

The excitation spectrum of ethidium bromide showed maxima at 310 nm and 480 nm under the conditions used (Fig. 5.2). Emission spectra for excitation at both 310 nm and 480 nm are shown in Fig. 5.3 and an emission maximum at 590 nm was seen in both cases. These values for spectral parameters are in the same region as those of LePecq *et al.* (1964) which were obtained under slightly different experimental conditions of pH, ionic strength etc. ; their figures were 590 nm for emission maxima and 300 nm and 480 nm for excitation maxima. Subsequent modifications of the spectrophotofluorimeter caused changes in the observed wavelength maxima for emission and excitation.

5D1.2 Fluorescence of ethidium bromide in the presence of nucleic acids

Both RNA and DNA cause qualitatively similar effects on the fluorescence of associated ethidium bromide. The

effect of the presence of DNA and RNA on the excitation spectrum of ethidium bromide is seen in Figs. 5.4 and 5.5. With increasing nucleic acid concentration, there was a shift to longer wavelengths of excitation for both the 310 nm and the 480 nm excitations, although the shift for the latter was more pronounced. Further, there was a marked increase in the fluorescent intensity as nucleic acid concentration increased.

The presence of nucleic acids affected the emission spectrum of ethidium bromide by causing an increase in fluorescent intensity without altering the wavelength of the emission maximum at 590 nm.

5D1.3 Quantitative relationship between ethidium bromide fluorescence and nucleic acid concentration

The increase in ethidium bromide fluorescence with increasing nucleic acid concentration can be described quantitatively and Fig. 5.6 shows the linear relationship between fluorescent intensity and DNA concentration, in the presence of excess ethidium bromide. Fig. 5.7 shows the fluorescent intensity change due to RNA, in the presence of DNA of various concentrations, and this forms the basis for the measurement of RNA in RNA polymerase assays. The set of curves in Fig. 5.7 was used to determine a standard curve that was employed in several experiments.

5D1.4 Effect of other factors on the fluorimetric determination of RNA

The composition of the RNA polymerase reaction mixture was described in Section 5B. Of the components, the concentrations of DNA and nucleoside triphosphates seemed to be most likely to vary in the course of subsequent experiments. Thus, the dependence of fluorescent intensity on RNA concentration was observed at a number of concentrations of

DNA and NTP. Each such experiment yielded a linear response of fluorescent intensity to RNA concentration and the gradient of these curves, d(FI)/d(RNA), is shown as a function of DNA and NTP concentrations in Fig. 5.8 . These results showed a detectable influence of both DNA and NTP on RNA measurement by this method.

Besides changes in the components of the RNA polymerase reaction mixture, all measurements of fluorescent intensity were subject to variations in the instrumental response, whether due to deliberate alterations to the fluorimeter or the fluctuations normally associated with a single beam instrument.

5D1.5 Quantitation of fluorimetric RNA measurement

The preceding experiments established that the observed fluorescent intensity of the dye can be a linear function of RNA concentration under defined conditions. The sensitivity of the spectrophotofluorimeter has varied as xenon lamps and photomultiplier tubes have been changed, and also as changes to the instrumental settings have been made. It is estimated from various experiments, however, that an RNA concentration of about 1 μM RNA (with respect to monomer) can be detected. The accuracy of the measurements decreases when RNA is measured against a background of the fluorescence caused by DNA. This has been one of the problems in the practical application of this technique of RNA estimation. Partial resolution of the problem was achieved by the use of a photometer with a blank subtract facility (Aminco solid-state, blank-subtract photomultiplier microphotometer, Cat.No. 10-280) , thus allowing electronic subtraction of the fluorescence caused by the presence of DNA.

A commercial preparation of tRNA was used in the preceding

experiments and has also been used as an RNA standard. The RNA synthesized in the presence of RNA polymerase will most likely be of a different type than yeast tRNA. Hence, it can be argued that this is not a good choice of RNA standard. The ideal RNA standard would be the actual product of the RNA polymerase catalyzed reaction under investigation. This would be difficult to obtain in quantity and besides may vary in composition with reaction conditions. Yeast tRNA, then, was selected as a readily available standard which will yield self-consistent results and to which other RNA standards could be referred if desired.

5D2 MEASUREMENT OF RNA SYNTHESIS - FLUORESCENCE METHOD

A reaction mixture of 1 ml volume was incubated at 37°C, the reaction being started by the addition of RNA polymerase. At various times, 0.1 ml aliquots were removed and mixed thoroughly with 1.9 ml of ethidium bromide solution (5 mg/l ethidium bromide ; 0.01 M-tris-HCl, pH 7.9 ; 0.05 M-NaCl ; 0.001 M-EDTA). The reaction ceased after mixing with ethidium bromide reagent. The fluorescence of the ethidium bromide was measured in an Aminco-Bowman spectrophotofluorimeter (excitation at 310 nm or 480 nm ; emission at 590 nm). These wavelengths have varied slightly (Section 5D1.1).

In preliminary experiments, fluorescent intensity was related to RNA concentration by a calibration curve (Fig. 5.7). The interference of DNA and NTP was neglected and effectively the following equation was used.

$$r_t = K.(FI_t - FI_o).D \qquad \text{eqn. 5.1}$$

where

K is a constant, and the gradient of the lines in Fig.5.7.

r_t is the RNA concentration at time, t.

FI_o is the fluorescent intensity at time zero, FI_t at time, t.

D accounts for the dilution of the aliquot of the reaction mixture on addition to the ethidium bromide reagent.

However, variations in the constant K occured for reasons discussed in Section 5D1.4 and so, in most experiments, a sample of a standard RNA solution was measured under the conditions of each assay. If the increase in fluorescent intensity caused by the presence of the RNA standard is S, and the concentration of the RNA standard is r_s, then eqn. 5.1 becomes

$$r_t = \frac{r_s}{S} . (FI_t - FI_o).D \qquad \text{eqn. 5.2}$$

r_s was chosen to be within the linear region of the dependence of fluorescent intensity on RNA concentration. Since normally the initial rate was wanted, eqn. 5.2 can be conveniently written as

$$(\frac{dr}{dt})_{t=0} = (\frac{dFI}{dt})_{t=0} . \frac{r_s}{S} . D \qquad \text{eqn. 5.3}$$

Eqn. 5.3 enables initial rates to be derived directly from plots of fluorescent intensity against time.

5E CONTINUOUS MEASUREMENT OF RNA BY A FLUORIMETRIC METHOD

5E1 CONTINUOUS ASSAY FOR RNA POLYMERASE

If RNA is produced in the presence of ethidium bromide, then its synthesis could be followed by measuring the increase in the fluorescent intensity of the dye.

Fig. 5.9 shows an RNA polymerase catalyzed reaction, in the presence of 1 μg/ml ethidium bromide. A readily detectable increase in fluorescent intensity with time was observed. An experiment lacking poly(dA-dT) as template gave no such increase. Evidence that this RNA synthesis was not caused by the presence of polynucleotide phosphorylase in the enzyme preparation was twofold. Firstly, the presence of 1 mM

phosphate did not inhibit the reaction, conditions which have been shown to severely inhibit polymerization catalyzed by polynucleotide phosphorylase (Burgess, 1969a). Secondly, the ATP and UTP had been purified and contained little contaminating diphosphates (Sections 4D2.3 and 6C2). Also, when ADP was the only nucleotide present, the rate of increase of fluorescent intensity was well below that observed when ATP and UTP were present (Fig. 5.9).

From the above results, it is concluded that poly(dA-dT) could direct the synthesis of poly(A-U) by RNA polymerase in the presence of 1 μg/ml ethidium bromide. The reaction rate in the presence of ATP and UTP at various poly(dA-dT) concentrations is shown in Fig. 5.10 and the results were as expected on the basis of standard radioactive assays (Fig. 7.2). However, the validity of assessing RNA polymerase activity by this method is questionable, since ethidium bromide is known to interfere with RNA polymerase activity (Elliott, 1963 ; Waring, 1964).

5E2 CONTINUOUS ASSAY FOR POLYNUCLEOTIDE POLYMERASE

The same measurement system as described in the previous section can be used to observe polynucleotide phosphorylase activity, provided that ethidium bromide does not totally inhibit the activity of this enzyme. A reaction catalyzed by a commercial preparation of polynucleotide phosphorylase is shown in Fig5.11. This enzyme required an oligonucleotide primer. The application of this assay method to the measurement of polynucleotide phosphorylase is described in Section 6C2.

Fig. 5.2 Excitation spectrum of ethidium bromide.

The excitation spectrum of ethidium bromide (5 mg/l ethidium bromide ; 0.01 M-tris-HCl, pH 7.9 ; 0.05 M-NaCl ; 0.001 M-EDTA) was measured with an Aminco-Bowman spectrophotofluorimeter at room temperature (about 20°C). The vertical axis of the figure is the fluorescent intensity and is in arbitrary units. For an emission wavelength of 590 nm, excitation maxima were observed at 310 nm and 480 nm, the 590 nm peak being caused by scattering.

Instrument details (see Section 2B1)

Xenon lamp : No.1

Photomultiplier tube : No.1

Slit arrangement : Slits 2,5, and 7 all 3/16 in
Other slit positions unoccupied.

Sensitivity : 40

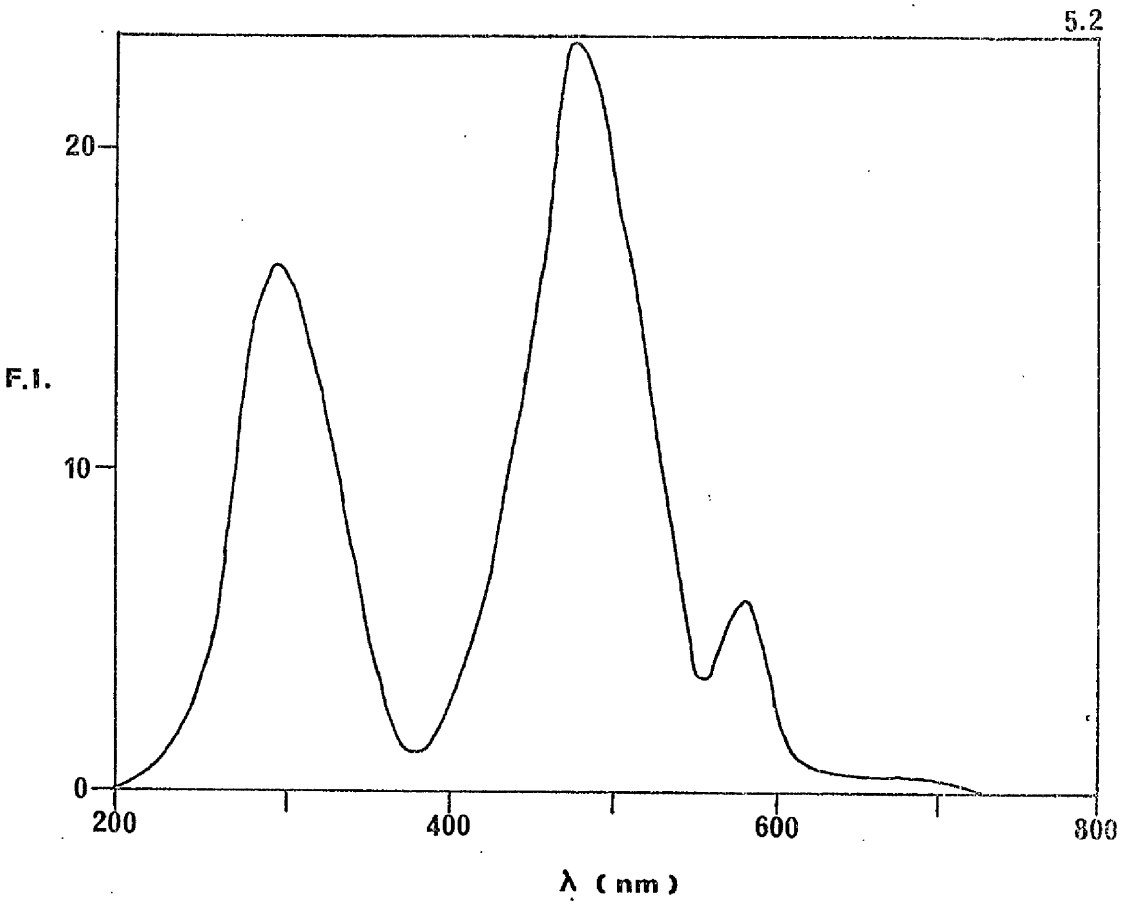

5.2
20
10
0
F.I.
200
400
600
800
λ (nm)

Fig. 5.3 Emission spectra of ethidium bromide.

Emission spectra of ethidium bromide (5 mg/l ethidium bromide ; 0.01 M-tris-HCl, pH 7.9 ; 0.05 M-NaCl ; 0.001 M-EDTA) were recorded using an Aminco-Bowman spectrophotofluorimeter at room temperature (about 20°C). The vertical axis is fluorescent intensity in arbitrary units.

Spectrum 1 : Excitation at 310 nm giving emission maxima at 590 nm, 410 nm and 310 nm, the last probably being caused by scattering.

Spectrum 2 : Excitation at 480 nm giving emission maxima at 590 nm and 480 nm, the latter caused presumably by scattering.

Instrument details (see Section 2B1)

Xenon lamp : No.1

Photomultiplier tube : No.1

Slit arrangement : Slits 2,5, and 7 all 3/16 in
Other slit positions unoccupied

Sensitivity : 40

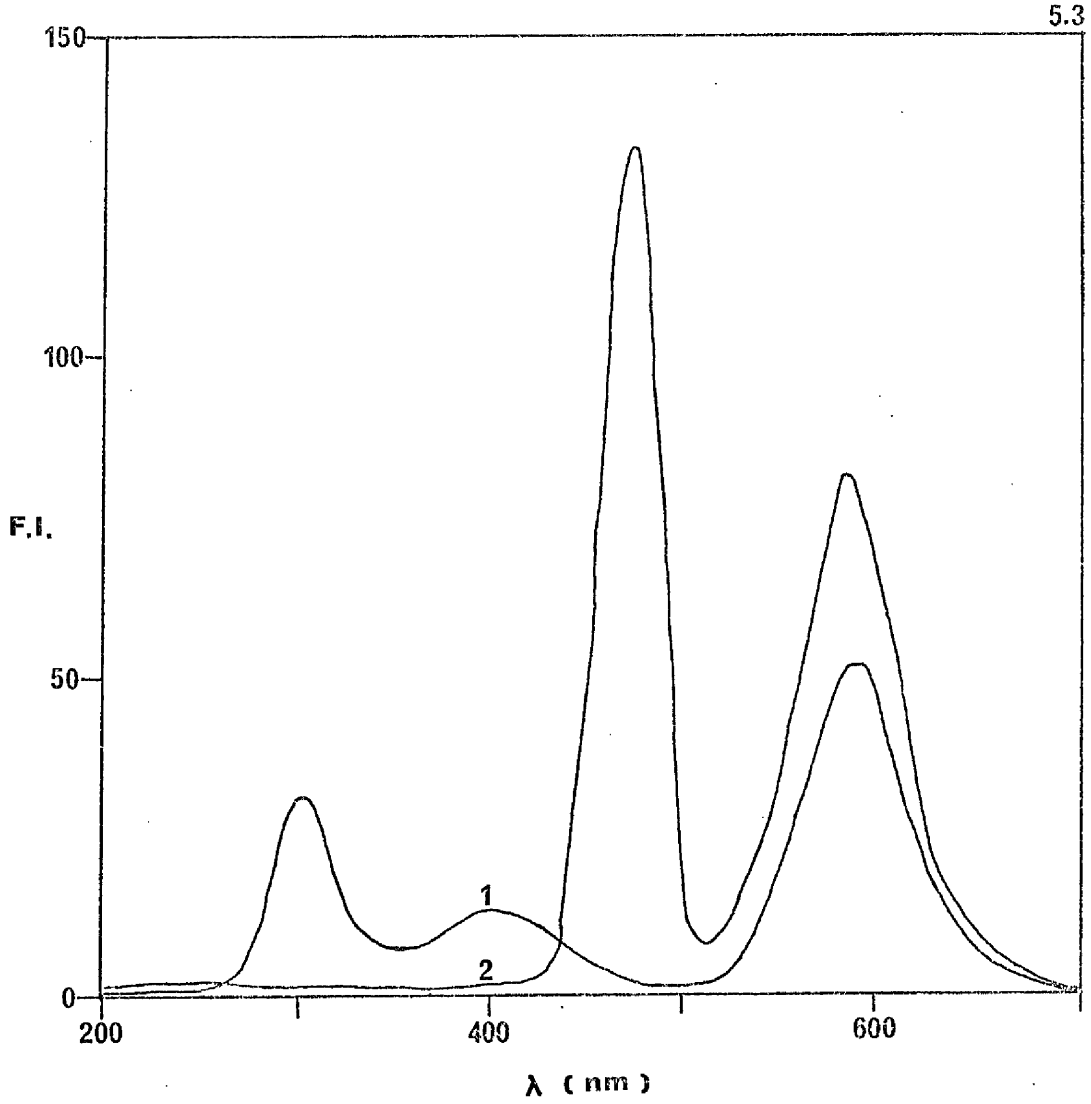

150
100
50
0
F.I.
200
400
600
λ (nm)
1
2

Fig. 5.4 Excitation spectra of ethidium bromide in the presence of RNA.

Excitation spectra of ethidium bromide (5 mg/l ethidium bromide ; 0.01 M-tris-HCl. pH 7.9 ; 0.05 M-NaCl ; 0.001 M-EDTA) were recorded using an Aminco-Bowman spectrophotofluorimeter at room temperature (about 20°C) in the presence of various concentrations of yeast tRNA. The emission wavelength was 590 nm and the vertical axis of the figure is fluorescent intensity in arbitrary units. The RNA concentrations were (with respect to monomer) : 1) 0.0 μM; 2) 10 μM ; 3) 40 μM ; 4) 80 μM .

Instrument details (see Section 2B1)

Xenon lamp : No.1

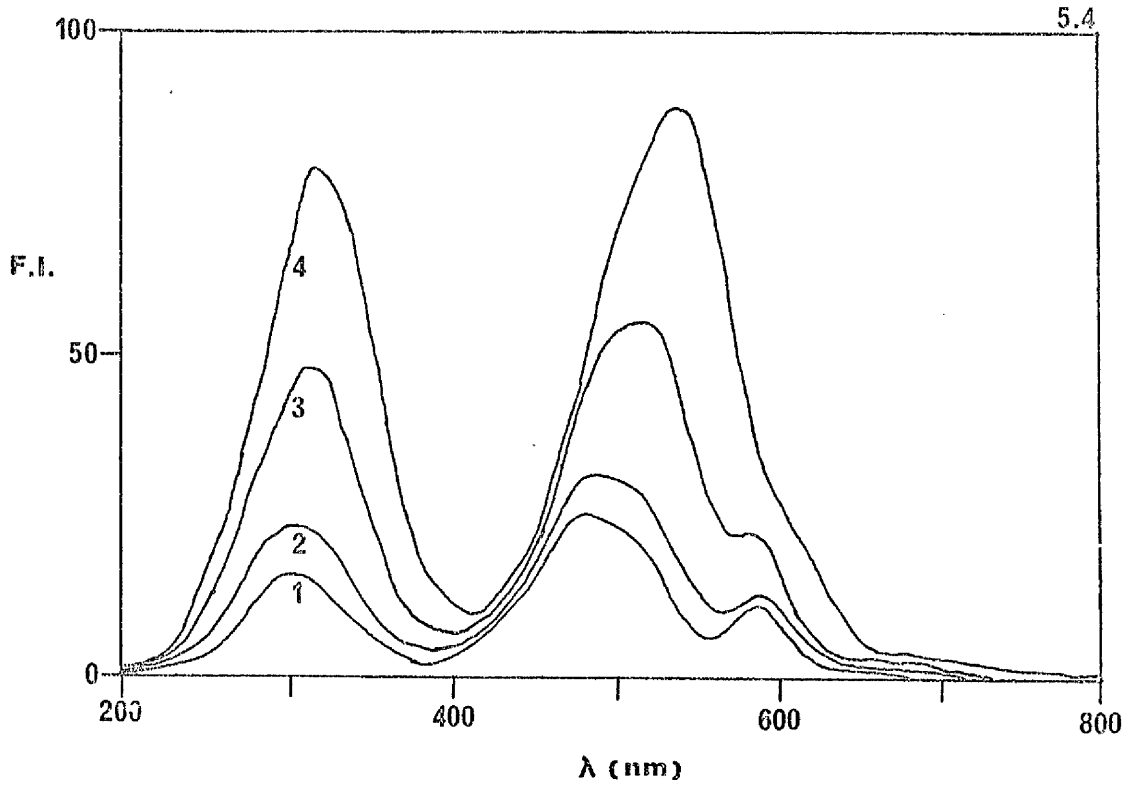
Photomultiplier tube : No.1

Slit arrangement : Slits 2,5, and 7 all 3/16 in
Other slit positions unoccupied

Sensitivity : 40

Fig. 5.5 Excitation spectra of ethidium bromide in the presence of DNA.

Excitation spectra of ethidium bromide were recorded in the presence of various concentrations of calf thymus DNA, experimental details being identical with those for Fig. 5.4 . The DNA concentrations (with respect to monomer) were : 1) 0.0 μM ; 2) 5.9 μM ; 3) 11.8 μM ; 4) 23.6 μM.


5.4
100
50
0
F.I.
4
3
2
1
200
400
600
800
λ (nm)

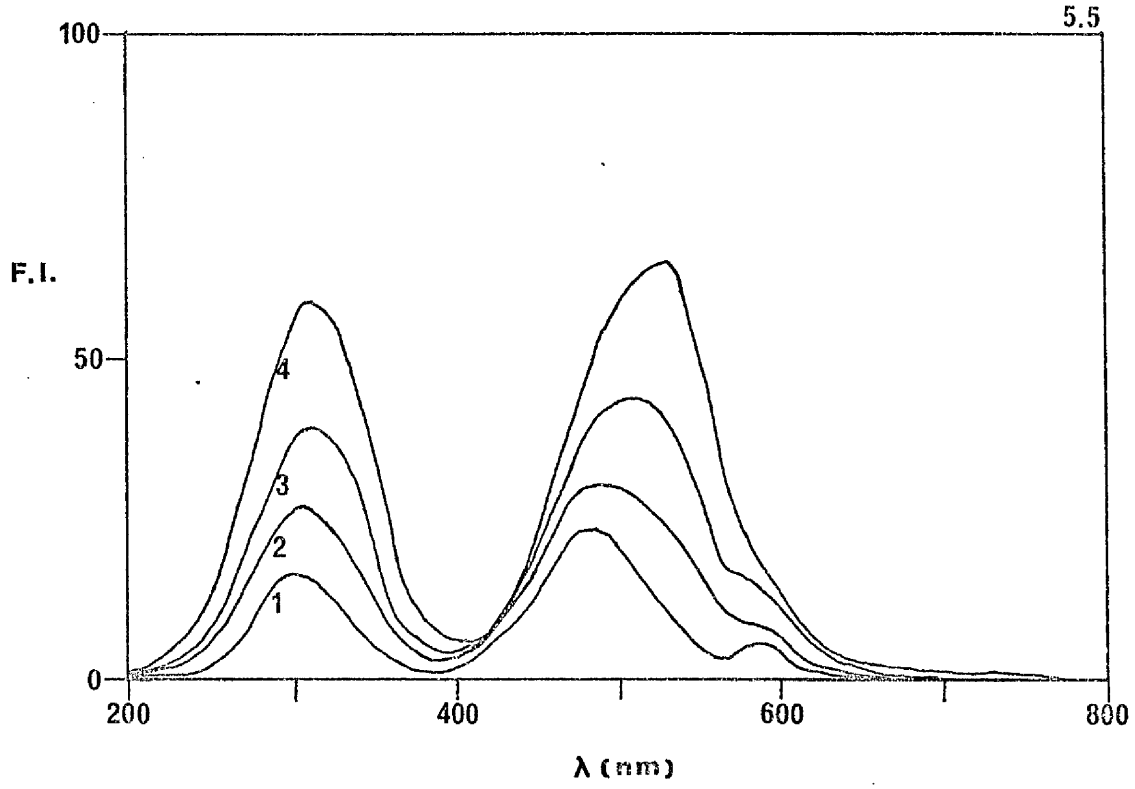

5.5
100
50
0
F.I.
4
3
2
1
200
400
600
800
λ (nm)

Fig. 5.6 Relationship between fluorescent intensity of ethidium bromide and DNA concentration.

The fluorescent intensity of ethidium bromide (5 mg/l ethidium bromide ; 0.01 M-tris-HCl, pH 7.9 ; 0.05 M-NaCl ; 0.001 M-EDTA) was measured in an Aminco-Bowman spectrophotofluorimeter in the presence of various calf thymus DNA concentrations. Measurements were at room temperature (about 20°C) with excitation at 310 nm and emission at 590 nm.

Instrument details (see Section 2B1)

Xenon lamp : No.1

Photomultiplier tube : No.1

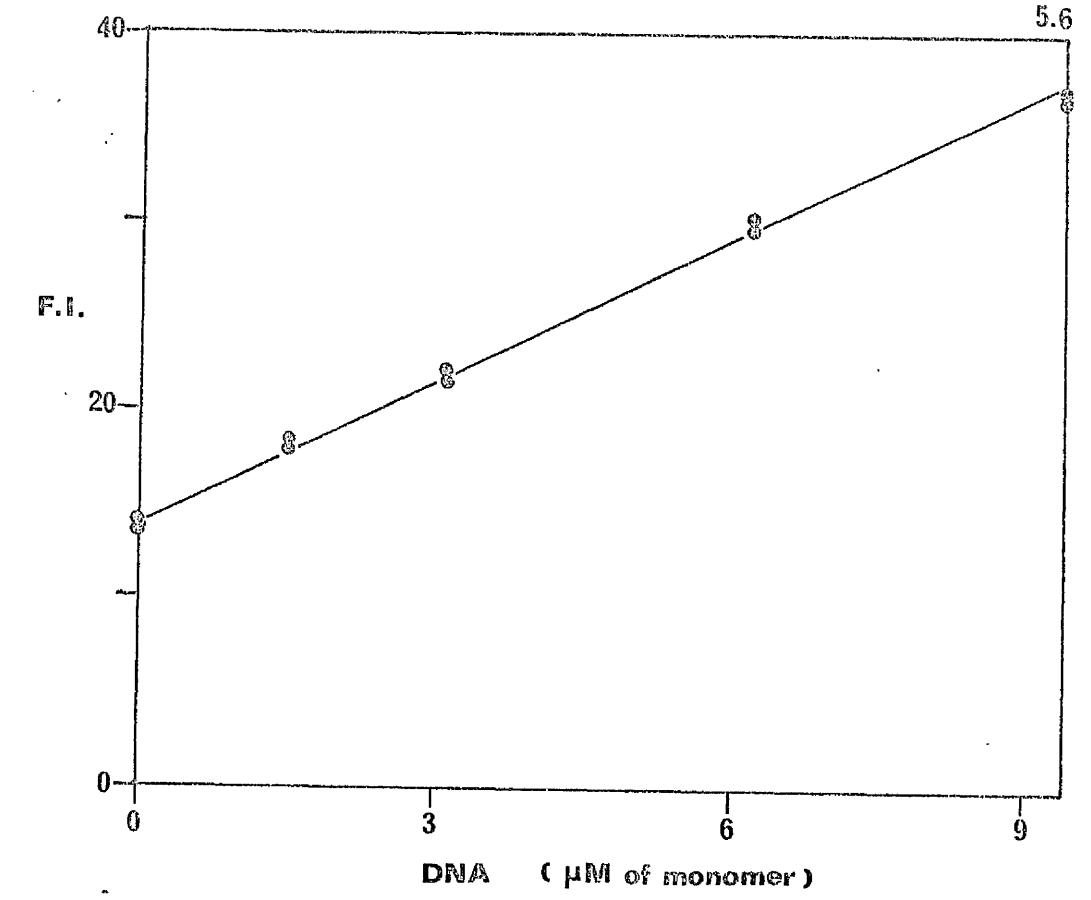
Slit arrangement : Slits 2,5, and 7 all 3/16 in
Other slit positions unoccupied

Sensitivity : 40

Fig. 5.7 Relationship between fluorescent intensity of ethidium bromide and RNA concentration.

The fluorescent intensity of ethidium bromide at various yeast tRNA concentrations was measured in the presence of several calf thymus DNA concentrations. Excitation was at 310 nm and emission was at 590 nm. The DNA concentrations were 1) 0.0 μM ; 2) 1.56 μM ; 3) 3.12 μM ; 4) 6.24 μM ; 5) 9.36 μM.

All other details were as for Fig. 5.6 .


5.6
40
20
0
F.I.
0
3
6
9
DNA (μM of monomer)

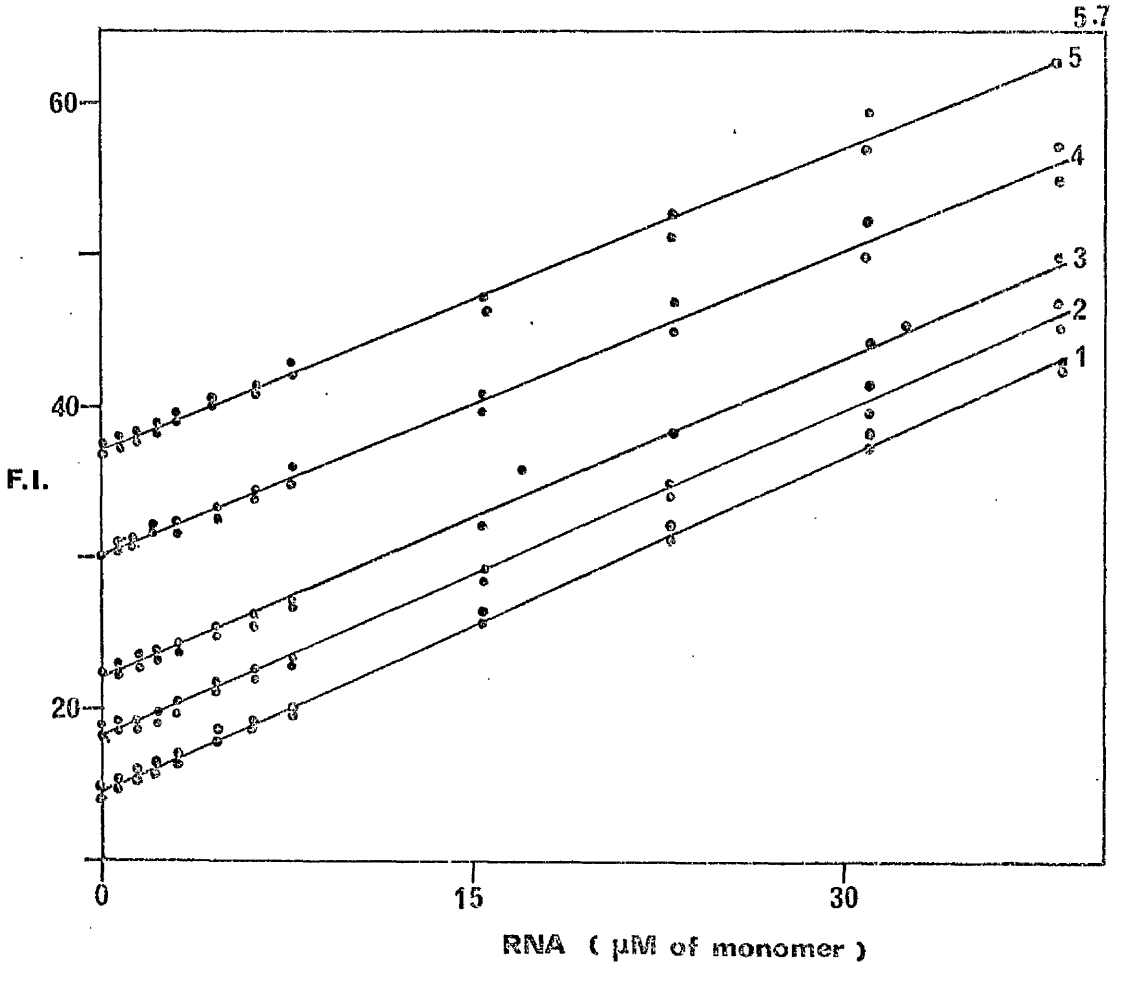

5.7
60
40
20
0
F.I.
5
4
3
2
1
0
15
30
RNA (μM of monomer)

Fig. 5.8a The effect of DNA concentration on the relationship between the fluorescent intensity of ethidium bromide and RNA concentration.

The change of fluorescent intensity of ethidium bromide fluorescence (excitation at 530 nm and emission at 590 nm) with yeast tRNA concentration was measured at several calf thymus DNA concentrations using an Aminco-Bowman spectrophotofluorimeter. Measurements were at room temperature (about 20°C). The error bars represent the standard errors in the slope of each plot of fluorescent intensity against RNA concentration. The ethidium bromide solution was 10 mg/l ethidium bromide ; 0.01 M-tris-HCl, pH 7.9 ; 0.05 M-NaCl ; 0.001 M-EDTA. In this experiment, the higher wavelength excitation of ethidium bromide was used.

Instrument details (see Section 2B1)

Xenon lamp : No.2

Photomultiplier tube : No.1

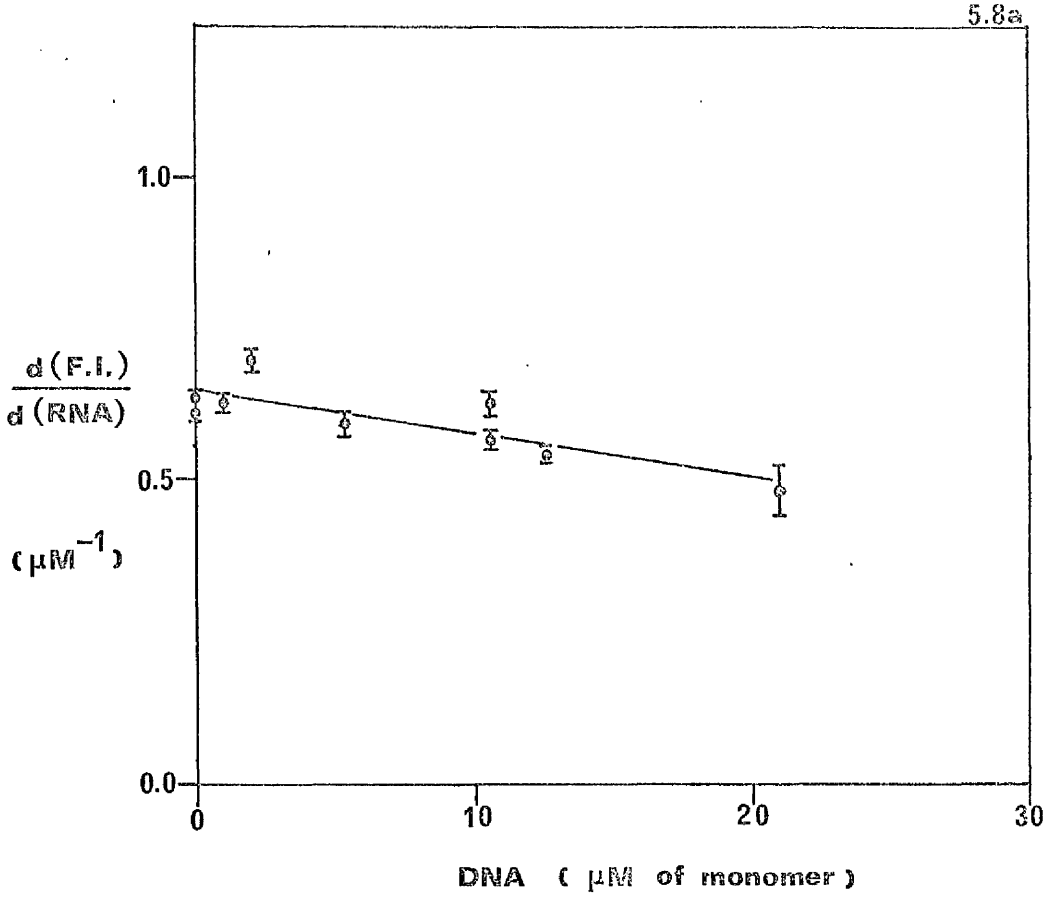
Slit arrangement : Slit 2, 4 mm ; slit 5, 3 mm ; slit 7, 1/16 in ; all other slit positions were unoccupied.

Sensitivity : 100

Fig. 5.8b The effect of nucleoside triphosphate concentration on the relationship between the fluorescent intensity of ethidium bromide and RNA concentration.

The experiment of Fig. 5.8a was repeated, with NTP instead of DNA (NTP represents here equimolar ATP, CTP, GTP, and UTP). All details were as in Fig. 5.8a except that the excitation wavelength was 310 nm.

5.8a


1.0
0.5
0.0
0
10
20
30
d(F.I.) / d(RNA)
(μM⁻¹)
DNA (μM of monomer)

5.8b

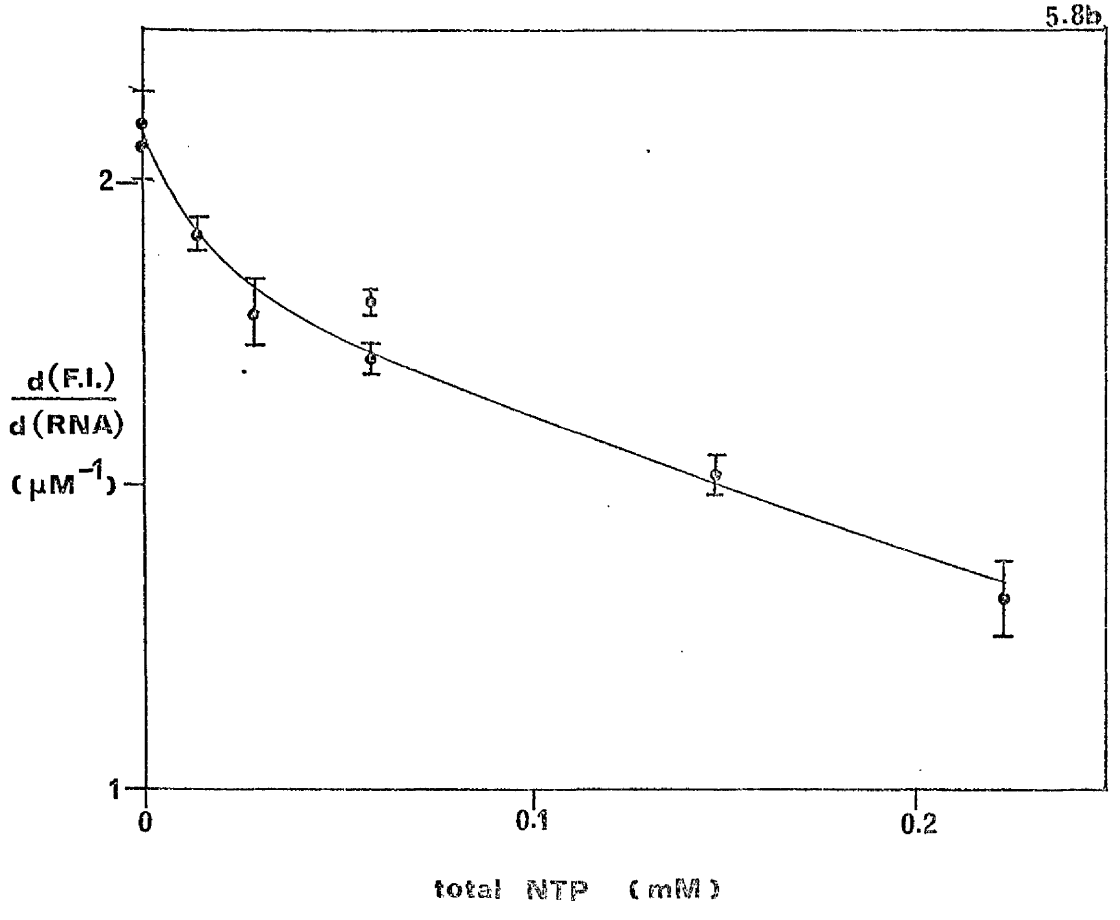

2
1
0
0.1
0.2
d(F.I.) / d(RNA)
(μM⁻¹)
total NTP (mM)

Fig. 5.9 Fluorimetric detection of poly(dA-dT) directed poly(A-U) synthesis by RNA polymerase in the presence of ethidium bromide.

Reaction mixtures, total volume 1 ml, were set up in quartz fluorescence cells and contained 0.04 M-tris-HCl, pH 7.9 ; 0.1 mM-EDTA ; 0.1 mM-dithiothreitol : 0.02 M-$MgCl_2$; 0.2 M-KCl and 1 mg/l ethidium bromide. In addition, individual reactions contained

1) 0.075 mM poly(dA-dT) ; 1 mM ATP and UTP (purified, Section 4D)... o-o
2) 0.075 mM poly(dA-dT) ; 1 mM ADP ... ⊙-⊙
3) 1 mM ATP and UTP (purified)... ⊙-⊙

Each reaction was started by the addition of 110 µg of RNA polymerase after which the reaction was monitored by following the fluorescence of ethidium bromide (excitation at 310 nm, emission at 615 nm) with an Aminco-Bowman spectrophotofluorimeter. The reaction took place at room temperature (about 20°C). In the figure, the vertical axis is fluorescent intensity in arbitrary units, and the arrow indicates the addition of 1 mM phosphate.

Instrument details (see Section 2B1)

Xenon lamp : No.2

Photomultiplier tube : No.2

Slit arrangement : Slit 2, 5 mm ; slit 5, 5 mm ; slit 7, 5 mm ; all other slit positions were unoccupied.

Sensitivity : 100

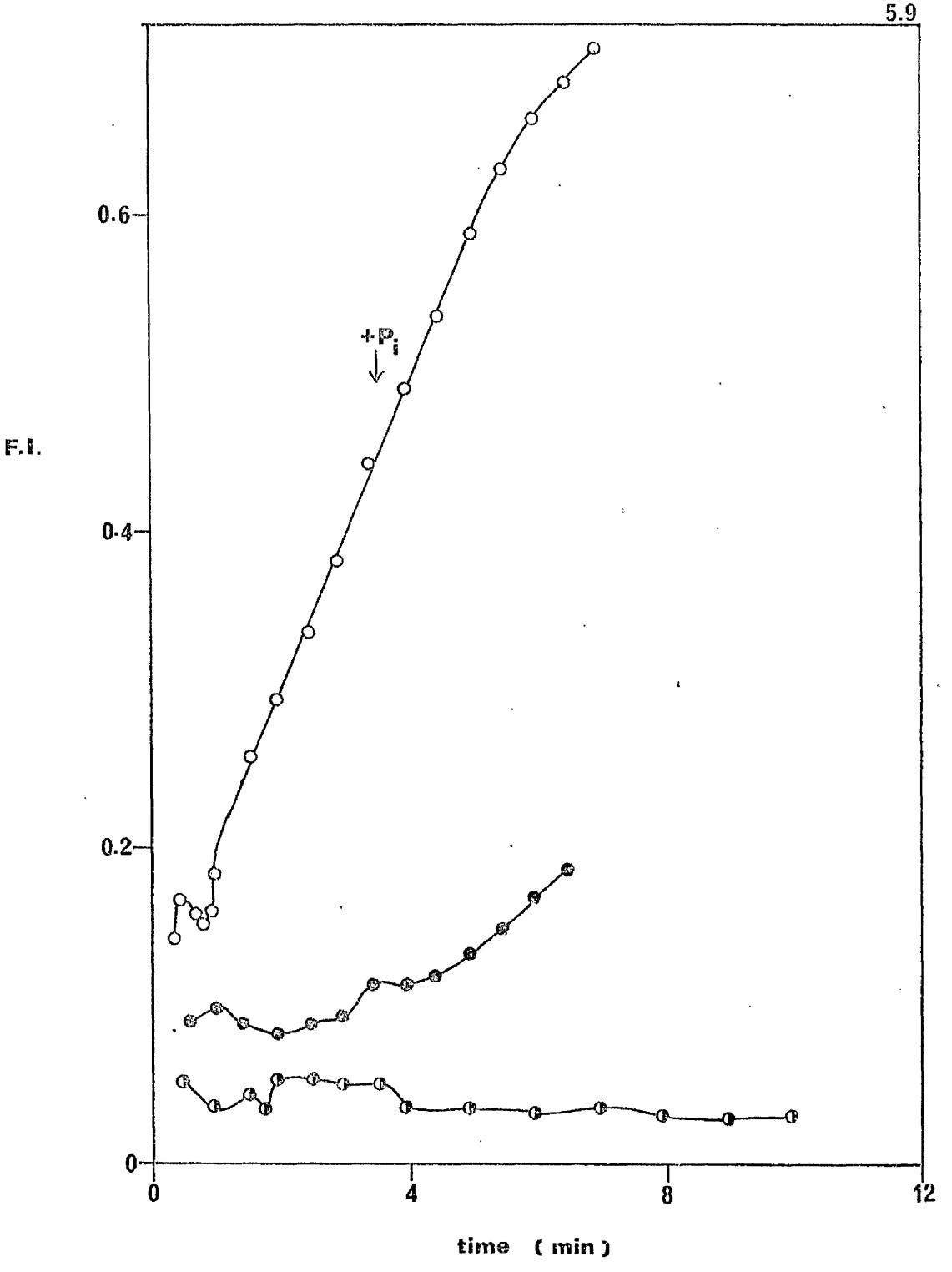

F.I.
0.6
0.4
0.2
0
+Pi
0
4
8
12
time (min)

Fig. 5.10 Dependence of the rate of RNA polymerase catalyzed poly(A-U) synthesis, in the presence of ethidium bromide, on poly(dA-dT) concentration.

Reaction mixtures, total volume 1 ml, were set up in quartz cells and contained 0.04 M-tris-HCl, pH 7.9 ; 0.1 mM-EDTA ; 0.1 mM-dithiothreitol ; 0.02 M-$MgCl_2$; 0.2 M-KCl ; 1 mg/l ethidium bromide ; 1 mM-ATP ; 1 mM-UTP; (both nucleotides were purified); and various concentrations of poly(dA-dT). The reaction was started by the addition of 110 μg of RNA polymerase, $E_{\sigma\theta}$, and the fluorescence of the ethidium bromide was followed with an Aminco-Bowman spectrophotofluorimeter (excitation at 310 nm and emission at 615 nm). The reaction took place at room temperature (about 20°C).

Instrument details (see Section 2B1)

Xenon lamp : No.2

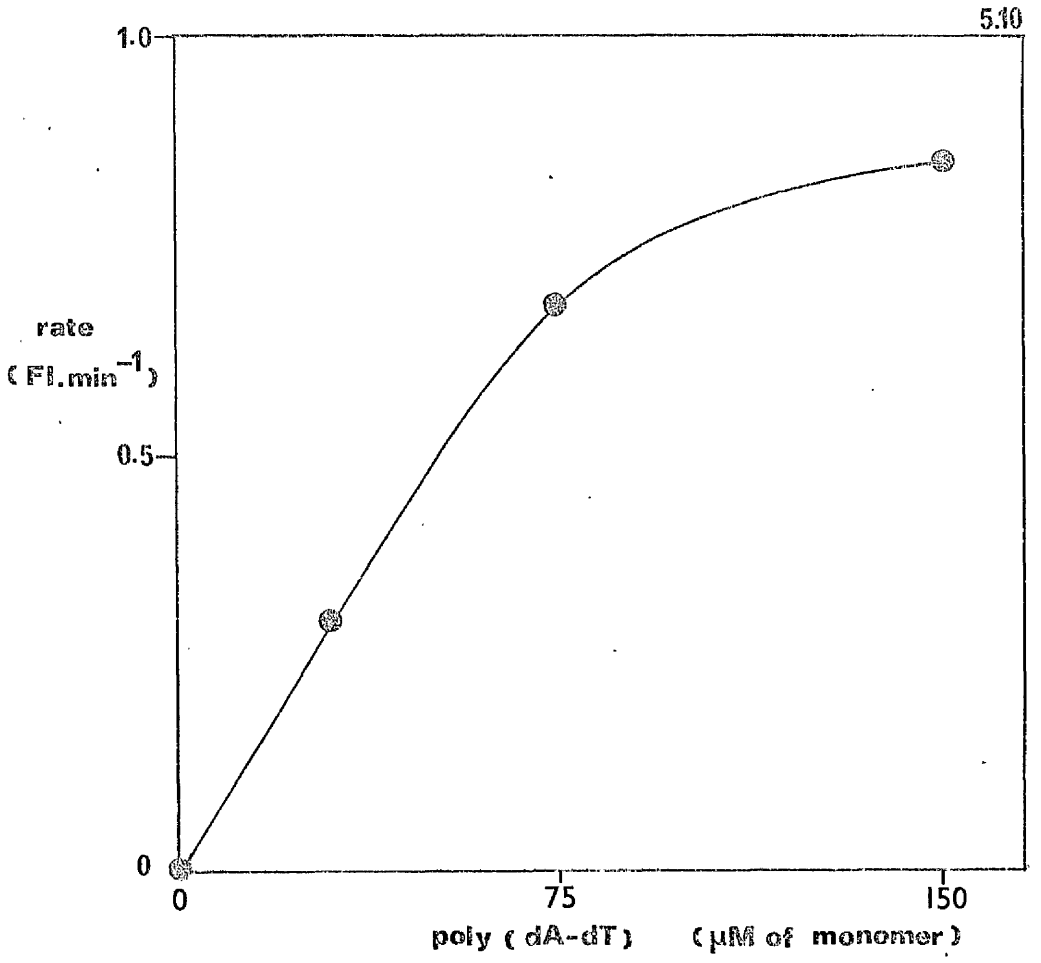
Photomultiplier tube : No.2

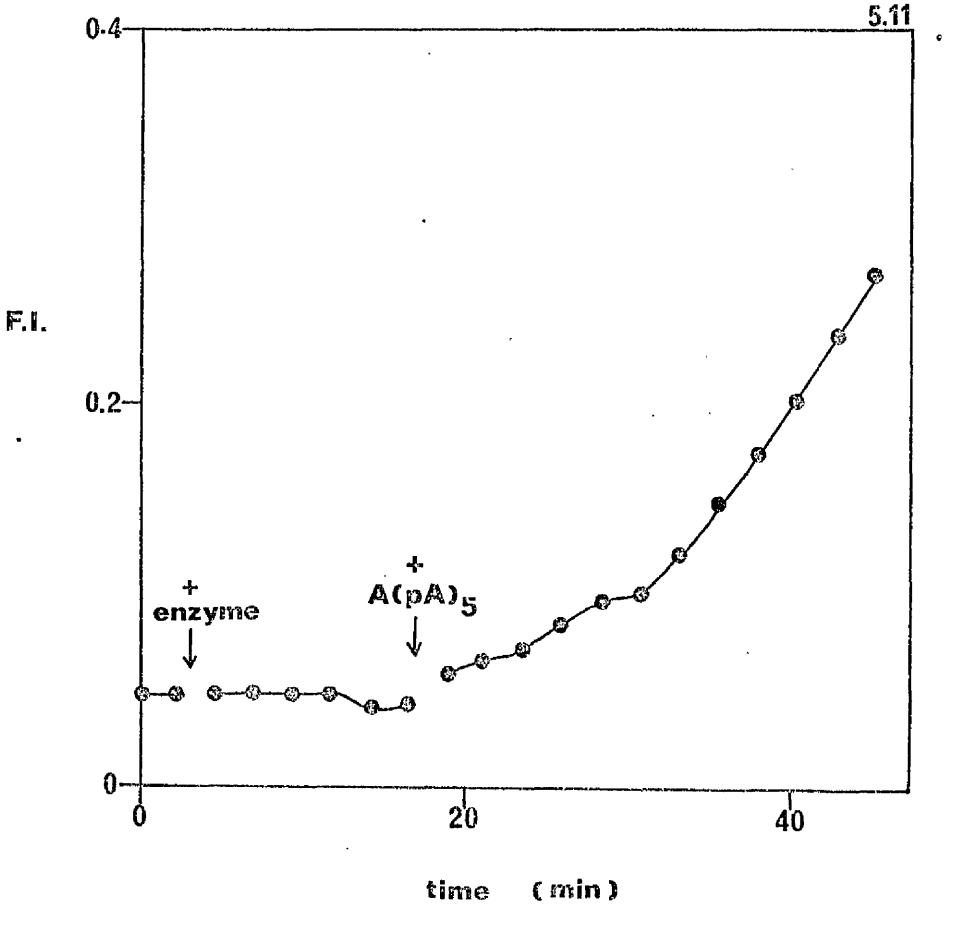
Slit arrangement : Slits 2, 5, and 7, all 5 mm ;
All other slit positions unoccupied

Sensitivity : 100

Fig. 5.11 Fluorimetric detection of polynucleotide phosphorylase activity.

Reaction conditions were as for Fig. 5.10 except that 1 mM ADP replaced the ATP and UTP, and that there was no poly(dA-dT). The vertical axis is fluorescent intensity in arbitrary units and the arrows indicate additions to the reaction system. All other experimental conditions were as for Fig. 5.10. 10 μg of polynucleotide phosphorylase from *M. luteus* were added initially, but the reaction only started after 25 μg of $A(pA)_5$ were added.


5.10
1.0
0.5
0
rate
(Fl.min^{-1})
0
75
150
poly (dA-dT) (μM of monomer)


5.11
0.4
0.2
0
F.I.
+
enzyme
+
A(pA)$_5$
0
20
40
time (min)

SECTION 6

CHARACTERIZATION OF RNA POLYMERASE

For the reaction catalyzed by RNA polymerase, it is particularly important to define the enzyme used, since RNA polymerase is a complex protein compared with most enzymes. This section describes the characterization of RNA polymerase preparations used in this work and includes some considerations of the structure of the enzyme.

6A ROUTINE CHARACTERIZATION OF RNA POLYMERASE PREPARATIONS

RNA polymerase purified by the method described in Section 4A was routinely analysed by u.v. spectroscopy, SDS-polyacrylamide gel electrophoresis and assays for enzyme activity.

1) Specific activities ranging from about 100 - 800 nmol./10 min/mg of nucleotide incorporated into RNA have been observed with calf thymus DNA as template. The ratio of activity with T7 DNA to that with calf thymus DNA has varied from 1 - 5, presumably reflecting a variation in the σ content of different enzyme preparations.

2) E_{280}/E_{260} ratios (the ratio of the absorption at 280 nm to that at 260 nm) have ranged from 1.3 to 1.7 .

3) SDS-polyacrylamide gel electrophoresis was considered a useful means of analysing RNA polymerase samples. A photograph of a typical gel analysis is shown in Plate 1. Bands corresponding to the β, β', and α subunits were invariably seen. Although σ was always present, its proportion relative to the subunits of core enzyme varied, in agreement with 1) above. In addition to β, β', α, and σ, another polypeptide species, designated θ, was present in gels of RNA polymerase preparations and such enzyme will be denoted $E_{\sigma\theta}$.

6B THETA

The polypeptide θ has mol.wt. of about 58 000 as determined by SDS-polyacrylamide gel electrophoresis and was consistently present in RNA polymerase prepared in this laboratory by the method described in Section 4A (Lochhead, 1972). In the course of this present work, further knowledge of θ's behaviour was sought.

6B1 PREPARATION OF RNA POLYMERASE CONTAINING NO THETA

Enzyme that was essentially devoid of θ was prepared by a modification of the normal purification procedure. Instead of the two agarose gel filtration steps described in Section 4A, glycerol gradient centrifugation was used, as had been given originally by Burgess (1969a) as a preparative method. After DEAE-cellulose chromatography, the enzyme fraction (fraction 4) was subjected to centrifugation on a low salt 10-30% (v/v) glycerol gradient for 24 h at 25 000 rev./min in a Spinco SW25.2 rotor at 4°C (Section 4C). The analysis of the gradient is shown in Fig. 6.1. The fractions containing enzyme activity were pooled, precipitated by the addition of 1.5 volumes of Buffer C saturated with ammonium sulphate and centrifuged at 30 000 rev./min in a Spinco 30 rotor at 4°C. The pellet was dissolved in Buffer A + 1.0 M-KCl (fraction 5a) and dialysed against this same buffer for 2 h. The protein was then centrifuged in a high salt 10-30% (v/v) glycerol gradient in a Spinco SW25.2 rotor (Section 4C). The rotor was operated at 25 000 rev./min and 4°C for 36 h. Analysis of the gradient is shown in Fig. 6.2. Two fractions were analysed by SDS-polyacrylamide gel electrophoresis (Plate 2), but the fractions containing maximal enzyme activity were pooled and the enzyme collected as for low salt gradients. SDS-polyacrylamide gel electrophoresis of the final enzyme was

compared with enzyme prepared simultaneously from the same fraction 4 using the normal gel filtration steps (Plate 2). The enzyme purified via the glycerol gradients can be seen to contain only traces of θ. The immediate conclusions from the above experiment are :

1) It is possible to separate θ from RNA polymerase.

2) It is known that RNA polymerase has a mol.wt. of the order of 450 000 in solutions of high ionic strength and yet θ had the greater sedimentation coefficient at 1.0 M-KCl. Since θ has mol.wt. of about 58 000 in the presence of SDS and mercaptoethanol, the θ-containing species in the high salt glycerol gradient must represent an aggregate of 58 000 mol. wt. polypeptide.

3) Analysis of the high salt glycerol gradient showed three incompletely resolved peaks of u.v. absorbing material and a fourth peak of more slowly sedimenting material. Peak 1 contained θ and peak 3, β, β', σ, and α. Peak 2 comprised β, β', and θ. This could be caused by the formation of a complex of β and β' with one or more molecules of θ ; perhaps, $\beta\beta'\theta_n$. The presence of α cannot be totally excluded, however, since in equimolar proportions α stains much less strongly than β. Thus, peak 2 could contain core enzyme plus θ.

6B2 SEPARATION OF THETA FROM THETA-CONTAINING RNA POLYMERASE

The results of the previous section prompted the following experiment. RNA polymerase, prepared in the normal way and containing θ, was centrifuged in a high salt glycerol gradient as described in Section 4C. As might be predicted from the results in Section 6B1, separation of θ from the other components of the enzyme was achieved, although in this case only two protein peaks were observed (Fig. 6.3). One peak

contained predominantly θ and the other β, β', α, and σ. The intervening fractions displayed a gradation in the ratio of θ to β or β' (Plate 3). This experiment again illustrates the isolation of θ which is in a high state of purity (fraction 12 in Plate 3). The difference in the profiles of Fig. 6.2 and Fig. 6.3 may only be a question of resolution but could result from the different source of the enzyme sample applied to the gradient. In this case, the enzyme used had already been purified by agarose gel filtration steps, and the above result suggests that any sample of $E_{\sigma\theta}$ can be separated into E_{σ} and θ by this density gradient treatment.

6B3 DEAE-CELLULOSE CHROMATOGRAPHY

In the normal RNA polymerase preparation, elution of the DEAE-cellulose was with stepped increases of KCl concentration (Section 4A5). In one instance, elution was carried out using a gradient (0.13-0.4 M) of KCl (Fig. 6.4). Fractions were selected for analysis by SDS-polyacrylamide gel electrophoresis (Plate 4). Although the enzyme is relatively impure at this stage, certain features of the distribution of RNA polymerase polypeptides was observed.

1) The amount of θ increased as the KCl concentration became greater.

2) A prominent band with similar mobility to σ was observed in a gel arising from fraction 80, with concomitantly low levels of β, β', and α. This might indicate an alternative route for preparing σ.

RNA polymerase eluting from the DEAE-cellulose column at different KCl concentrations had different proportions of the components σ and θ. In the case of θ, whether this represented different enzyme-θ complexes at high and low salt concentration cannot be decided at this time, but this experiment does serve to illustrate how the preparative procedure can influence the

final composition of the enzyme ; the enzyme-containing fractions from DEAE-cellulose chromatography were pooled and carried through the normal purification procedure. The resulting RNA polymerase contained less than the usual proportion of θ and also had a prominent component of mol.wt. about 52 000 (Plate 4).

6B4 EFFECT OF THETA ON ENZYME AVTIVITY

Enzyme preparations G3 and G3' resulted from the agarose gel filtration procedure and the glycerol gradient method respectively so that G3 contained a normal level of θ whilst G3' was defficient in θ. The activities of these two enzyme samples were compared with a view to observing an effect arising from the presence of θ. The results are shown in Table 6.1. The ratio of the activity of G3 to G3' was approx. 1, with both T7 DNA and calf thymus DNA templates. Also, the ratio of activity with T7 DNA to that with calf thymus DNA was the same for both enzyme samples. The substitution of Mn^{2+} for Mg^{2+} in the reaction mixtures did not significantly alter the results. By the admittedly coarse criterion of this experiment, then, no difference was observed between enzyme lacking θ and enzyme containing θ.

6B5 SUMMARY

The polypeptide θ of mol.wt. 58 000 co-purifies with RNA polymerase in the purification procedure described in Section 4A. The reason for θ co-purifying is not so clear. If θ is an integral part of the transcription machinery, then its role has still to be elucidated. Glycerol gradient centrifugation at 1.0 M-KCl allowed the separation of θ from RNA polymerase, under which conditions θ existed as a species with mol.wt. in excess of 500 000, presumably an aggregate of θ. In both glycerol gradient and DEAE-cellulose chromatography experiments,

fractions were obtained that contained θ and RNA polymerase in widely varying relative proportions. Whether this resulted from effects on the stability of specific enzyme-θ complexes or was the reflection of θ's intrinsic properties, is not clearly established in the preceding experiments. The specific association of θ with RNA polymerase cannot be ruled out at this time.

6C OTHER COMPONENTS OF RNA POLYMERASE PREPARATIONS

Besides β, β', α, σ, and θ, other polypeptide species have been observed in the RNA polymerase prepared in several laboratories (Section 1A2.5). SDS-polyacrylamide gel electrophoresis of various RNA polymerase samples, purified in this laboratory, also indicated the presence of several minor components. These components may be co-purifying contaminants, but in view of the present lack of knowledge concerning the transcription machinery, it is worthwhile to catalogue their appearance in RNA polymerase samples prepared in this laboratory.

6C1 CATALOGUE OF MINOR COMPONENTS

This section lists a number of polypeptide species which have occurred in different enzyme preparations that included various procedural changes. These species were characterized by the estimation of their mol.wt.'s by SDS-polyacrylamide gel electrophoresis as described in Section 3F5. A compilation of these minor components is given in Table 6.2 .

The mol.wt.'s are based on β', β, α, σ, and θ as markers, taking the mol.wt.'s for these from Lochhead (1972) - see Section 1A2.2 . These values are lower, especially for β, β', and σ than those commonly quoted (Chamberlin, 1970 ; Burgess, 1971) but the choice of standard mol.wt.'s will introduce systematic rather than random changes in derived estimates of

mol.wt.'s.If in fact, Lochhead's values for $\beta'+\beta$ and α are taken, and the data of Burgess et al. (1969) treated as described in Section 3F5, σ then is given a mol.wt. of 80 000 and τ, 94 000. Of the components listed, x3 is most likely τ but no positive identification of any of the others has been possible although there may be some correlation with the bands observed in a commercial preparation of polynucleotide phosphorylase when similarly analysed (Section 6C2). The polypeptide ω, frequently observed in RNA polymerase (Section 1A2.5), would not have been detected by the gel electrophoresis system used as it would have run off the end of the gel.

6C2 PRESENCE OF POLYNUCLEOTIDE PHOSPHORYLASE IN RNA POLYMERASE PREPARATIONS

The presence of polynucleotide phosphorylase activity in RNA polymerase preparations has been noted previously (Burgess, 1971). Using the assay system described in Section 5E2, polynucleotide phosphorylase activity has been detected in all four enzyme preparations that were analysed. Commercial Nucleoside triphosphates contained enough diphosphates to support detectable activity, but with further purified ATP and UTP no activity was observed (Fig. 6.5). This finding, along with the inhibition of the reaction by P_i (Fig. 6.6), was taken as evidence that the reaction observed was the result of polynucleotide phosphorylase activity.

The polynucleotide phosphorylase activity of various enzyme preparations is shown in Table 6.3 . Core enzyme was devoid of such activity but the wash-through (peak A) from phosphocellulose column was particularly active, indicating that β, β', and α are not implicated in the polynucleotide phosphorylase activity.

Because of the above results, a commercial preparation of polynucleotide phosphorylase from E. coli was subjected to SDS-polyacrylamide gel electrophoresis, both on its own and mixed with RNA polymerase (Plate 5). This sample of polynucleotide phosphorylase gave rise to several bands on electrophoresis, and the mol.wt.'s of some of these were estimated as in Section 3F5. It is not known which of these polypeptides were impurities and which genuine subunits of polynucleotide phosphorylase. As judged by mol.wt., some of the polypeptides found in the polynucleotide phosphorylase preparation are equivalent to some of the minor components of RNA polymerase listed in Table 6.2 (τ , x4,x6 and x7). Also, a species denoted x10 had a similar mol.wt. to σ . For all these polypeptides, this does not constitute proof of identity but does suggest that there are polypeptides that co-purify with both RNA polymerase and polynucleotide phosphorylase.

6D CHARACTERIZATION OF CORE ENZYME

Core enzyme, prepared by phosphocellulose chromatography (Section 4B) contained only β, β', and α as assessed by SDS-polyacrylamide gel electrophoresis. E_{280}/E_{260} ratios were greater than 1.5 and the activity of the enzyme was much lower with T7 DNA than calf thymus DNA (Table 6.4). Activity on the T7 DNA template was greatly increased in the presence of the peak A (which contained σ) from phosphocellulose chromatography.

The results of SDS-polyacrylamide gel electrophoresis of a typical core enzyme preparation are seen in Plate 6, and enzyme of this structure will be denoted by E_c.

Fig. 6.1 Glycerol gradient centrifugation of RNA polymerase fraction 4 at low KCl concentration

Fraction 4 enzyme of the normal preparative method was centrifuged at 25 000 rev./min in a Spinco SW25.2 rotor for 24 h on a 10-30% (v/v) glycerol gradient (Section 6B1). The closed circles are the results assays for the incorporation of ^{3}H-ATP as measured by the standard assay procedure (calf thymus DNA 150 µg/ml)-Section 5C. The continuous line represents the u.v. absorption at 280 nm as measured by passage of the gradient through the flow cell of a Gilford spectrophotometer. The volume contained in each centrifuge tube was 60 ml and the fractions were collected starting from the bottom of the tube.

6.1

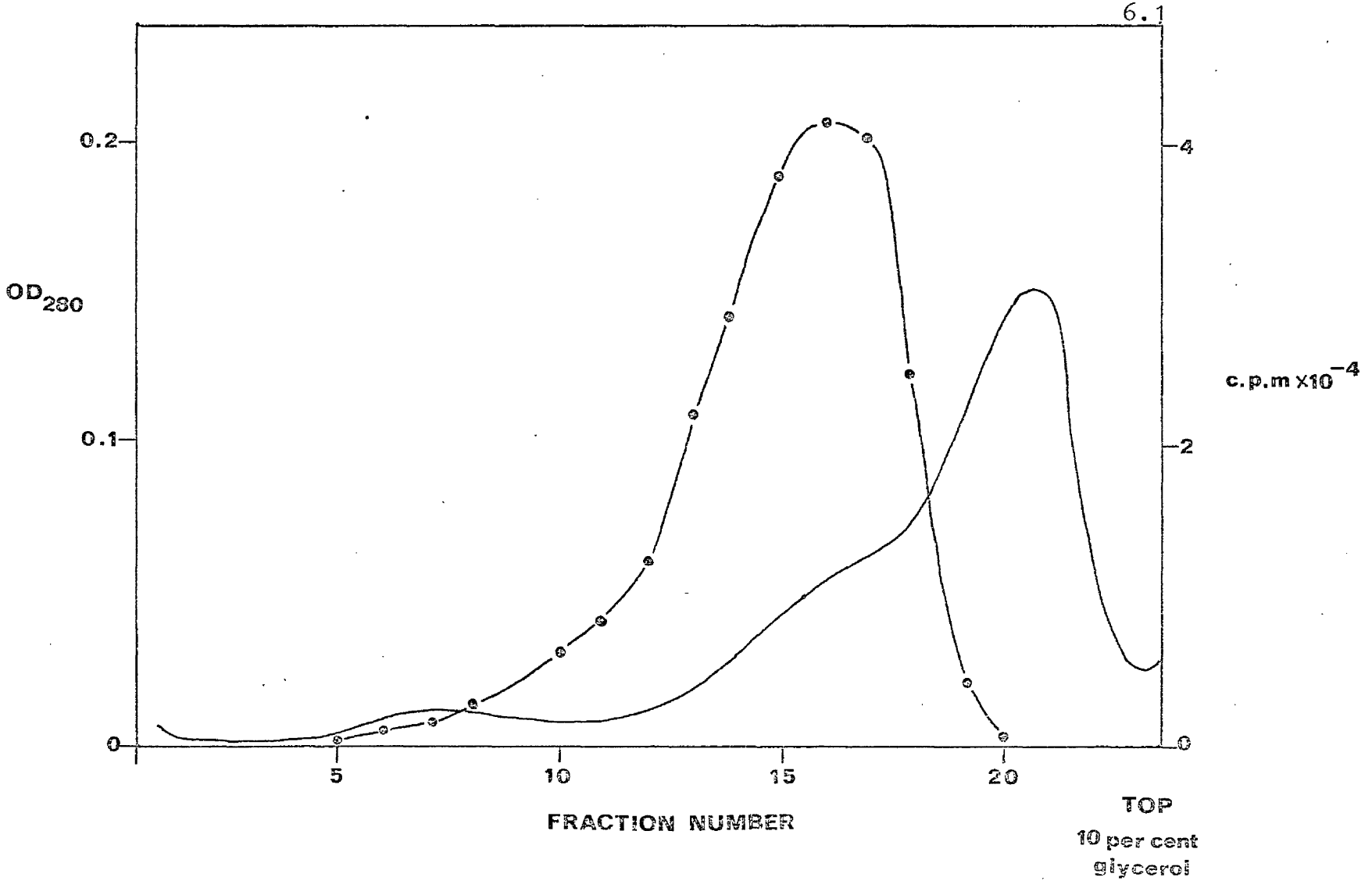

0.2
0.1
0
OD_{280}
4
2
0
c.p.m $\times 10^{-4}$
5
10
15
20
FRACTION NUMBER
TOP
10 per cent
glycerol

Fig. 6.2 Glycerol gradient centrifugation of RNA polymerase fraction 5a at high KCl concentration

Fraction 5a enzyme was centrifuged at 25 000 rev./min in a Spinco SW25.2 rotor for 36 h on a 10-30% glycerol gradient at 1.0 M-KCl (Section 6B1). The histogram represents the incorporation of ^{3}H-ATP as measured by the standard assay procedure (Section 5C). The broken line is the u.v. absorption at 280 nm as measured by the passage of the gradient through the flow cell of a Gilford spectrophotometer. The volume contained in the centrifuge tube was 60 ml and fractions were collected starting at the bottom of the tube. SDS-polyacrylamide gel electrophoresis was used to analyse tubes 11 & 13, as well as the enzyme finally purified by the glycerol gradient method (Plate 2).

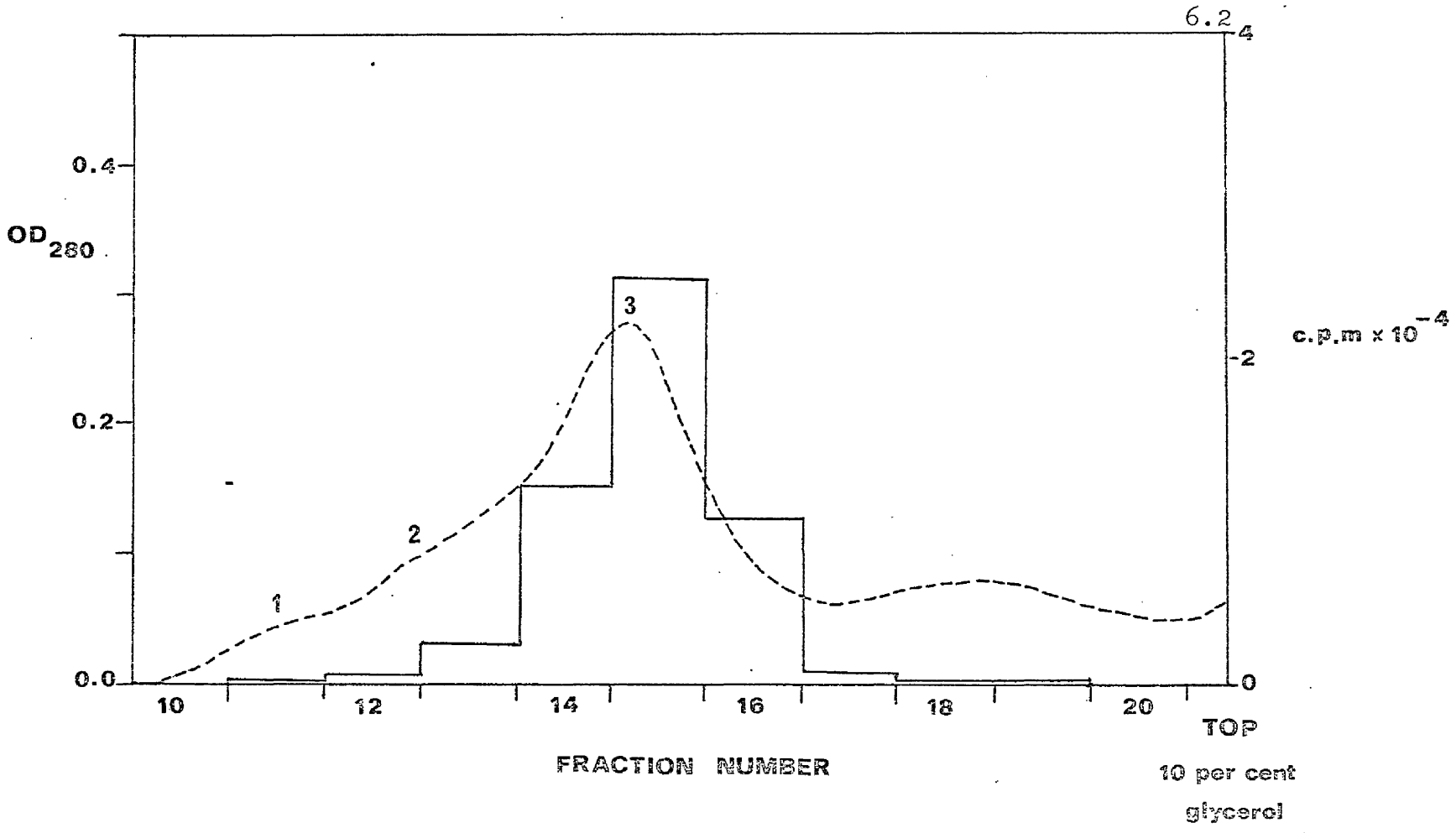

6.2
4
0.4
OD_{280}
3
c.p.m x 10^{-4}
2
0.2
1
0.0
0
10
12
14
16
18
20
TOP
FRACTION NUMBER
10 per cent
glycerol

Fig. 6.3 Glycerol gradient centrifugation of RNA polymerase $E_{\sigma\theta}$ at high KCl concentration

RNA polymerase, fraction 6 of a normal preparation, was centrifuged in a high salt (1.0 M-KCl) glycerol gradient in a Spinco SW25.2 rotor at 25 000 rev./min for 36 h. The profile in the figure is that of the u.v. absorption at 280 nm as measured by passage of the gradient through the flow cell of a Gilford spectrophotometer. The volume contained in the centrifuge tube was 60 ml and fractions were collected starting from the bottom of the tube. Fractions 10, 12, 14, 16, 18, and 19 were analysed by SDS-polyacrylamide gel electrophoresis (Plate 3)

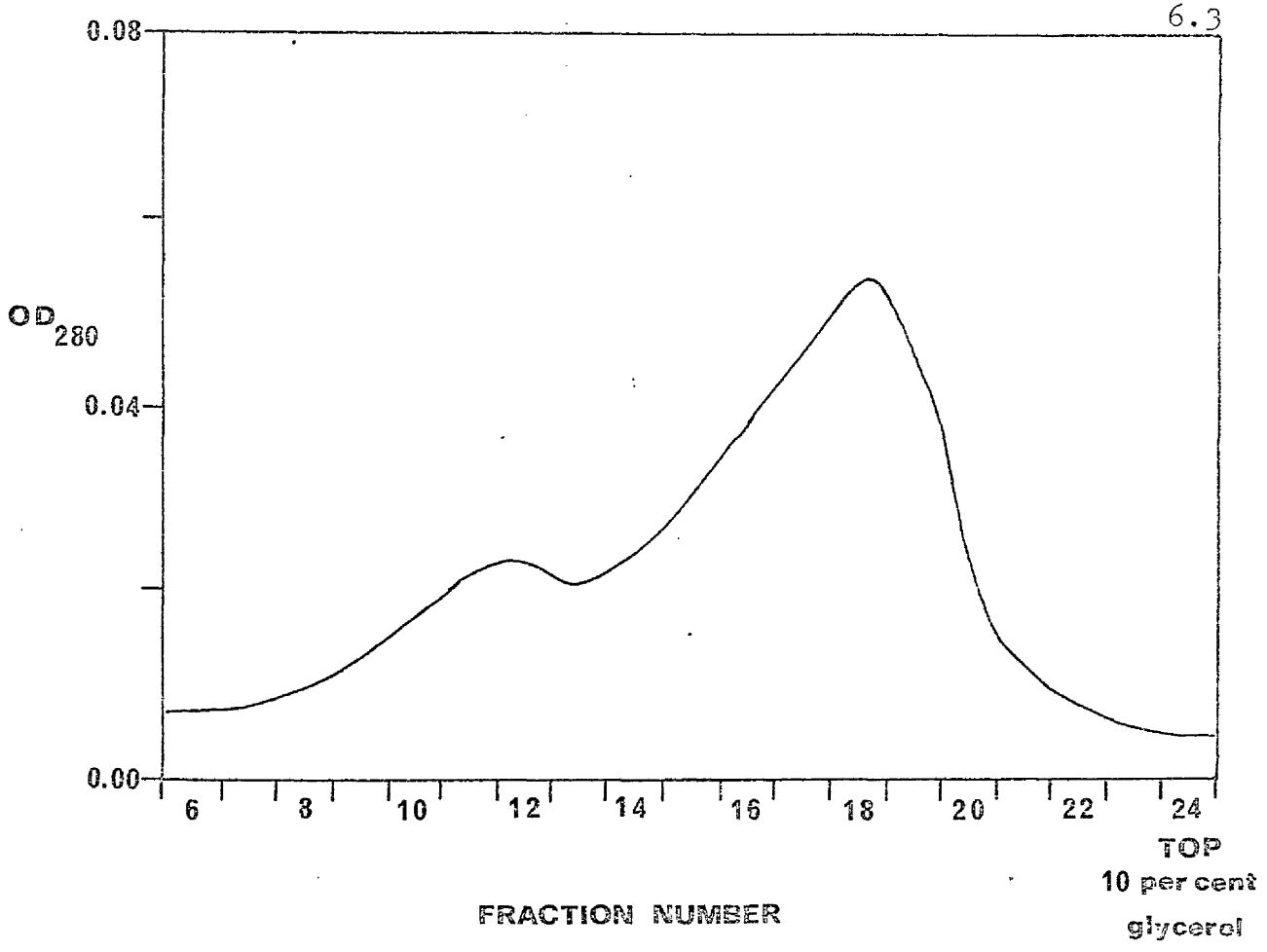

0.08
0.04
0.00
OD_{280}
6
8
10
12
14
16
18
20
22
24
TOP
FRACTION NUMBER
10 per cent
glycerol

Fig. 6.4 DEAE-cellulose chromatography in RNA polymerase preparation - KCL gradient elution

RNA polymerase fraction 3 (Section 4A4) was applied to a column of DEAE-cellulose as described for the normal enzyme purification in Section 4A5. Elution was initially stepwise, as normal, with the successive passage of Buffer A and Buffer A + 0.13 M-KCl. Then, instead of eluting the column with Buffer A + 0.23 M-KCl, a KCl gradient was used (0.13-0.4 M-KCl in Buffer A). Finally, the column was eluted with Buffer A + 1.0 M-KCl. Flow rate was 90 ml/h.

The figure shows only the elution profile after the start of the KCl gradient - the initial phase was as in Fig. 4.1. The continuous line indicates the u.v. absorption at 280 nm in the 3 mm pathlength flow cell of an LKB Uvicord. The points show the incorporation of ^{3}H-ATP measured by the standard assay procedure (Section 5C) for some of the fractions. Fractions 80, 100, and 130 were analysed by SDS-polyacrylamide gel electrophoresis (Plate 4)

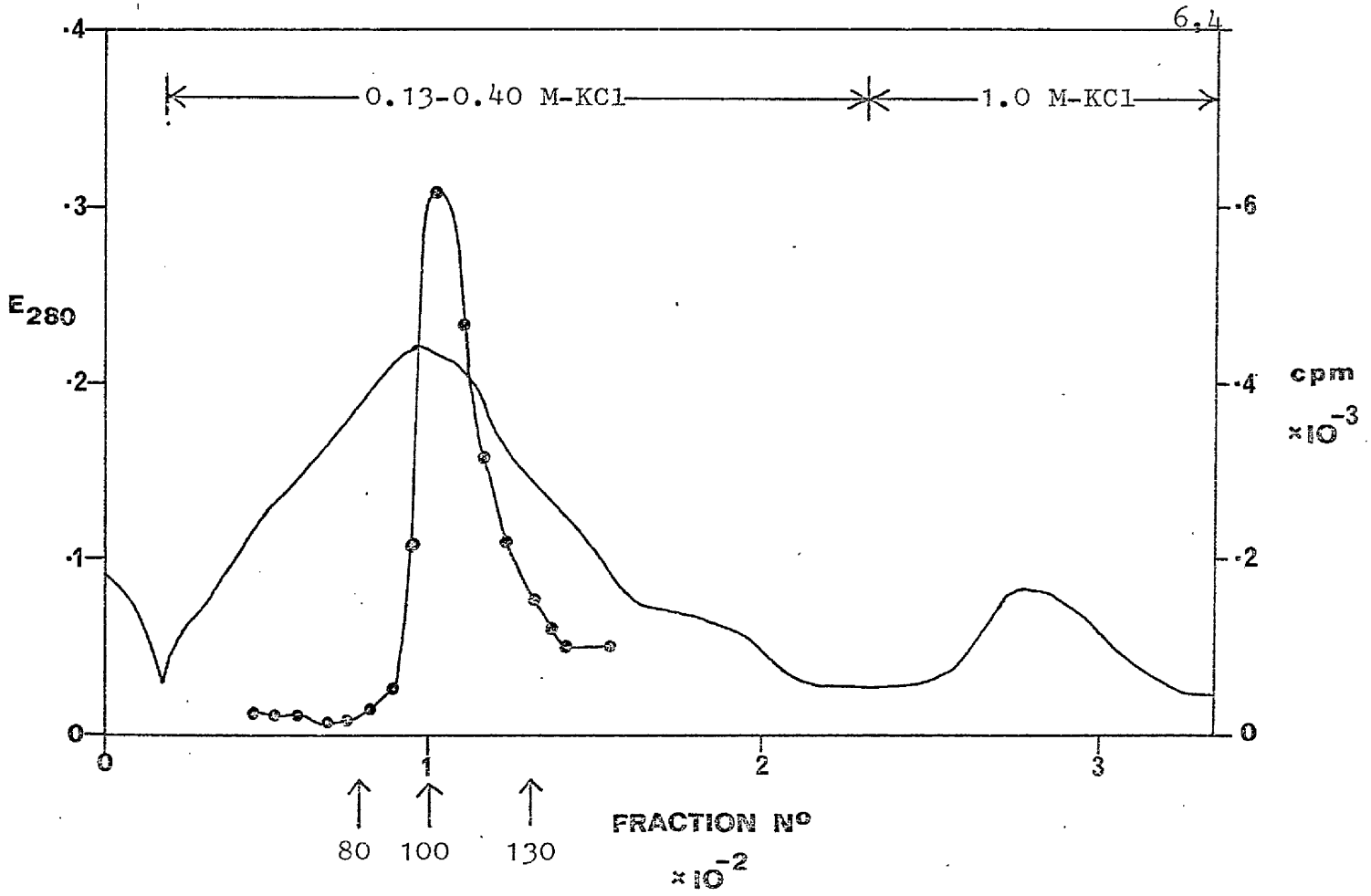

0.13-0.40 M-KCl
1.0 M-KCl
E_{280}
·4
·3
·2
·1
0
·6
·4
·2
0
cpm
$\times 10^{-3}$
0
1
2
3
80
100
130
FRACTION Nº
$\times 10^{-2}$

Fig. 6.5 Polynucleotide phosphorylase activity in RNA polymerase preparations (1)

Assays for polynucleotide phosphorylase activity in a normal RNA polymerase preparation were carried out essentially as described in Section 5E2. Standard RNA polymerase reaction conditions were used (Section 5B) except that 1 μg/ml ethidium bromide was also present and only two nuclectides, ATP and UTP, were included (●-● : commercial preparations of ATP and UTP, 0.75 mM each ; o-o : further purified ATP and UTP, 1 mM each). The reaction, 1 ml volume in a quartz cell, was started by the addition of 110 μg/ml $E_{\sigma\theta}$ and the fluorescence of the ethidium bromide followed (excitation at 310 nm and emission at 615 nm). The reaction took place at room temperature (about 20°C)

Instrument details (see Section 2B1)

Xenon lamp : No. 2

Photomultiplier tube No. 2

Slit arrangement : Slits 2, 5, and 7, all 5 mm

All other slit positions unoccupied

Sensitivity : 100

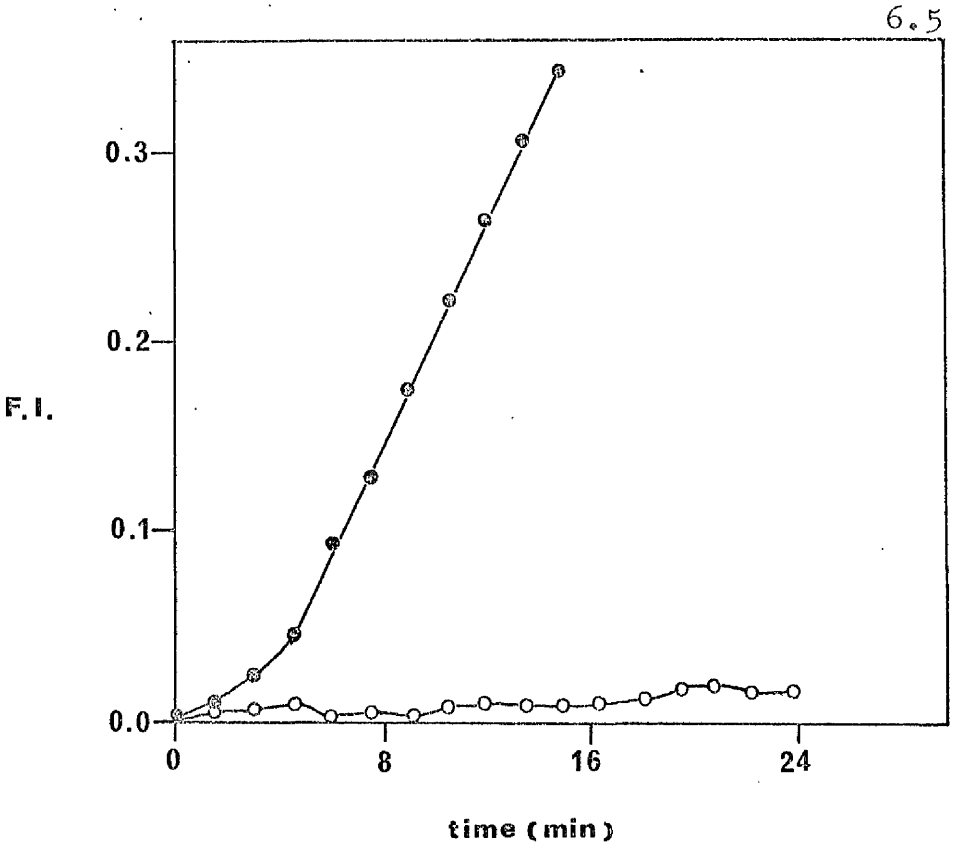
Fig. 6.6 Polynucleotide phosphorylase activity in RNA polymerase preparations (2)

Polynucleotide phosphorylase activity was assayed in a normal RNA polymerase preparation as described in the above legend, using standard RNA polymerase assay conditions, except that 1 μg/ml ethidium bromide was present and :

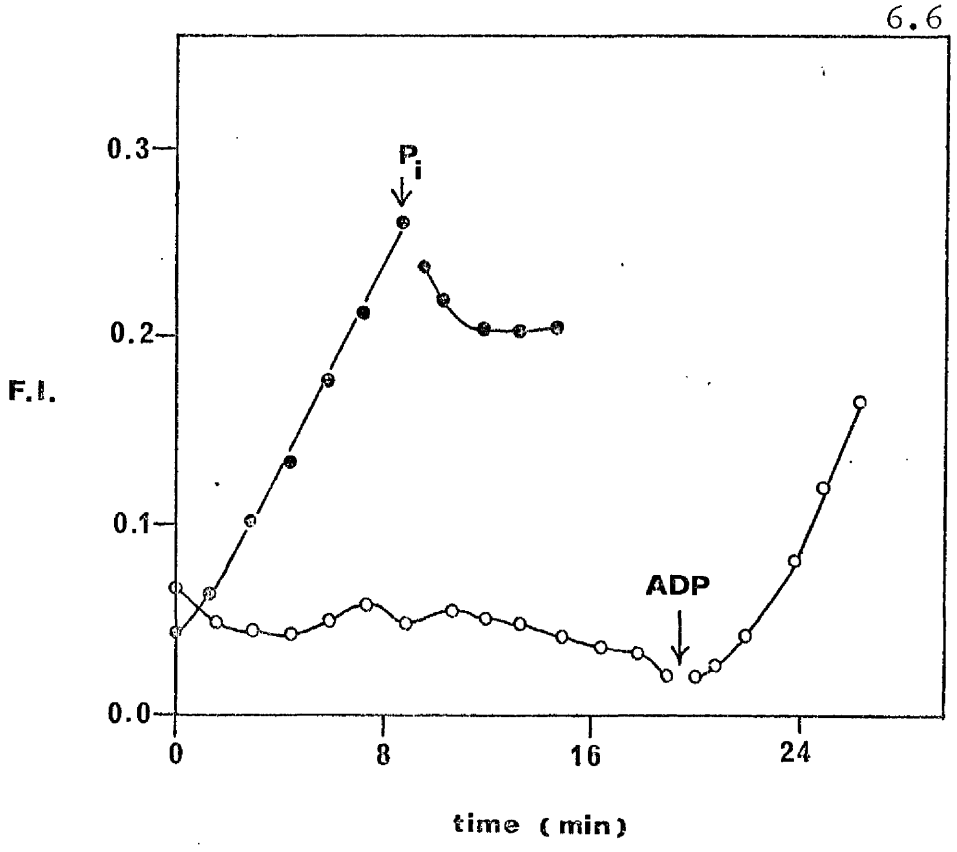
●-● : the nucleotides present were ATP and UTP (commercial), 0.75 mM each. The addition of 1 mM phosphate is noted.

o-o : no nucleotides initially present and the addition of 1 mM-ADP is indicated, after which the reaction started.

6.5


F.I.
0.3
0.2
0.1
0.0
0
8
16
24
time (min)

6.6


F.I.
Pi
ADP
0.3
0.2
0.1
0.0
0
8
16
24
time (min)

enzyme	ion	T7 DNA	calf thymus DNA
$E_{\sigma\theta}$ (G3)	Mg^{2+}	733 EU/mg	187
	Mn^{2+}	490	210
E_{σ} (G3')	Mg^{2+}	600	175
	Mn^{2+}	425	177

Table 6.1 Specific activities of RNA polymerase, $E_{\sigma\theta}$ and E_{σ}, in the presence of different templates and divalent cations

The numbers in the table are the values for the specific activities of RNA polymerase, measured by the standard radioactive technique (Section 5C), and expressed as EU/mg. Standard reaction conditions were used (Section 5B) except where Mn^{2+} (4 mM) replaced Mg^{2+} (20 mM). The concentrations of DNA's and enzyme (μg/ml) were : T7 DNA and calf thymus DNA, 60 ; $E_{\sigma\theta}$, 15.4 ; E_{σ}, 27.2. The assays were for 5 min at 37°C, and controls were run which contained no DNA.

Table 6.2 Catalogue of polypeptides found in RNA polymerase preparations (using the SDS-polyacrylamide gel electrophoresis technique)

IDENTITY	MOL.WT. ($x10^{-3}$)	A	B	C	D	E	F	G	H	I
β'	150	***	***	***	***	***	***	***	***	
β	140	***	***	***	***	***	***	***	***	
x2	130							*		
x3(τ)	93				**	*		**	**	*
x4	80		**		**	**	*			*
σ	78	**	**	**	**	***	**	***	***	
x10	78									*
x6	66			*		*	*	*		*
θ	58	***	***		**	*	*	*	***	
x7	52		*			**		**	***	**
x8	48							***	**	
x9	44							**	*	
α	37	**	**	**	**	**	**	**	**	

The relative intensity of staining of polypeptide species after SDS-polyacrylamide gel elctrophoresis (Section 3F) is represented on a three point scale ; ***, strong, **, moderate, and * weak.

A,B : normal RNA polymerase preparations

C : enzyme prepared using glycerol gradients (Section 6B1 and Plate 2)

D : normal enzyme preparation, except that caproic acid was present, during the purification procedure.

E : enzyme prepared using KCl gradient elution at DEAE-cellulose chromatography stage (Section 6B3 and Plate 4).

F : fraction 16 of high salt glycerol gradient experiment using a normal enzyme preparation (Section 6B2 and Plate 3)

G,H : fractions of KCl gradient DEAE-cellulose chromatography when used in enzyme purification. G & H are fractions 100 and 130 respectively (Section 6B3 and Plate 4)

I : *E. coli* polynucleotide phosphorylase (Section 6C2 and Plate 5)

sample assayed	activity ΔFI/min/mg protein
(1)	0.68
$E_{\sigma\theta}$ (2)	0.65
(3)	1.11
E_{σ}	3.18
peak C	0
peak A	27.5

Table 6.3 Polynucleotide phosphorylase activity in several RNA polymerase samples

Polynucleotide phosphorylase activity in several RNA polymerase preparations was measured by the method described in Section 5E. Standard RNA polymerase reaction conditions (Section 5B) were used, except that 1μg/ml ethidium bromide was included and 1 mM-ADP replaced the four nucleoside triphosphates. There was, then, no DNA present. The addition of enzyme samples (20-100 μg) to the 1 ml of reaction mixture started the reaction which was followed fluorimetrically, the details being the same as for Fig. 6.5. The initial rate of increase of fluorescent intensity was taken as a relative measure of polynucleotide phosphorylase activity.

Peak A and peak C refer to protein fractions resulting from phosphocellulose chromatography of RNA polymerase (Section 4B).

	sample assayed	specific activity (EU/mg)	
		T7 DNA	calf thymus DNA
1	$E_{\sigma\theta}$	1588	760
2	peak C (E_c)	264	512
3	peak C + peak A	1096	-

Table 6.4 Comparison of the activities of E_c and $E_{\sigma\theta}$

Enzyme was assayed by the fluorescence technique (Section 5D2) at standard reaction conditions (Section 5B) and at 37°C with the following enzyme and DNA concentrations (all in μg/ml) : T7 DNA, 50 ; calf thymus DNA, 60 ; $E_{\sigma\theta}$, 41.7 ; peak C(in 2), 43.4 ; peak C (in 3), 32.5 ; peak A, 18.6 . Controls contained no DNA.

PHOTOGRAPHS OF SDS-POLYACRYLAMIDE GEL ELECTROPHORETIC ANALYSES

The following Plates contain photographs of the results of analysing various protein samples by SDS-polyacrylamide gel electrophoresis. The details of the electrophoresis technique are described in Section 3F, and the following plates contain only identification and references to the text.

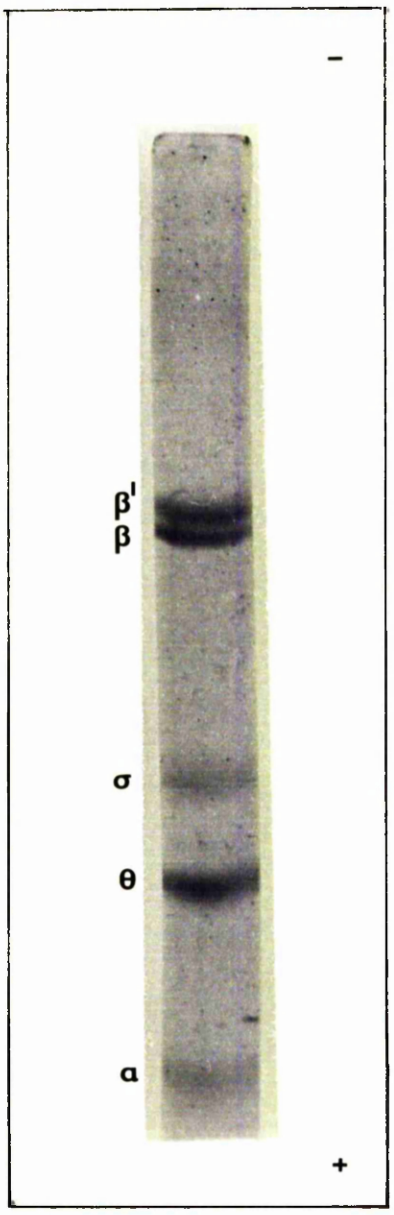
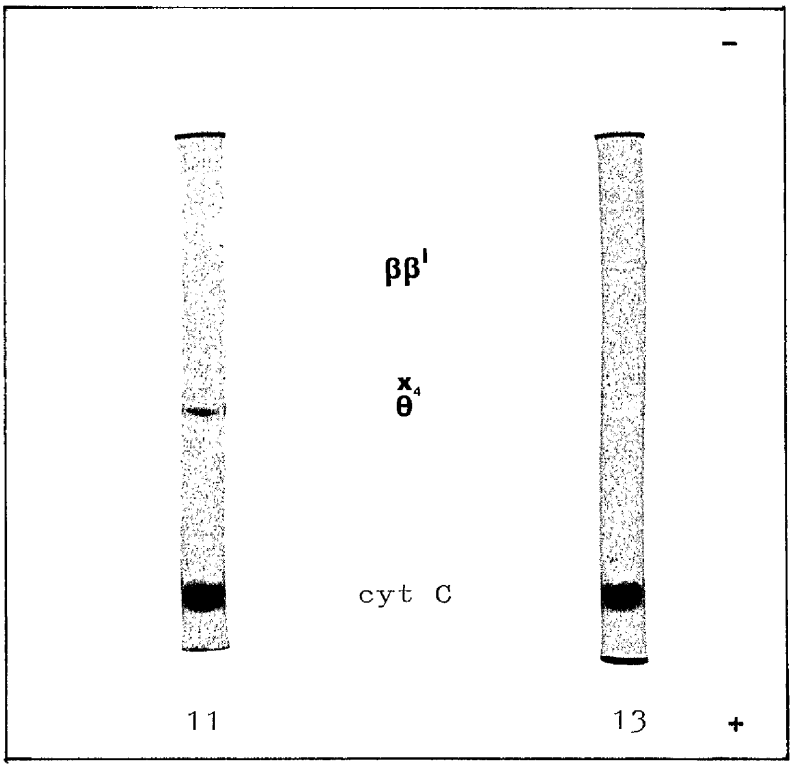
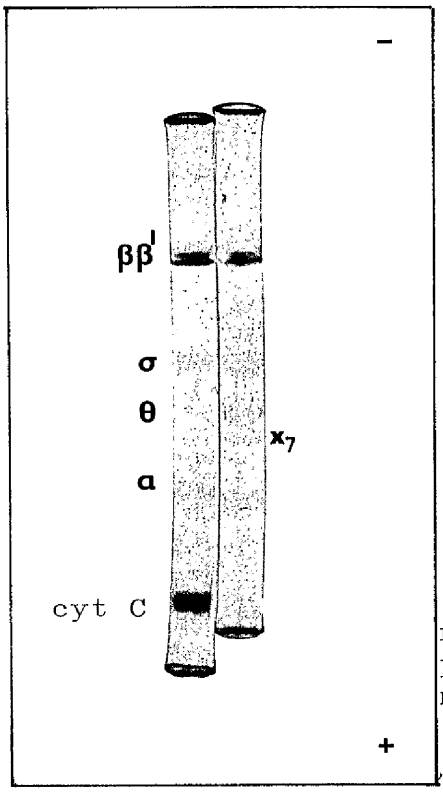


Plate 1

SDS-polyacrylamide gel electrophoresis of a normal RNA polymerase preparation



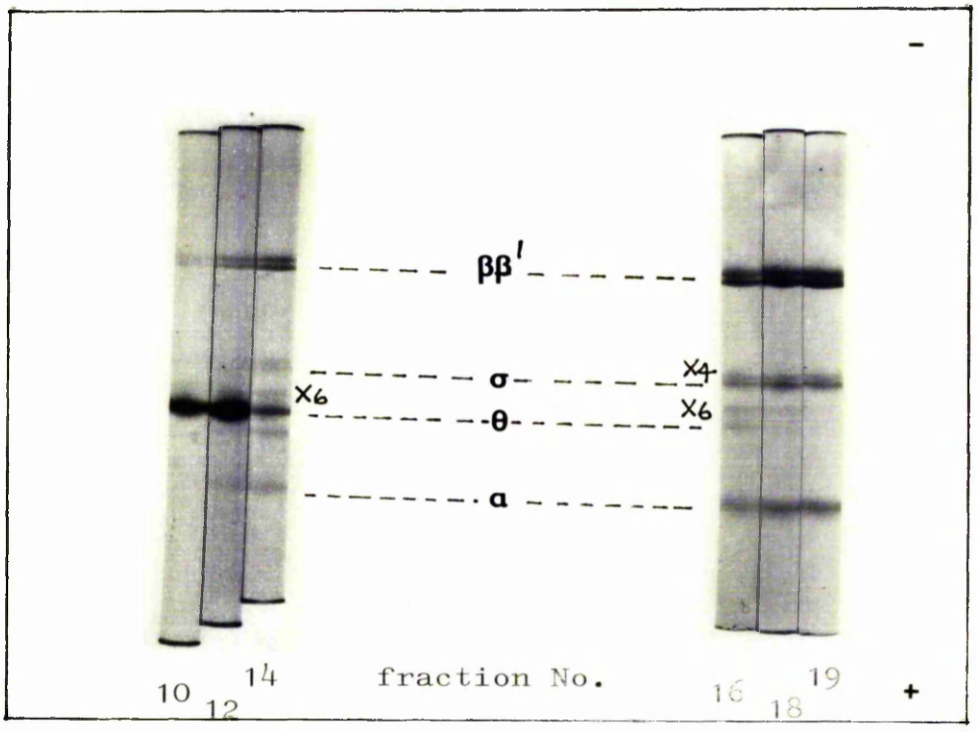
fractions from high salt glycerol gradient (Fig. 6.2)



enzyme peak from high salt glycerol gradient

normal purification method

Plate 2. Analysis of high salt glycerol gradient (Section 6B1) of RNA polymerase



Palte 3. Analysis of high salt glycerol gradient fractions (Fig. 6.3)

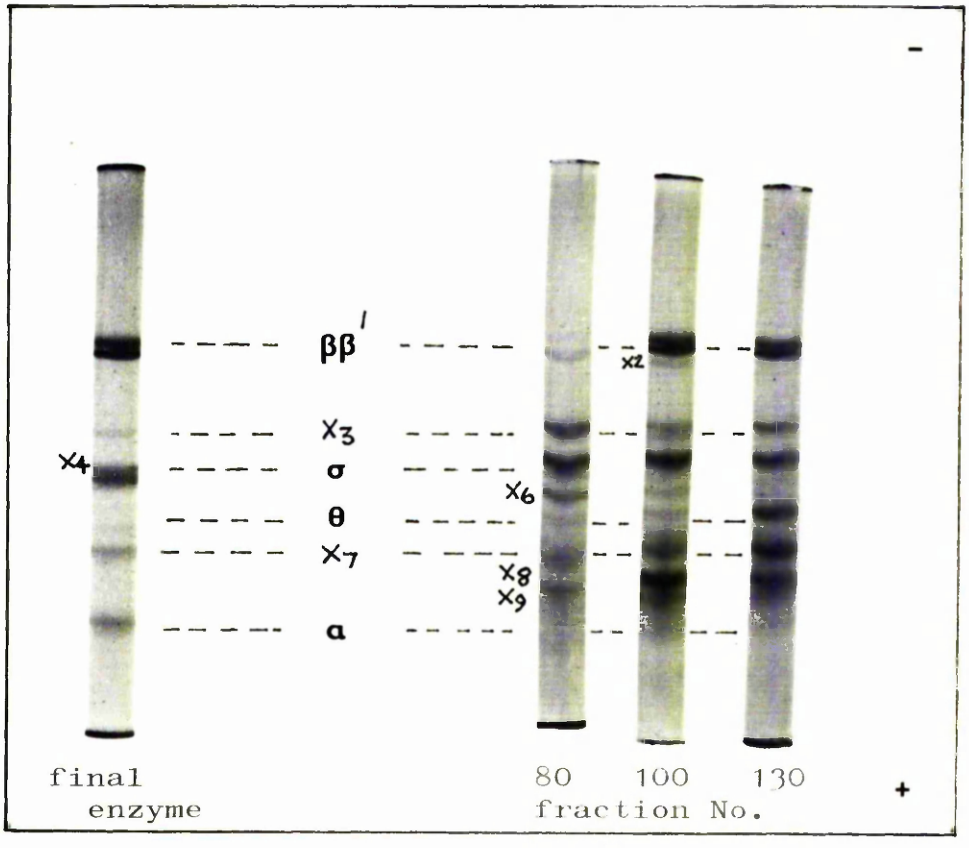


Plate 4. Purification of RNA polymerase using KCl gradient elution of DEAE-cellulose (Section 6B3 ; Fig. 6.4). Fractions are from DEAE-cellulose chromatography

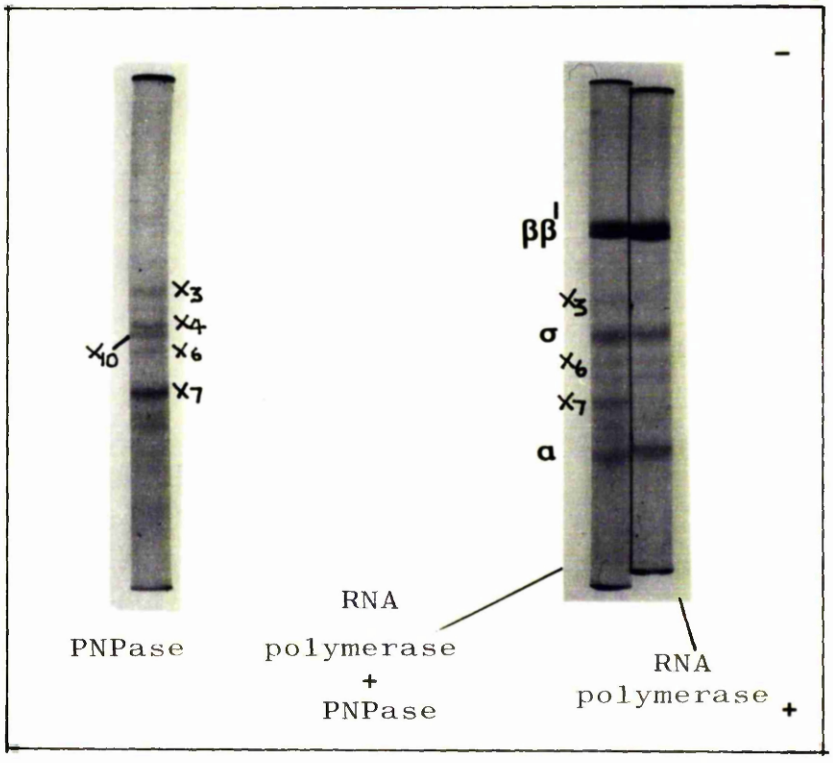


Plate 5. polynucleotide phosphorylase (PNPase)

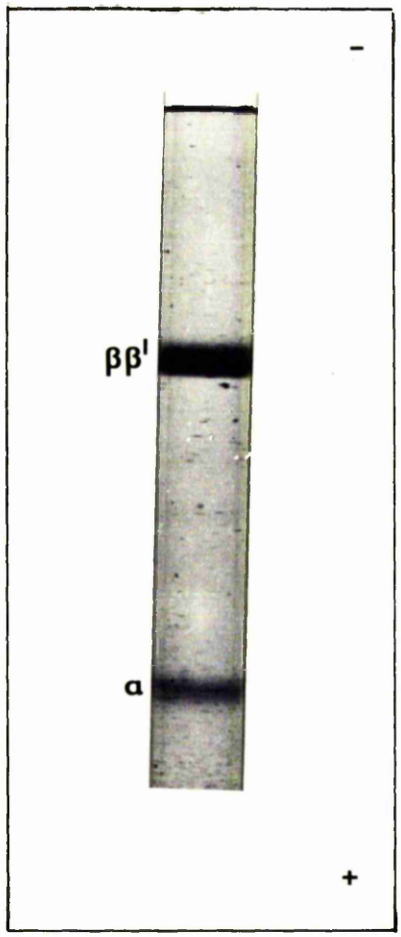


Plate 6

RNA polymerase core enzyme

SECTION 7

THE DEPENDENCE OF RNA POLYMERASE ACTIVITY ON REACTION CONDITIONS

7A DEPENDENCE ON ENZYME CONCENTRATION

Fig. 7.1 illustrates the relationship between the rate of RNA synthesis and RNA polymerase concentration. The curve approaches a saturation level, presumably due to saturation of the DNA template by enzyme. In subsequent experiments, the enzyme concentration was usually chosen to correspond to the linear region of the curve where DNA is not limiting.

7B DEPENDENCE ON TEMPLATE TYPE AND CONCENTRATION

Under the conditions used, RNA synthesis by RNA polymerase in the absence of DNA was very low, and enzyme assays lacking DNA were the most commonly used control. The activity of the enzyme with several different DNA's is shown in Table 7.1 . The enzyme contained σ, which is reflected in the high activity with T7 DNA as compared to that with calf thymus DNA. The effect of varying template concentration is illustrated in Fig. 7.2 for core enzyme with poly(dA-dT).

7C DEPENDENCE ON IONIC CONDITIONS

RNA polymerase is known to be influenced by ionic conditions, as discussed in Section 1C5. Consequently, some investigation of ionic effects was undertaken.

7C1 MONOVALENT CATIONS

RNA polymerase showed a maximum of activity with the variation of KCl concentration (Figs. 7.3 & 7.4) at about 0.2 M-KCl in two experiments ; in one, $E_{\sigma\theta}$ was used with a T7 DNA template, and in the other, E_c was used with a poly(dA-dT) template. The two experiments also differed as to the concentrations of $MgCl_2$, enzyme and template. Thus, at 0.2 M-KCl, the ionic strength differed between the two experiments.

However, the value of about 0.2 M-KCl agrees with that reported by So et al. (1967), and was taken as 'standard' KCl concentration. No detailed explanation of the effects of KCl on the reaction was sought.

7C2 DIVALENT CATIONS

The requirement of divalent cations for RNA polymerase activity is well-known (for example, Furth et al., 1962).

7C2.1 Dependence of the reaction rate on $MgCl_2$ concentration

The effect of the variation of $MgCl_2$ concentration on the initial rate of the RNA polymerase catalyzed reaction is shown in Figs. 7.5 & 7.6, for two enzyme-template systems. With $E_{\sigma\theta}$ and T7 DNA, the maximal rate was at 0.02 M-$MgCl_2$, and with E_c and poly(dA-dT) the maximum was at approx. 0.03 M-$MgCl_2$. In both cases, there was a very low rate in the absence of $MgCl_2$, and the optimal $MgCl_2$ concentrations were both somewhat higher than that reported by So et al. (1967) who quoted 0.012 M-$MgCl_2$ as optimal. 0.02 M-$MgCl_2$ was taken as 'standard' and used in most experiments.

The overall effect of $MgCl_2$ is probably a summation of the effects of Mg^{2+} on the various reaction components. With the poly(dA-dT)-E_c system, the specific requirement is clearly seen. Between 0 and 0.3 M-KCl, the activity of the enzyme was never below about 50% of the maximum (Fig. 7.3). At zero $MgCl_2$ concentration, however, when the ionic strength was in the above range (0.2 M-KCl), no poly(A-U) synthesis was detected. $MgCl_2$ is not required solely for its contribution to the ionic strength of the reaction mixture.

The concentration of Mg^{2+} in assays, as detected by the 8-hydroxyquinoline method (Section 3E), was lower than the total Mg^{2+} concentration added (Table 7.2). The difference was approximately the sum of the nucleoside triphosphate

concentrations, indicating, as would be expected, that complexes of Mg^{2+} and nucleoside triphosphates were present in the reaction mixtures (Section 1A4). It seems unlikely, however, that Mg^{2+} was required only to combine with the substrates since the $MgCl_2$ concentration that gave maximum activity was 3 to 5 times that needed to titrate the nucleoside triphosphates, the formation constants for Mg^{2+}-nucleotide complexes being high (Section 1A4). Further, the Mg^{2+} optima for the two enzyme-template systems differed by about 0.01 M, the higher optimum being observed, in fact, when the total nucleotide concentration was lower. This would not be explained if the effect of Mg^{2+} was only on the substrate.

7C2.2 Dependence of the time course of the reaction on $MgCl_2$ concentration

Fig. 7.7 illustrates the effect of $MgCl_2$ on the time course of RNA synthesis by RNA polymerase with a T7 DNA template. Elevation of the $MgCl_2$ concentration prolonged the period of linear RNA production with time. Above 0.05 M-$MgCl_2$ there was a lag phase in the time course. Both of these findings are in accord with the results of Fuchs et al. (1967). The time course of the reaction is considered in more detail in Section 8A. At 0.02 M-$MgCl_2$, RNA synthesis was linear for at least 10 min in a number of experiments, and this was part of the reason for selecting 0.02 M-$MgCl_2$ as standard.

7C2.3 Effect of Mn^{2+} on the reaction

Mn^{2+} is known to be able to replace Mg^{2+} in the RNA polymerase reaction (Furth et al., 1962). The effect of Mn^{2+} on the reaction was not investigated in detail but it was shown that the enzyme purified in this laboratory could function in the presence of Mn^{2+}. Table 6.1 shows the activity of RNA polymerase with Mn^{2+} and Mg^{2+} on both T7 DNA and calf thymus DNA templates. Little can be said on the basis of this

experiment concerning the effects of Mn^{2+} but the substitution of Mn^{2+} for Mg^{2+} did not markedly alter the ratio of the activities on T7 DNA and calf thymus DNA. Some investigators include both Mg^{2+} and Mn^{2+} in their standard assay system for RNA polymerase (for example, Anthony et al.,1969). This can give rise to greater RNA synthesis than in the presence of Mg^{2+} alone (Fuchs et al., 1967), but these assay conditions were not adopted on the grounds that they introduce a further component into an already complex reaction system.

7C2.4 Effect of Zn^{2+} on the reaction

The effect of Zn^{2+} on the activity of RNA polymerase has not received a great deal of attention but Scrutton et al., (1971) recently described experiments that provided evidence for the presence of Zn in RNA polymerase. This prompted the following experiments. RNA polymerase activity was measured in the presence of 0.5 mM-Zn^{2+} (zinc acetate). The results are in Table 7.3, from which it can be seen that this level of Zn^{2+} totally inhibited the action of both E_c and $E_{\sigma\theta}$ on a calf thymus template. When the Zn^{2+} concentration was varied, the results in Fig. 7.8 were obtained. Half maximal inhibition occurred at about 0.2 mM-Zn^{2+}. Added Zn^{2+} either had a completely inhibitory effect on the reaction or else the concentration range studied did not extend low enough to see any stimulation.

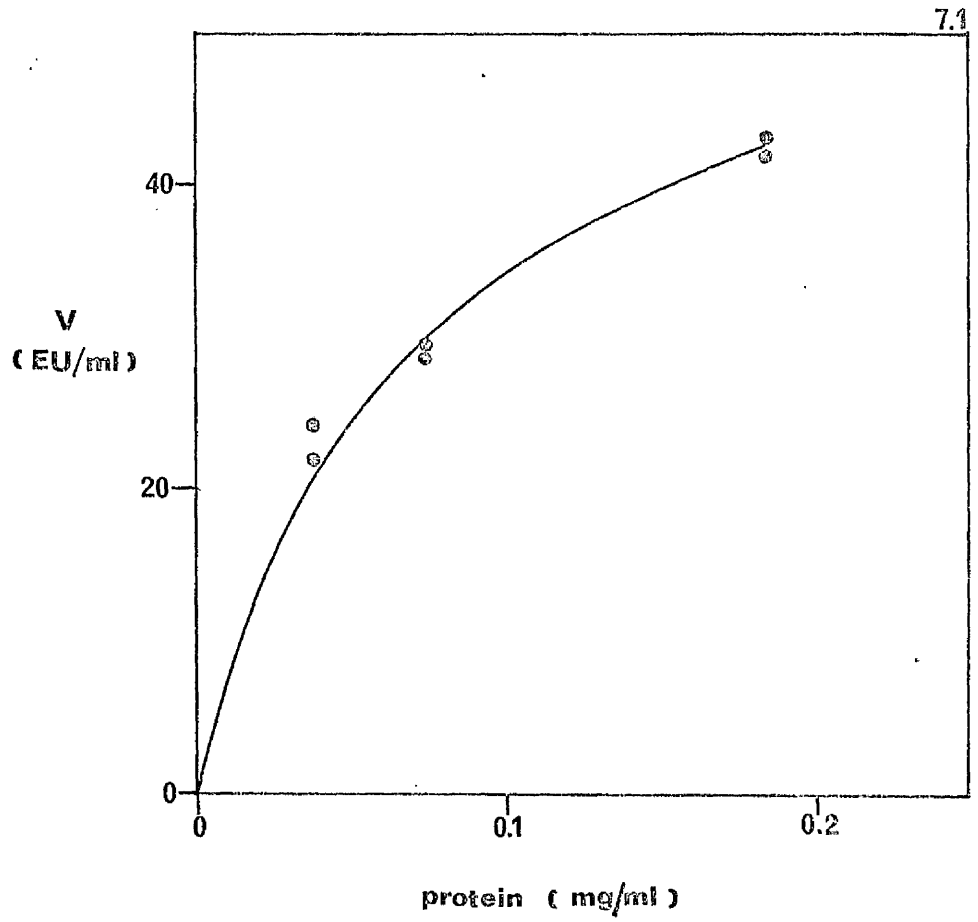


Fig. 7.1 Variation of RNA polymerase activity with enzyme concentration

RNA polymerase activity was assayed by the radioactive method (Section 5C) at standard reaction conditions (Section 5B) except that $MgCl_2$ and KCl were 0.015 M and 0.15 M respectively. Calf thymus DNA was present at a concentration of 60 µg/ml and RNA polymerase, $E_{\sigma\theta}$, was varied as indicated on the axis of the figure. The assays were for 10 min at 37°C.

Fig. 7.2 Variation of RNA polymerase E_c activity with poly(dA-dT) concentration

The activity of RNA polymerase core enzyme was determined by the radioactive technique (Section 5C) at standard reaction conditions (Section 5B). Each assay contained 13.4 μg/ml of E_c and the indicated concentration of poly(dA-dT). The assays lasted 5 min and were carried out at 37°C.

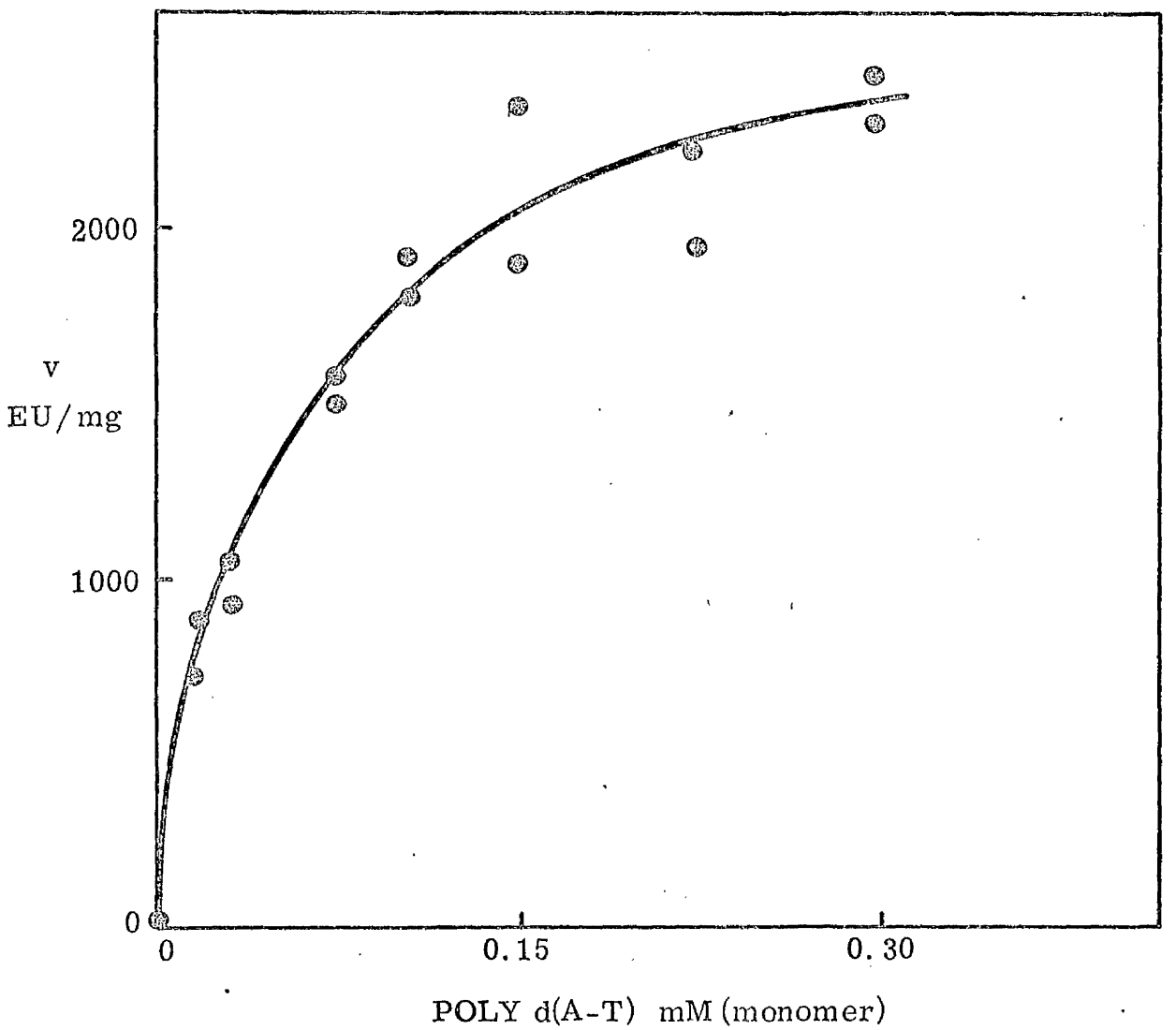

2000
1000
0
v
EU/mg
0
0.15
0.30
POLY d(A-T) mM (monomer)

Fig. 7.3 Dependence of the rate of RNA synthesis on KCl concentration with a T7 DNA-$E_{\sigma\theta}$ system

RNA polymerase activity was measured, at several KCl concentrations, using the fluorimetric assay described in Section 5D2. Other reaction components were at standard concentrations (Section 5B) except that $MgCl_2$ was 0.015 M. Each reaction mixture contained 20μg/ml of $E_{\sigma\theta}$ and 50 μg/ml of T7 DNA. The reactions took place at 37°C, and controls had no DNA.

Spectrophotofluorimeter details (see Section 2B1)

Xenon lamp : No. 2

Photomultiplier tube : No. 2

Slit arrangement : Slit 2, 5 mm ; slit 5, 5 mm, slit 7, 3/16 in, all other slit positions were unoccupied

Sensitivity : 100

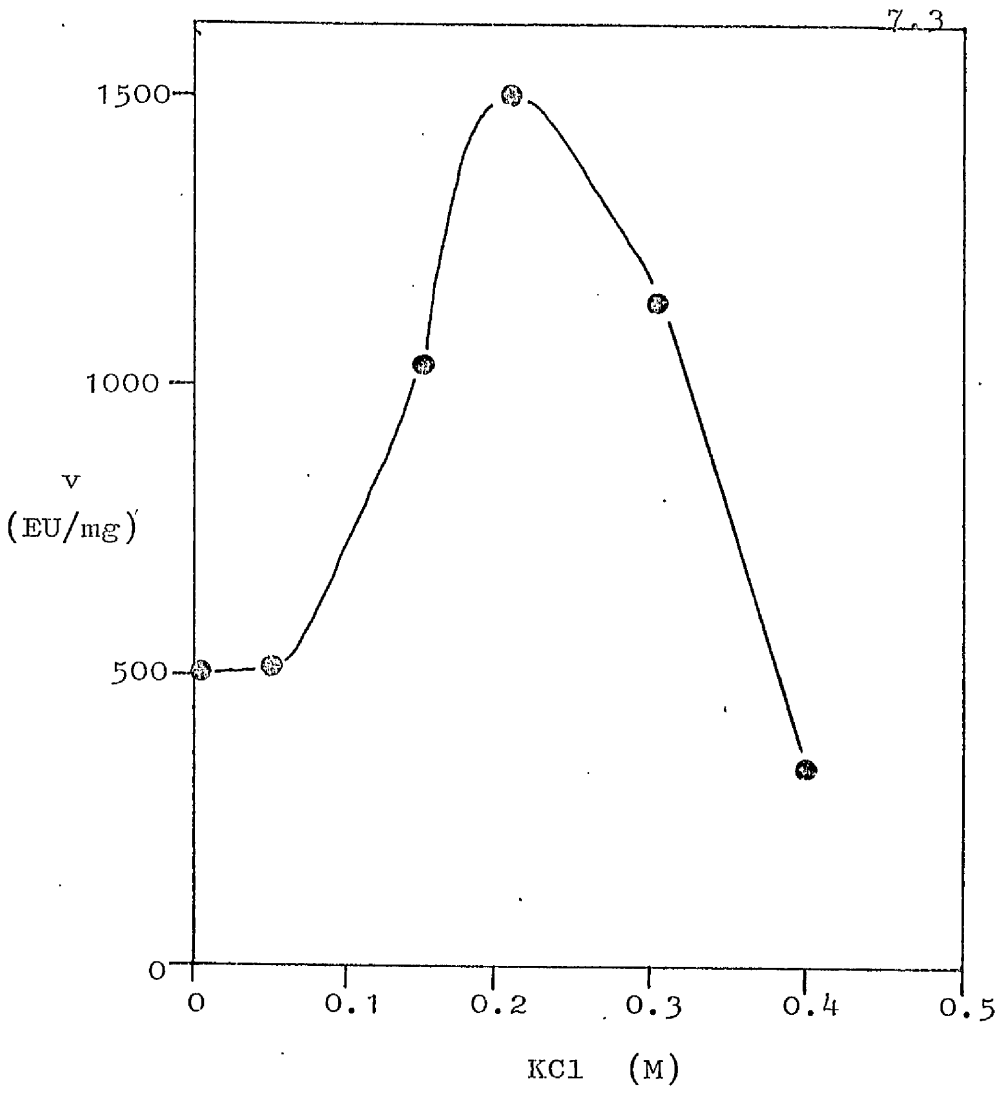

7.3
1500
1000
500
0
v
(EU/mg)
0
0.1
0.2
0.3
0.4
0.5
KCl (M)

Fig. 7.4 Dependence of the rate of RNA synthesis on KCl Concentration with a poly(dA-dT)-core enzyme system

RNA polymerase activity was measured, at several KCl concentrations, using the radioactive assay described in Section 5C. Other reaction components were at standard concentrations (Section 5B). Each assay contained 6.7 μg/ml of E_c and 0.075mM(with respect to monomer) poly(dA-dT). The assays were of 5 min duration and were carried out at 37°C. The observed rates were corrected for controls containing no DNA.

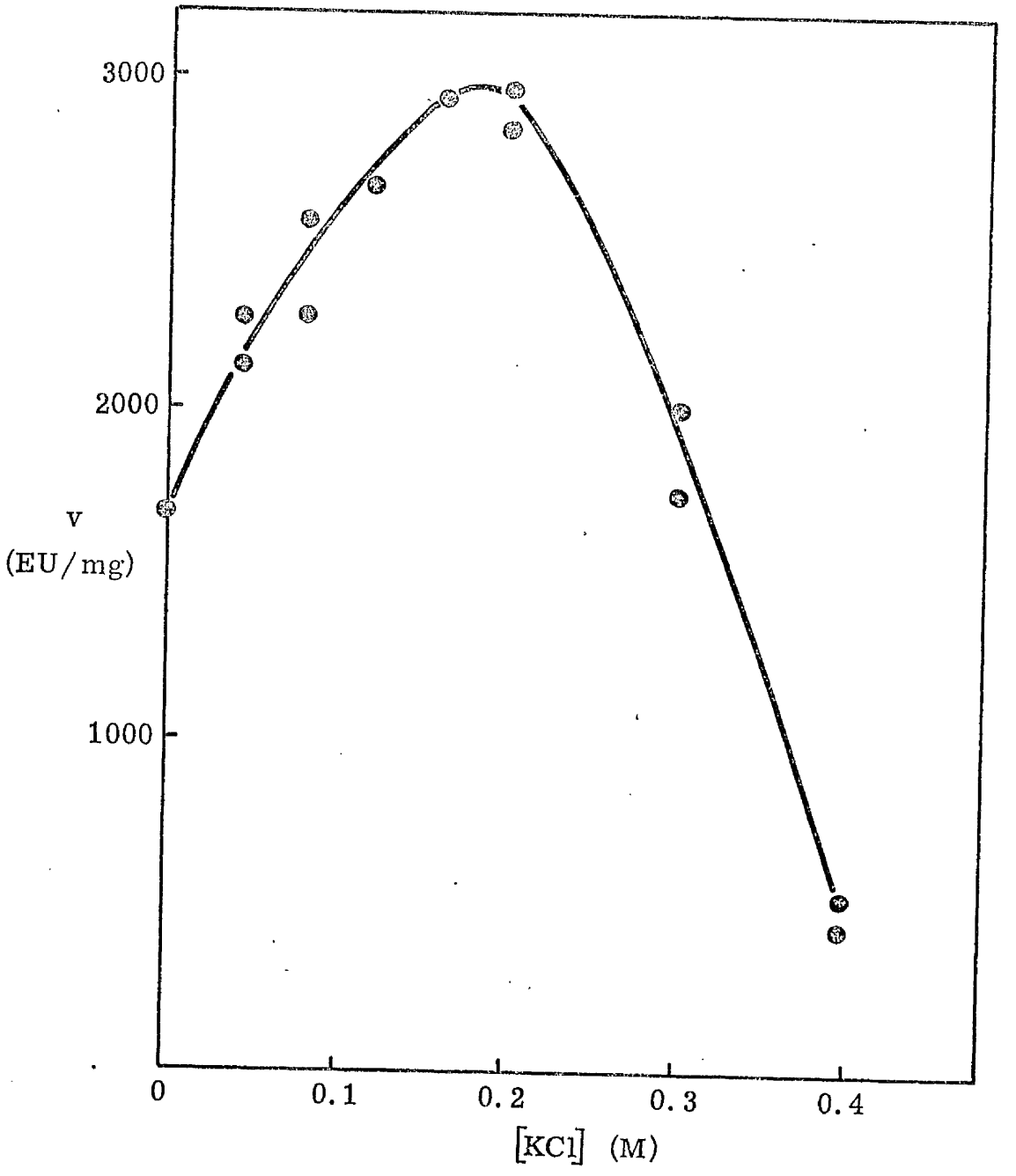

3000
2000
1000
v
(EU/mg)
0
0.1
0.2
0.3
0.4
[KCl] (M)

Fig. 7.5 Dependence of the rate of RNA synthesis on $MgCl_2$ concentration with a T7 DNA-$E_{\sigma\theta}$ system

RNA polymerase activity was measured, at several $MgCl_2$ concentrations, using the fluorimetric assay described in Section 5D2. Other reaction components were at standard concentrations (Section 5B). All other details were as in the legend to Fig. 7.3

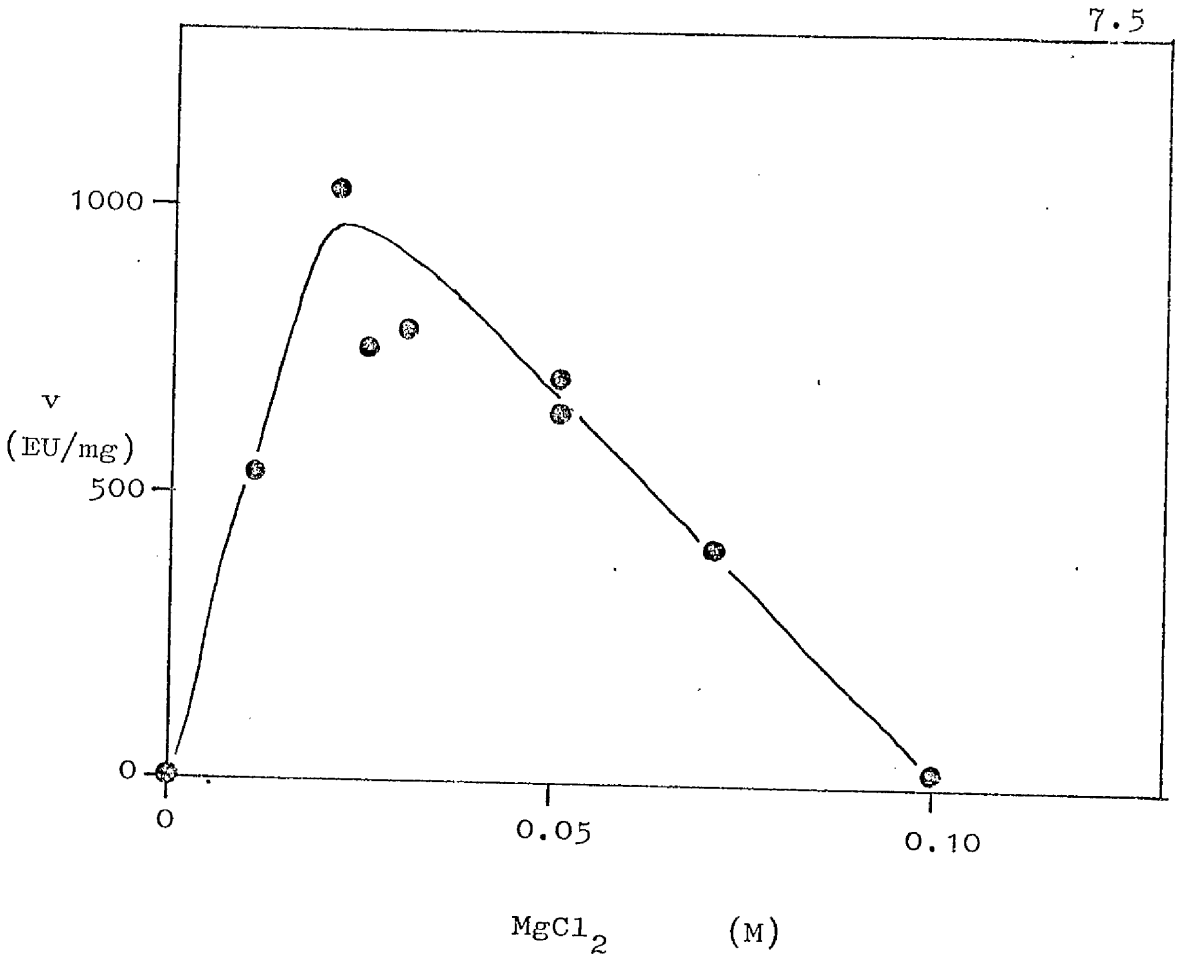

7.5
1000
v
(EU/mg)
500
0
0
0.05
0.10
MgCl2 (M)

Fig. 7.6 Dependence of the rate of RNA synthesis on $MgCl_2$ concentration with a poly(dA-dT)-core enzyme system

RNA polymerase activity was measured, at several KCl concentrations, using the radioactive assay technique described in Section 5. Other reaction components were at standard concentrations (Section 5B). Each assay contained 6.7 μg/ml of E_c and 0.075 mM (with respect to monomer) poly(dA-dT). The assays were of 5 min duration and were carried out at 37°C. The observed rates were corrected for controls which contained no DNA.

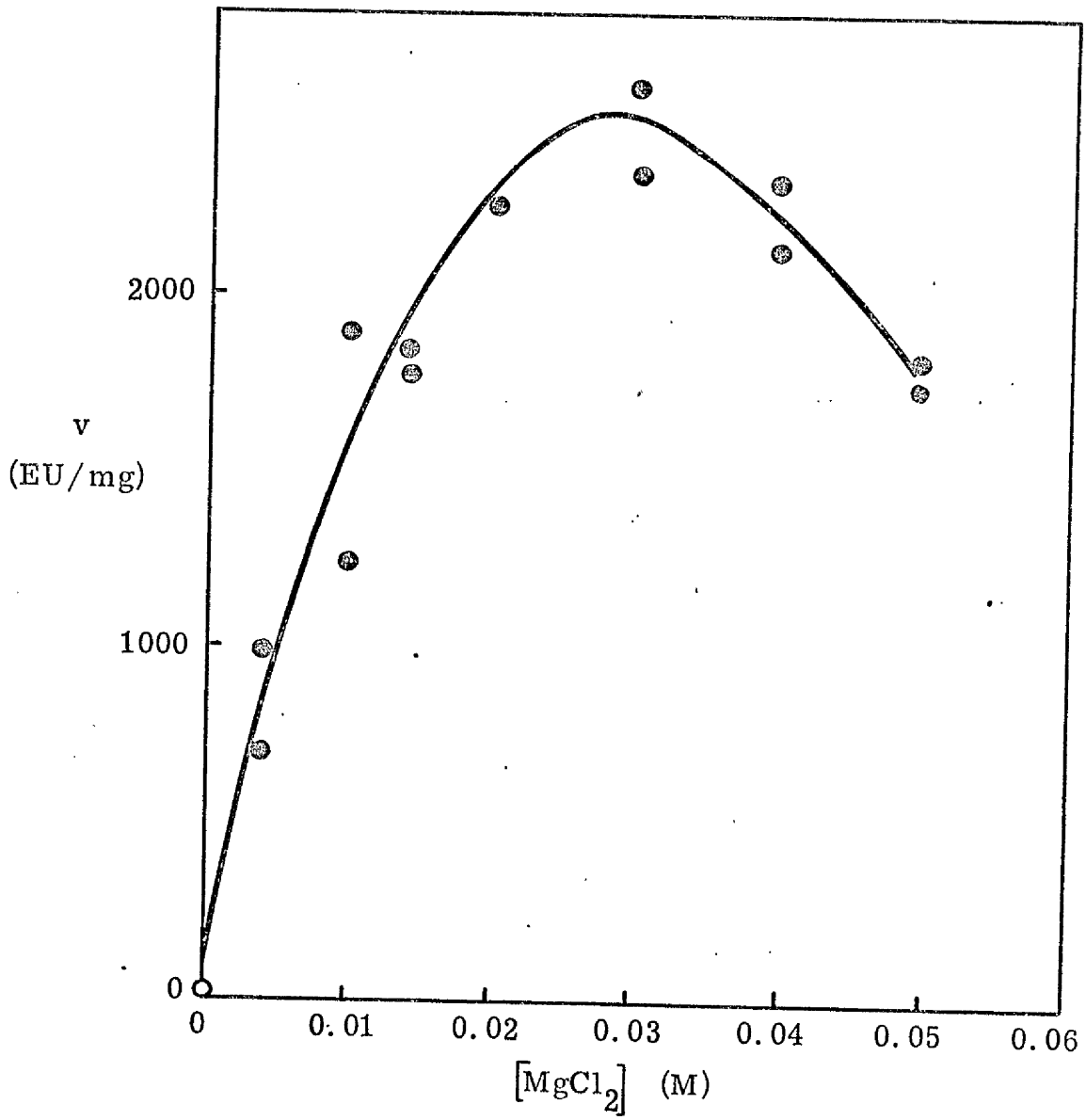
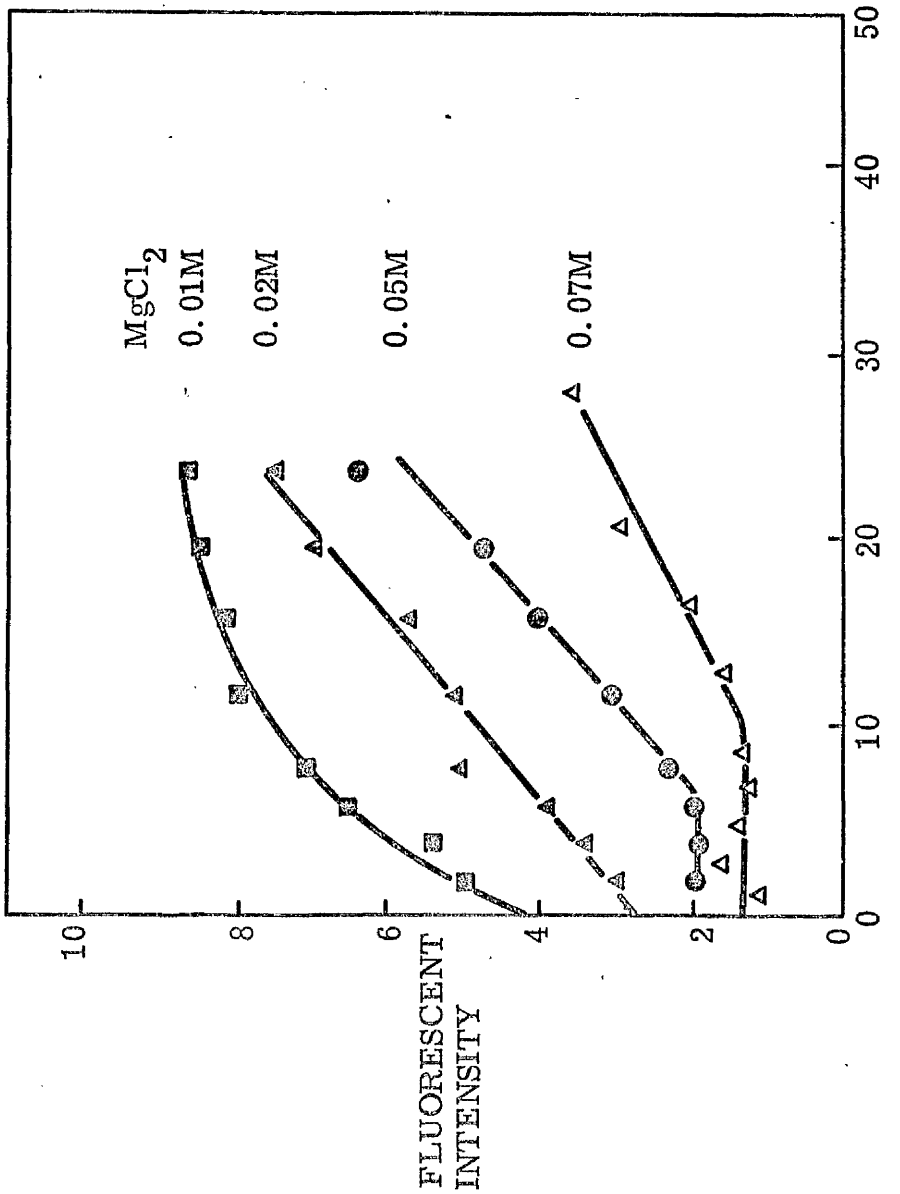

2000
1000
0
v
(EU/mg)
0
0.01
0.02
0.03
0.04
0.05
0.06
[MgCl2] (M)

Fig. 7.7 Effect of $MgCl_2$ concentration on the time course of RNA synthesis by RNA polymerase with a T7 DNA template

The time course of RNA production by a T7 DNA - $E_{\sigma\theta}$ system was observed, at various $MgCl_2$ concentrations, using the fluorimetric assay technique described in Section 5D2. Other reaction components were at standard concentrations (Section 5B), and all other experimental details were as in the legend to Fig. 7.3.


$MgCl_2$
0.01M
0.02M
0.05M
0.07M
FLUORESCENT INTENSITY
TIME (MIN)
0
2
4
6
8
10
0
10
20
30
40
50

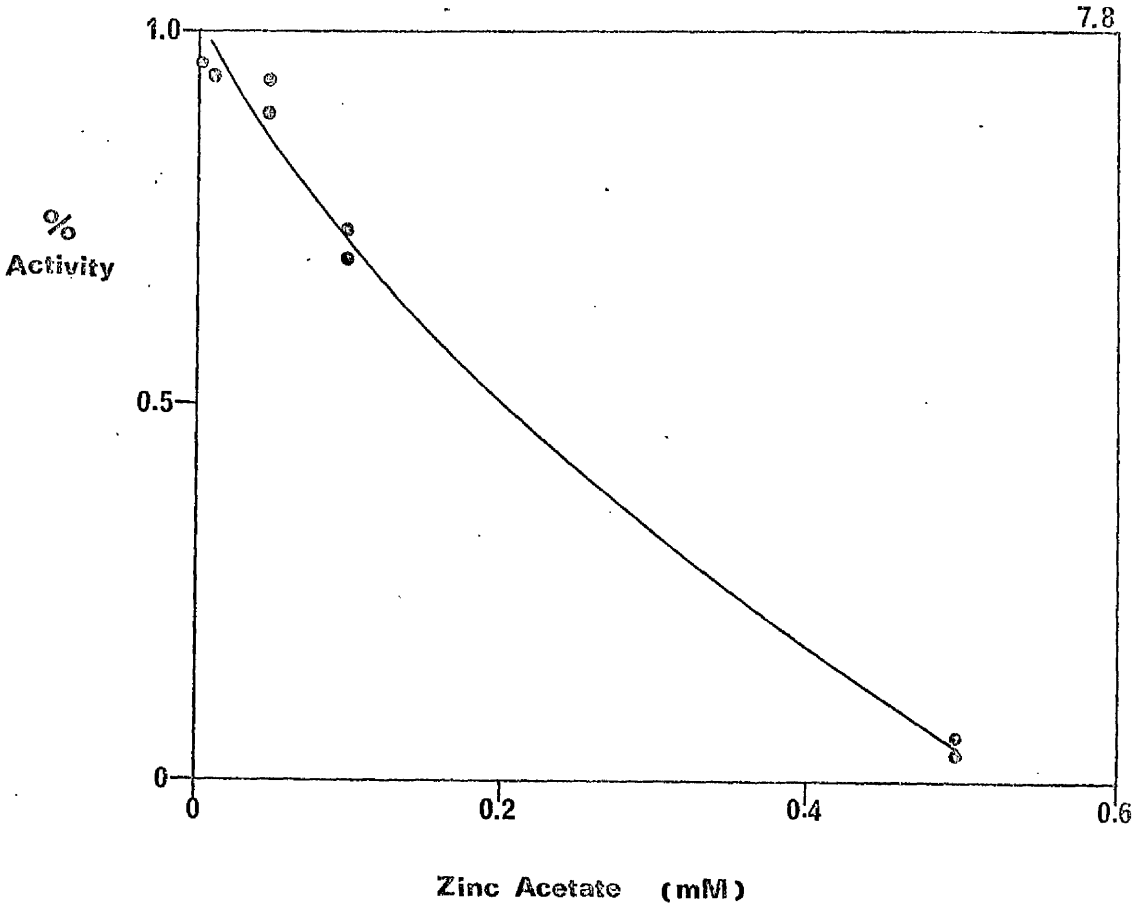


Fig. 7.8 Inhibition of RNA polymerase activity by the presence of Zn^{2+} of varying concentration

The figure shows the effect of varying the concentration of zinc acetate on the activity of RNA polymerase measured by the radioactive assay method (Section 5C). $E_{\sigma\theta}$ with a calf thymus DNA template was used, and all other details were as in the legend to Table 7.3, except that the assay duration was 5 min in this case.

DNA source	specific activity (EU/mg)
T7	1294
calf thymus	483
λ	146
E. coli	196
Ascites	296
no DNA	5

Table 7.1 Activity of RNA polymerase with various DNA templates

RNA polymerase was assayed by the fluorescence technique (Section 5D2) at standard reaction conditions (Section 5B) and at 37°C. The DNA added gave a final concentration of about 60μg/ml and the enzyme concentration was 37.5μg/ml of $E_{\sigma\theta}$.

$MgCl_2$ added (M)	$MgCl_2$ - NTP (M)	observed $MgCl_2$ (M)
0	-	0.0
0.005	-	0.002
0.010	0.004	0.003
0.015	0.009	0.005
0.020	0.014	0.014
0.025	0.019	0.016
0.030	0.026	0.030
0.050	0.044	0.042
0.050	0.044	0.041

Table 7.2 Measurement of Mg^{2+} concentration in RNA polymerase reaction mixtures

The Mg^{2+} concentration of RNA polymerase reaction mixtures was determined at various concentrations of the total $MgCl_2$ added, by the method described in Section 3E. The reaction samples contained standard concentrations of the other components (Section 5B) which means a total NTP concentration of 0.006 M. In addition, 60 µg/ml of T7 DNA and 32 µg/ml of RNA polymerase were present.

enzyme	specific activity (EU/ml)	
	$-Zn^{2+}$	$+Zn^{2+}$
$E_{\sigma\theta}$	204	18
E_c	263	10

Table 7.3 Inhibition of RNA polymerase by the presence of Zn^{2+}

RNA polymerase samples were assayed by the standard radioactive method (Section 5C) at standard reaction conditions (Section 5B) except that 0.5 mM-zinc acetate was present in some cases. The concentrations of calf thymus DNA and enzyme (all in µg/ml) were : DNA, 60 ; E_c, 44 ; $E_{\sigma\theta}$, 60 . The assays were at 37°C for 10 min, and controls contained no DNA .

SECTION 8

KINETIC EXPERIMENTS WITH RNA POLYMERASE

8A TIME COURSE OF RNA SYNTHESIS BY RNA POLYMERASE

This section describes some investigations into the effect of $MgCl_2$ and the reaction products on the time course of RNA synthesis by RNA polymerase. The influence on the time course of variations in $MgCl_2$ concentration are shown in Fig. 7.7, and were twofold. Firstly, the duration of the linear phase of RNA synthesis was prolonged with increasing Mg^{2+} until, secondly, at higher Mg^{2+} concentrations, a lag phase was observed.

8A1 LAG PHASE

Under some reaction conditions, the onset of RNA synthesis by RNA polymerase did not occur immediately the reaction was started by adding enzyme ; there was a lag phase in RNA production. This phenomenon was not studied extensively, but was observed if a $MgCl_2$ concentration greater than about 0.05 M-$MgCl_2$ was used (Fig. 7.7). The duration of the lag increased with $MgCl_2$ concentration and was of the order of 1 to 10 min. In one experiment, no lag phases were found at high KCl concentrations.

8A2 LINEARITY OF THE REACTION

In a typical RNA polymerase catalyzed reaction, the rate of RNA synthesis decreases with time. With increasing $MgCl_2$ concentrations, the reaction rate remained constant for a longer period. Such behaviour is common for enzyme catalyzed reactions and may arise for several reasons, one of which being inhibition by the products of the reaction. Table 8.1 shows the result of adding PP_i, tRNA and some oligoadenylates to assays of RNA polymerase activity. The molarity of PP_i equalled that of the RNA (with respect to monomer), as would be the case if both were simultaneously produced during RNA synthesis. Under these conditions, the RNA had a much greater inhibitory effect than PP_i or the oligoadenylates. The inhibition by RNA

may have been due to a specific interaction of tRNA with the enzyme-template system (Richardson, 1966b ; So et al., 1967), rather than inhibition by RNA in general. Added tRNA will, normally, differ from the product of RNA polymerase activity, firstly as regards size and conformation and secondly, because it is not physically attached at the polymerizing centre. These results were, however, interpreted as implying that RNA in general inhibits RNA polymerase whereas comparable concentrations of PP_i or oligoadenylates do not. ApApA and ApApApA, in fact, appeared to cause a slight increase in the rate of RNA synthesis. Inhibition of the reaction by RNA has been reported (So et al., 1967).

If PP_i does not inhibit RNA synthesis, then the decrease in the rate of the reaction with time might be attributed to the RNA product, assuming that RNA polymerase did not lose activity for any other reason. In an experiment with calf thymus DNA as template, the initial rate of RNA synthesis was not significantly decreased after incubating RNA polymerase for 20 min at 37°C in the absence of substrates. To study inhibition by the RNA product of the reaction, RNA production was followed and the reaction rate computed at various times (and hence RNA concentration). This was done for several initial substrate concentrations (Fig. 8.1). In Fig. 8.2, the percentage inhibition is expressed as a function of RNA concentration. Above a substrate concentration of 1.5 mM (total nucleotide concentration), the curves were nearly coincident indicating that high levels of substrate could not alleviate the inhibition. In general, though, a given RNA concentration caused a greater percentage inhibition at lower substrate concentration. Fig. 8.3 shows the same data displayed as the variation in reaction rate, at different RNA concentrations, with substrate concentration. This, and the

double reciprocal plots, indicated that the velocity-substrate curves became more sigmoid as the product concentration increased. Over the total substrate range, there is a complex pattern of inhibition which does not readily conform to any of the more common types (Section 1B1.4). At high substrate concentration, however, the pattern is that of non-competitive inhibition (in Cleland's classification ; Section 1B1.4), there being both intercept and slope effects in this substrate range (Fig. 8.4). At lower substrate concentrations, where the double reciprocal plots were clearly non-linear, the inhibition was obviously more complex (Section 1B1.4). As mentioned earlier, $MgCl_2$ concentration influenced the character of the time course. A similar experiment at 0.04 M-$MgCl_2$ with the time range of the above experiment (at 0.02 M-$MgCl_2$) indicated no change in the rate of RNA synthesis as the reaction progressed.

8B KINETIC EXPERIMENTS WITH CALF THYMUS DNA AND T7 DNA TEMPLATES

8B1 INTRODUCTION

The complexity of the RNA polymerase system precludes, at this time, investigations with the rigour normally associated with enzyme kinetic studies. It may not be easy to understand kinetic results within the framework of established enzyme kinetic formulations, in particular, steady state kinetics, but it is of interest to see how far this is possible. Also, characterization of RNA polymerase kinetically is of value in itself, especially in view of the wide range of K_m values reported and the lack of definition of the enzyme preparations used.

For the experiments described in this section, enzyme activity was measured fluorimetrically in some experiments, but

mostly the radioactive technique was used on account of its greater sensitivity. When the latter method was used, the incubation time was 5 min, and in order to relate the measured quantity to the initial rate of the reaction, the rate of RNA synthesis was taken to be constant over this period. In the experiments where the fluorimetric assay was used, this was in fact observed to be the case.

In the following experiments, apparent K_m's and V_{max}'s were calculated by computer using the statistical procedure of Wilkinson (Appendix 1). Where there were deviations from a hyperbolic rate law, in which case the Wilkinson method is not applicable, only the apparently hyperbolic regions were analysed statistically.

Two forms of RNA polymerase were used in these experiments. The first, $E_{\sigma\theta}$, resulted from normal preparations of RNA polymerase and contained the polypeptide θ' (Section 6B). A typical SDS-polyacrylamide gel electrophoretic analysis of this enzyme is shown in Plate 1, although as noted in Section 6, the precise composition of the enzyme was subject to some variation, and particularly as regards σ. The second enzyme form was the core enzyme, for which the gel electrophoresis pattern is seen in plate 6.

8B2 VARIATION OF THE FOUR SUBSTRATE CONCENTRATIONS

For the velocity-substrate curve shown in Fig. 8.5, the concentrations of ATP, CTP, GTP, and UTP were equal, and were varied together. In this experiment, $E_{\sigma\theta}$ was used with a T7 DNA template. At either 0.02 M or 0.04 M-$MgCl_2$, the apparent K_m was about 0.5 mM (with respect to the concentration of each nucleotide). This value agrees quite well with that of 0.63 mM found by Bremer under similar reaction conditions. With core enzyme and calf thymus DNA, the apparent K_m from a

similar experiment was 0.45 mM (for each nucleotide) and the double reciprocal plot was non-linear (Fig. 8.6)

8B3 COMPARISON OF THE KINETICS WITH RESPECT TO PURINE NUCLEOTIDES AND PYRIMIDINE NUCLEOTIDES

Anthony at al. (1969) had described a differential response of the reaction velocity to the variation of nucleotides involved in initiation (5'-terminal in RNA transcripts) compared with nucleotides that participated only in elongation reactions. It is also known that most RNA transcripts are in fact 5'-terminal in a purine residue. Hence, the four nucleotides were divided into two substrate sub-groups, comprising the two purine nucleotides (ATP and GTP, denoted PuTP) and the two pyrimidine nucleotides (CTP and UTP, denoted PyTP). A series of experiments was carried out in which either PuTP or PyTP was fixed, while the other was varied. All combinations of T7 DNA or calf thymus DNA with $E_{\sigma\theta}$ or E_c were used. A compilation of the apparent K_m's is shown in Table 8.2. The following general points were noted.

1) In all instances, the K_m with respect to PyTP (which will be denoted K_m(PyTP)) was less than the corresponding value when PuTP was variable (K_m(PuTP)) (for example, Fig.8.7).

2) Hyperbolic behaviour was always observed when PyTP was variable, but, in several experiments, the double reciprocal plots of 1/v against 1/PuTP were concave to the 1/v axis at low PuTP concentrations, indicating sigmoid velocity-substrate curves (for example, Fig. 8.7)

3) In general, the K_m(PyTP)'s were less sensitive to the nature of the enzyme and template used.

Points 1) and 2) are qualitatively in agreement with the results of Anthony et al. (1969), although different reaction conditions were used in their experiments. Quantitatively,

the differences between K_m(PyTP) and K_m(PuTP) were less pronounced in the present experiments ; K_m(PuTP)/K_m(PyTP) was of the order of 3 here whilst Anthony et al. (1969) reported a value of about 10 for this ratio. Also, the absolute values for all the K_m's quoted here are a factor of 2-4 higher than those cited by Anthony et al. (1969).

Further information can be derived from the results summarized in Table 8.2 by comparing experiments on the basis of the enzyme and template used.

8B3.1 Effect of calf thymus DNA concentration

Fig. 8.8 compares the double reciprocal plots for experiments in which two different ratios of enzyme to calf thymus DNA concentrations were used. The enzyme/template ratio appeared to affect K_m(PuTP) more than K_m(PyTP). As the RNA polymerase/DNA ratio increases, the enzyme would be able to form complexes at progressively weaker binding sites on the template. Thus, depending on the relative amounts of enzyme and template, there will be a heterogeneous population of enzyme-DNA complexes of differing stabilities, and conceivably differing kinetic properties. Further, it is reasonable to suppose that the greatest heterogeneity in the kinetic behaviour would be with respect to initiation rather than elongation. If this argument is accepted, then the initiation stage had a greater influence on K_m(PuTP) than K_m(PyTP), since the former was most sensitive to the enzyme /template ratio.

8B3.2 Variations in the kinetics of different enzyme preparations

Two different preparations of core enzyme showed little difference in K_m(PyTP) and K_m(PuTP), although the V_{max}'s varied (Fig. 8.9). Between two normal preparations of $E_{\sigma\theta}$,however, greater variation in the apparent K_m's was observed, especially in K_m(PuTP). Again, the V_{max}'s were significantly different

(Table 8.2). These findings probably arose from the fact that RNA polymerase, $E_{\sigma\theta}$, varied in composition, notably with respect to σ, from one preparation to another. Core enzyme, on the other hand, was always of better defined composition, no other polypeptide species but β, β', and α being observed by SDS-polyacrylamide gel electrophoresis. The influence of σ on the observed kinetic parameters was further substantiated by the results of the next section.

8B3.3 Effect of sigma on RNA polymerase kinetics

The influence of σ on the kinetic behaviour of RNA polymerase is clearly seen when the results of experiments using $E_{\sigma\theta}$ and E_c are compared directly. With a calf thymus DNA template, the most pronounced effect of the presence of σ was on K_m(PuTP), and non-hyperbolic kinetics, with respect to PuTP, were observed whether σ was present or not (Fig. 8.10).

With T7 DNA (Fig. 8.11), however, σ caused marked changes in both K_m(PuTP) and K_m(PyTP). Again, non-hyperbolic behaviour with variable PuTP was irrespective of the presence of σ.

Thus, whilst σ decreased K_m(PuTP) with calf thymus DNA, it increased K_m(PuTP) with a T7 DNA template. With both templates, the V_{max} depended on σ.

The differential response to σ for the two DNA's used is, in general, to be expected, since σ is strongly implicated in the initiation process, and T7 DNA and calf thymus DNA differ greatly in their template properties, and, hence, their initiation signals. Kinetically, σ could be described as an activator of the overall rate of RNA synthesis *in vitro* with respect to the core enzyme. This was further demonstrated when the σ-containing fraction from phosphocellulose chromatography (Section 4B), peak A, was added to a reaction containing core enzyme (peak C). The velocity-σ curve was sigmoid, and approached a saturating velocity as σ increased (Fig. 8.12).

The observed maximum rate sometimes exceeded that of the preparation of RNA polymerase $E_{\sigma\theta}$ from which the σ and core enzyme had been prepared. This may be explained if the original enzyme contained less than stoichiometric amount of σ or else if some of the σ present was inactive. In order to obtain more detailed information about the kinetic effects of σ, more extensive experiments are required. A major problem in this context is the fact that σ is both difficult to isolate in a pure form and is very labile when purified. With calf thymus DNA, σ altered the slope and intercept of plots of 1/v against 1/substrate, but with T7 DNA, there appeared to be only a change in the position of the intercept with the 1/v axis. This latter case could be classified as uncompetitive activation (Section 1B1.4), but how useful or informative such a statement is with such a complex system is less clear.

8C KINETIC EXPERIMENTS WITH A POLY(dA-dT) TEMPLATE

The results of the previous section showed that the kinetic properties of any RNA polymerase preparation will be strongly dependent on the proportion of σ present. In general, the exact composition of an RNA polymerase sample will be an important determinant of its kinetic behaviour. Likewise, the kinetic characteristics will depend on the template used, its source, and hence base sequence, and also its physical state. It has been suggested for example that the activity of core enzyme with intact phage DNA's is sensitive to the number of single strand nicks in the DNA (Vogt , 1969). Some of these difficulties in defining the RNA synthesizing system used in experiments are avoided by using RNA polymerase core enzyme and a poly(dA-dT) template. In addition, from an enzyme kinetic point of view, the number of substrates is then reduced from

four to two. Such a system of course will be totally devoid of control features other than any inherent in the core enzyme and the repetitive sequence of poly(dA-dT). It is, however, a useful model for studying RNA synthesis. In the following experiments, commercial preparations of ATP and UTP were further purified by ion exchange chromatography before use (Section 4D).

8C1 PROPERTIES OF A CORE ENZYME-POLY(dA-dT) SYSTEM

The effect of KCl and $MgCl_2$ concentrations on the reaction rate for a system of core enzyme and poly(dA-dT) were described in Section 7 and 0.2 M-KCl and 0.02 M-$MgCl_2$ were taken as standard. Synthesis of poly(A-U), measured fluorimetrically, continued for at least 60 min, and the time course was linear for about 10 min (Fig. 8.13). These findings were not altered by the presence of P_i, corroborating the results of Section 6C2 where core enzyme was found to be lacking in polynucleotide phosphorylase activity. These latter investigations of the time course used enzyme containing σ.

8C2 VELOCITY-SUBSTRATE CURVES

Using RNA polymerase core enzyme with a poly(dA-dT) template, the reaction rate, at various levels of fixed substrate concentration, was measured as the other substrate was varied. The results are shown in Figs. 8.14-8.18. Examination of the double reciprocal plots shows that the enzyme followed a hyperbolic rate law for a portion of the substrate ranges used. At higher substrate concentrations, however, there were deviations from hyperbolic behaviour. With ATP as the variable substrate, the double reciprocal plots became increasingly concave down (that is, concave to the 1/UTP axis) as the concentration of the fixed substrate, UTP, increased (Fig. 8.15). With the results displayed for UTP as the variable substrate, the double reciprocal plots showed less deviation from linearity,

and at low levels of ATP, the plots were concave up at high UTP concentration (Fig. 8.17). When both ATP and UTP, at equal concentrations, were varied together, the double reciprocal plot was markedly concave down (Fig. 8.18).

8C2.1 Analysis of data

Because of the deviations from hyperbolic kinetics, the data mentioned above were carefully fitted by the least squares method to model rate equations of the form

$$v = \frac{p_1 a^2 + p_2 a + p_3}{q_1 a^2 + q_2 a + q_3}$$

as described in Appendix 2. The goodness of fit was assessed by the value of χ^2_ν . This form of equation is that of a general steady state rate equation (Section 1B2 ; Wong & Hanes, 1962) and this is the reason for its selection.

UTP as the changing fixed substrate ; The results of least squares analysis of various model equations for the dependence of the reaction on ATP are shown in Table 8.3 . An equation of the form

$$v = \frac{p_1 a^2 + p_2 a}{q_1 a^2 + q_2 a + q_3} \qquad \text{model 1}$$

was the simplest that described the data. As judged by χ^2_ν , little improvement of the fit was afforded by the inclusion of a constant term in the numerator. As the fixed substrate, UTP, decreased in concentration the simple hyperbolic equation became an increasingly good fit, but was never appreciably better than model 1. This equation also provided the best fit to the data obtained when the concentrations of ATP and UTP were equal and were varied together. The curves generated by model 1 are shown with the data points in Fig. 8.14, and present good descriptions of the data. It is concluded then that model 1 adequately described the experimental data. It should

be stressed at this point that the selection of a putative rate equation in this way provides no definite proof of the validity of the equation. As judged by χ^2_ν, model 1 was merely the best of the model equations tested. However, the equation selected was the simplest which had the general form of a steady state rate equation (Section 1B2) that fitted the experimental results.

ATP as the changing fixed substrate ; The analysis of the goodness of fit of various model equations for the dependence of the reaction rate on UTP concentration is shown in Table 8.4 . Again, model 1 gave the best overall description of the data, but both the hyperbolic model (2) and substrate inhibition model (3) were also good fits at some ATP concentrations. The curves derived from model 1 along with the experimental points are shown in Figs. 8.16 & 8.17.

8C2.2 Region of hyperbolic behaviour

As was stated previously, the enzyme obeyed a hyperbolic rate law over a portion of the substrate ranges examined and so the data from within these ranges were treated as for a normal two substrate enzymic reaction (Section 1B2.3). The slopes and intercepts of the computer-drawn double reciprocal plots were replotted against the changing fixed substrate concentrations (Figs 8.19 & 8.20). With UTP as the changing fixed substrate, the pattern was that of a two substrate ping-pong mechanism ; the slope against 1/UTP plot was a straight line of zero gradient and the intercept was linearly dependent on 1/UTP. The deviations from this pattern are seen to occur at high UTP levels. With ATP as the changing fixed substrate, a similar pattern was obtained except that the slope against ATP replot showed a linear increase in slope as ATP increased. Such behaviour would be anticipated if there was substrate

inhibition by ATP.

In summary, the behaviour of RNA polymerase core enzyme in the experiments described above could be represented by a simple rate equation of the following form, in the substrate ranges, ATP. 0.08 to 0.4 mM ; UTP, 0.04 to 0.4 mM ;

$$v = \frac{K_1 au}{K_2 a + K_3 u + au + K_{ia} a^2}$$

where a and u are the concentrations (in mM) of ATP and UTP respectively. This equation is of identical form to eqn. 1.11. The experimental values for the constants are ;

	From Fig. 8.20	From Fig. 8.19
K_1 (EU/mg)	1565	1590
K_2 (mM)	0.0596	0.0604
K_3 (mM)	0.0415	0.0419
K_{ia} (-)	-	0.0164

8C2.3 Consideration of non-hyperbolic behaviour

The equation above cannot explain double reciprocal plots that are concave down. It was found, however, that an equation of the form of model 1 could accommodate the data over the entire substrate range that was examined, if the reaction rate was considered as a function of one substrate concentration at a time. It is not easy to decide whether the applicability of this model equation merely reflects that it has enough inherent versatility to smooth the data, or implies a physical significance in a rate law of this form. If the latter were true, then the parameters p_1,p_2,q_1,q_2, and q_3 should vary in a non-random way as the fixed substrate concentration is changed. This is illustrated in Figs. 8.21 & 8.22, where, for the most part, the parameters varied smoothly with the fixed substrate concentration, the exact nature of the variation differing for each parameter. The results of this experiment

did not supply enough information for the unequivocal determination of the form of the complete rate equation, that is, the equation expressed in terms of ATP and UTP concentrations. It is evident nonetheless that such an equation would be complex, involving higher degree terms in both ATP and UTP concentrations. Also, since there are likely to be a fairly large number of constants, the elucidation of the complete rate equation would require a large number of experimental data.

The above results gave an empirical description of the dependence of the reaction on substrate concentrations. One possible physical model to explain non-hyperbolic behaviour would be the existence of alternative pathways and further experiments were carried out to explore this possibility.

8C2.4 5'-terminal residue determination

Using basically the method of Maitra et al. (1967), the 5'-terminal residues of poly(A-U) transcripts were determined. The results are shown in Table 8.5b, and with a core enzyme-poly(dA-dT) system, only adenine was detected in the 5'-terminal position. It is concluded, from these findings as well as previous reports (Maitra & Hurwitz, 1965), that little UTP is incorporated into the 5'-ends of poly(A-U) synthesized by RNA polymerase core enzyme under these conditions. In fact, a similar experiment, using the method described by Downey & So (1970), with $E_{\sigma\theta}$ gave the same results, so that σ had no influence on the selection of the first substrate in poly(A-U) synthesis (Table 8.5a).

The results in Table 8.5b also provide information on the chain length of the poly(A-U) product. The values reported here for chain length are, in some instances, rather higher than those quoted for similar experiments (Maitra et al., 1967 :

Downey & So, 1970). Nonetheless, comparison of the measured chain lengths at various ATP to UTP ratios in the reaction mixture indicated that the chain length was reduced by an increase in the concentration of either substrate. Initiation, though, was not inhibited by elevated substrate concentrations. It would seem, then, that the substrate inhibition by ATP noted in Section 8C2.2 was at the elongation stage of poly(A-U) synthesis and that UTP also exhibited substrate inhibition.

From analysis of the 5'-terminal residues of poly(A-U) transcripts, there was no evidence for alternative reaction pathways at the stage of the incorporation of the first nucleoside triphosphate. However, these same experiments showed that substrate inhibition was at elongation rather than the initiation stage of poly(A-U) synthesis. If substrates could bind to RNA polymerase and inhibit chain elongation, it is reasonable to postulate that this binding is at the active site and that there might be an override of the template-directed selection of the incoming nucleotide at some substrate concentrations. This would mean, kinetically, the presence of alternative reaction pathways at the stage of elongation.

8C2.5 Stoichiometry of base residues in poly(A-U) synthesized by RNA polymerase

To test the above hypothesis, UTP and ATP incorporation was measured under similar reaction conditions. These conditions included various ratios of ATP and UTP concentrations. Velocity-substrate curves were determined for the variation of ATP or UTP, as measured by the incorporation of either ATP or UTP. The corresponding double reciprocal plots are shown in Figs. 8.23, a & b. It can be seen that the kinetics were non-hyperbolic for variable ATP concentration, irrespective of whether the reaction rate was measured by ATP or UTP incorporation. The kinetics with respect to UTP were hyperbolic.

The stoichiometry of the polyribonucleotide product, expressed as the mole fraction of either of the nucleotide residues (for example, A/(A + U)) was insensitive to variations in the composition of the substrates, in terms of the relative proportions of ATP and UTP (Fig. 8.24). That the value of this mole fraction (about 0.55) was not 0.5, as might be expected, was either due to systematic experimental error or to the inherent stoichiometry of the poly(dA-dT) template. The main conclusion, however, was that the stoichiometry of the product poly(A-U) was independent, at the accuracy of this experiment, of the concentrations, relative and absolute, of ATP and UTP.

8C2.6 Nearest neighbour analysis of poly(A-U) synthesized by RNA polymerase

Table 8.6 shows the results of nearest neighbour analysis of poly(A-U) synthesized by RNA polymerase core enzyme, with a poly(dA-dT) template, under conditions of high and low substrate concentrations. The dinucleotide sequences ApU and UpA were predictably the most frequent but there was a significant proportion of UpU. Whether this deviation from a strictly alternating sequence was imparted by the template or the action of RNA polymerase cannot be inferred from this experiment. Certainly, the dinucleotide frequencies were not greatly altered by high, compared with low, substrate levels in this experiment, arguing against enzyme involvement. However, if the enzyme was responsible, then misreading of the template occurred about 3 % of the time, with the faulty incorporation of UTP.

8C2.7 Determination of the residue adjacent to the 5'-terminal base of poly(A-U) synthesized by RNA polymerase

The method is based on that described by Maitra et al. (1967)-see Section 5A6- and effectively the relative proportions

of pppApA and pppApU at the 5'-ends of a population of poly(A-U) molecules synthesized by RNA polymerase are measured. These measurements, then, were made at both high and low substrate concentrations in the RNA polymerase reaction mixture. The experiment was conducted on the premise that an adenine residue was 5'-terminal in the poly(A-U) transcribed from poly(dA-dT). The next residue, the penultimate one, should have been a uridine if the strict alternating sequence was observed. In fact, an appreciable proportion of the penultimate residues in the population of poly(A-U) molecules was adenine (Table 8.7). This proportion was lower at high substrate levels, but this experiment is insufficient to establish the dependence of the occurrence of pppApA on substrate concentrations. As with the experiment of the previous section, it is not possible to decide whether the observed departure from a strictly alternating sequence was caused by the enzyme or the template. The finding of pppApA is of some interest, since it may indicate the operation of alternative reaction pathways for poly(A-U) synthesis.

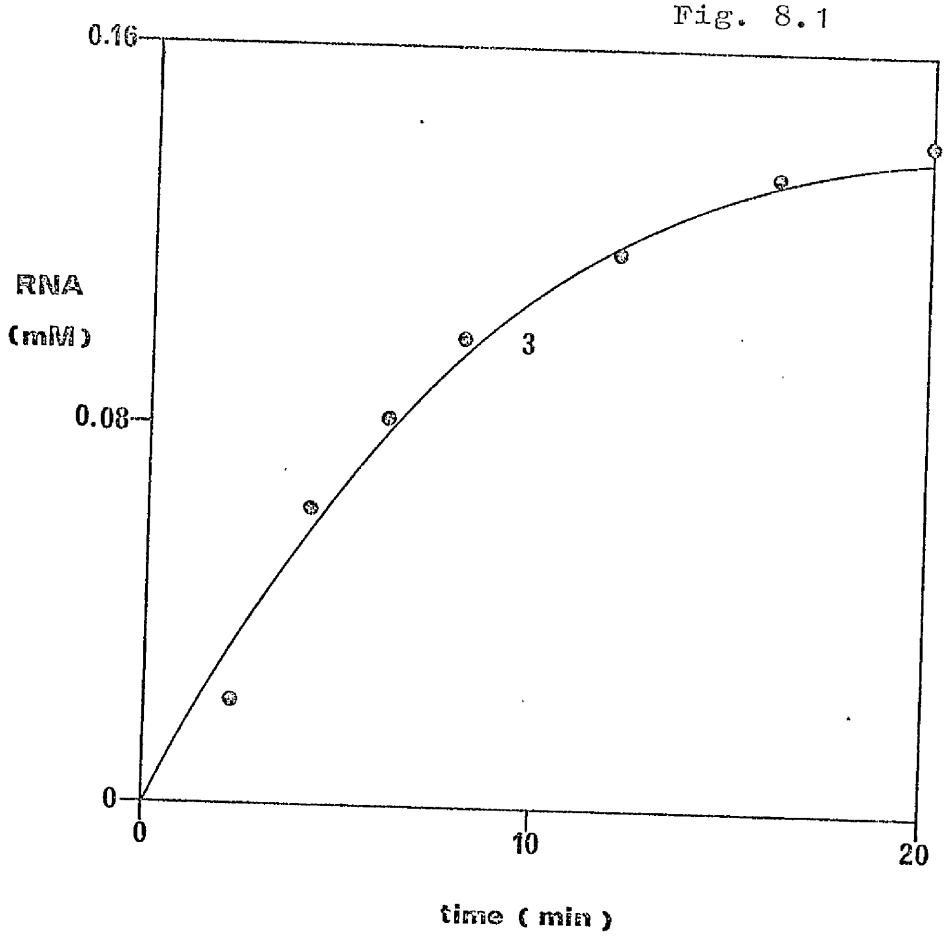
Fig. 8.1 Time course of RNA synthesis by RNA polymerase at various substrate concentrations

Several RNA polymerase catalyzed reactions were followed by the fluorimetric method described in Section 5D. Reaction conditions were standard except that the NTP concentration was varied (NTP represents the total nucleotide concentration of an equimolar mixture of ATP, CTP, GTP, and UTP). The reaction mixtures also contained 30 μg/ml of $E_{\sigma\theta}$ and 75 μg/ml of T7 DNA. Details of the measurement of fluorescence were as in the legend to Fig. 7.3 .

The experimental points are shown in the figure, the curves being the best least squares fit to a trinomial in time ($RNA = a_o + a_1 t + a_2 t^2 + a_3 t^3$ where RNA represents RNA concentration and t, time; a_o, a_1, etc. are constants). From the fitted curve, estimates of the reaction rate at any given time during the reaction can be made.

The NTP concentrations in the various reaction mixtures were: 1) 6.0 mM, 2) 3 mM, 3) 1.5 mM, 4) 0.6 mM, 5) 0.45 mM and 6) 0.15 mM.

Fig. 8.1


0.16
RNA
(mM)
0.08
0
0
10
20
3
time (min)

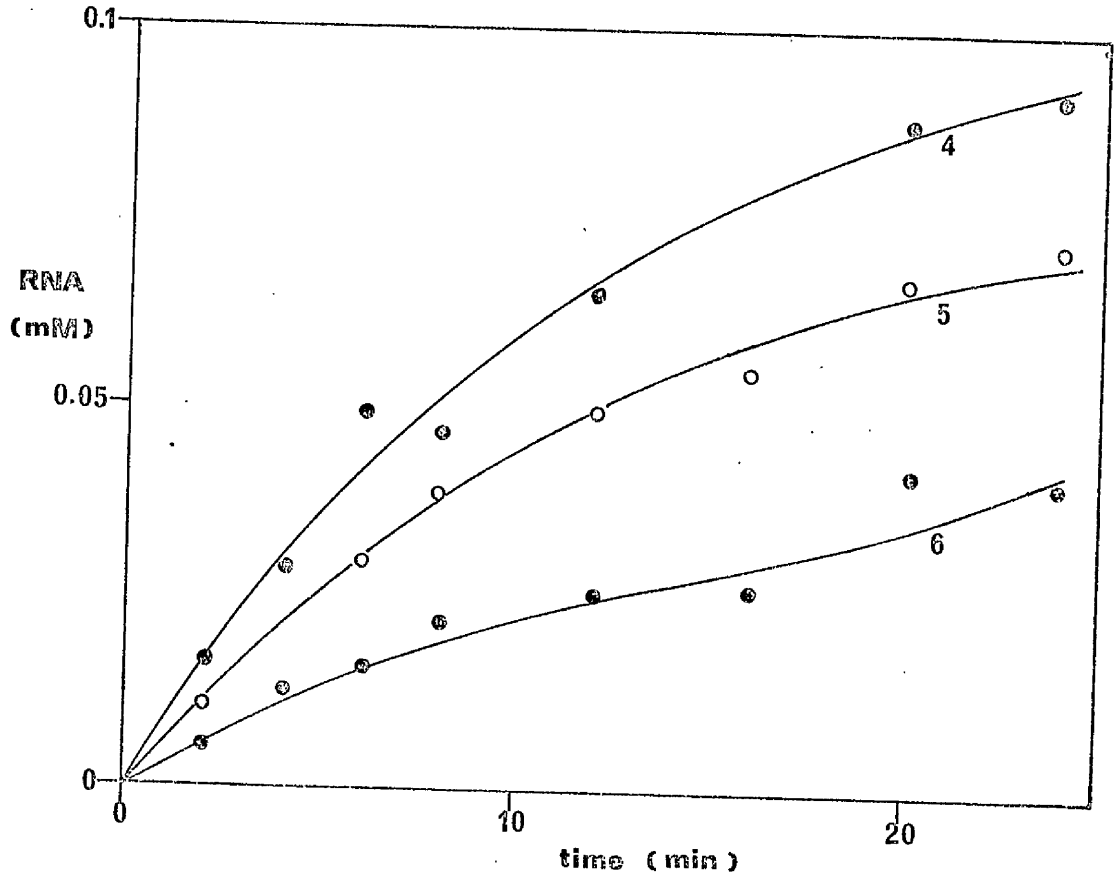
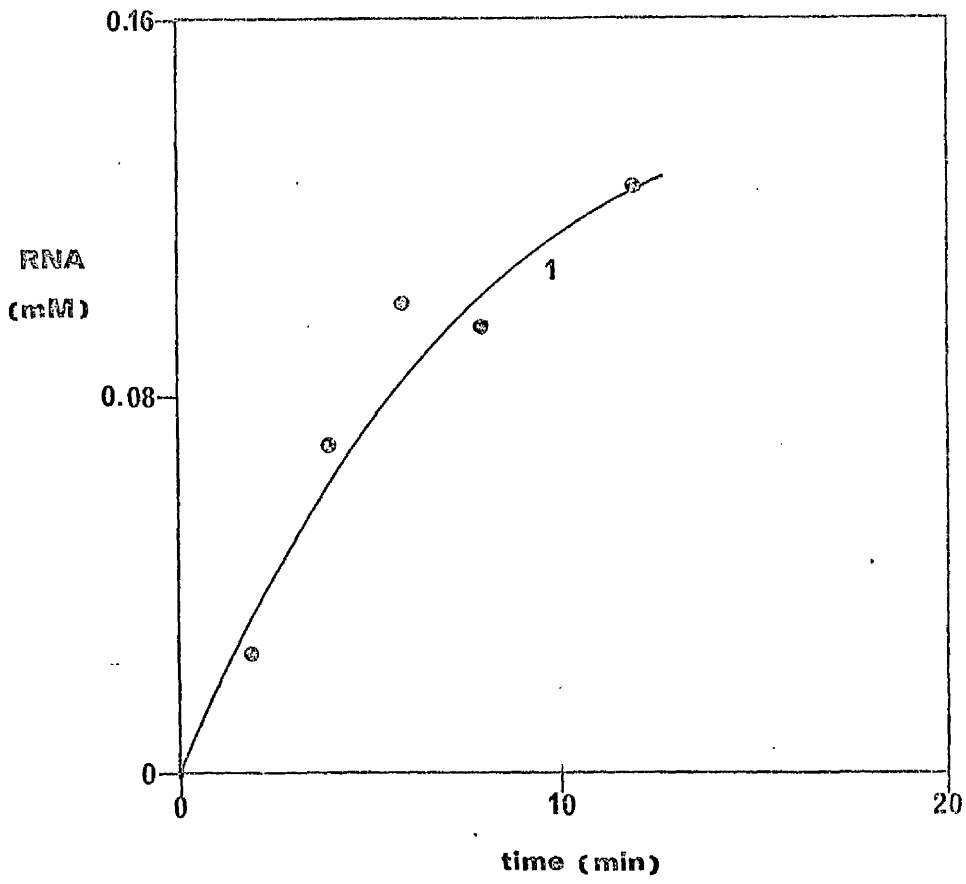

0.1
RNA
(mM)
0.05
0
0
10
20
4
5
6
time (min)

Fig. 8.1


0.16
RNA
(mM)
0.08
0
1
0
10
20
time (min)

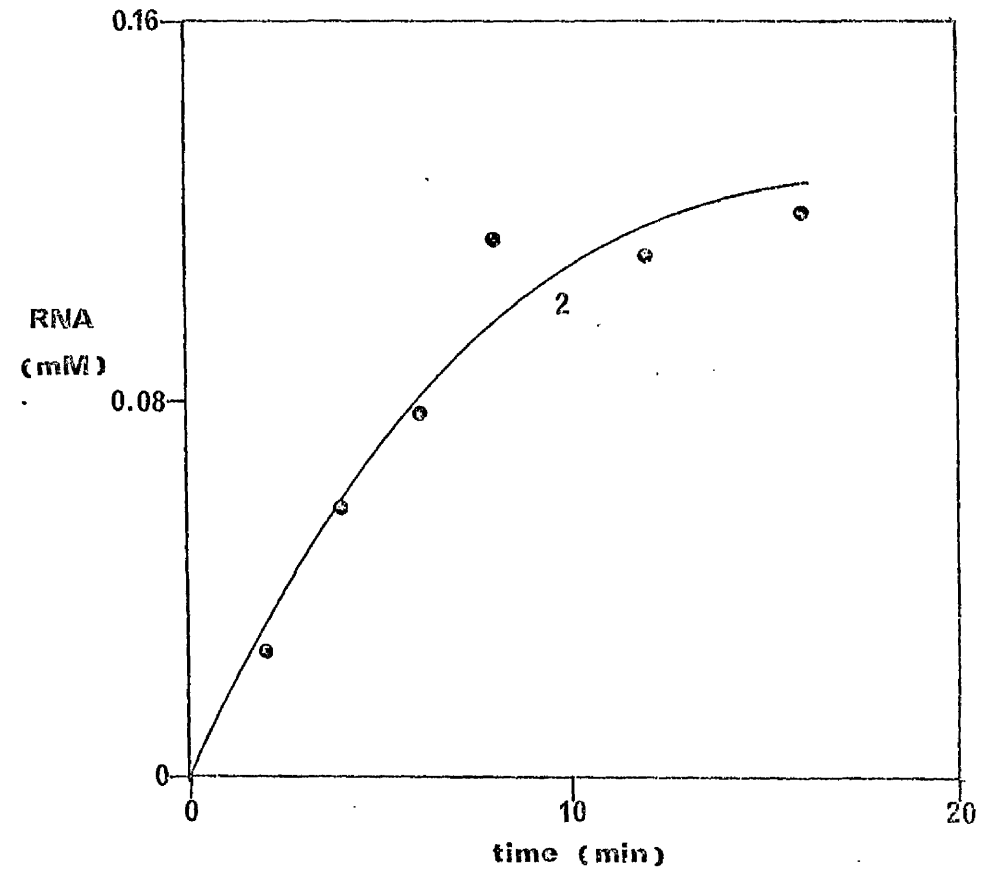

0.16
RNA
(mM)
0.08
0
2
0
10
20
time (min)

Fig. 8.2 Inhibition of RNA polymerase action as a function of the product RNA concentration

From the data displayed in Fig. 8.1, the reaction rate for RNA synthesis was calculated at various product RNA concentrations. The percent inhibition $(v_o - v_{rna}/v_o)$ was then plotted as a function of the RNA concentration. This was done for each initial NTP concentration:

o-o ,6.0 mM ; ●-● , 3 mM ; ▲-▲ , 1.5 mM ; △-△ , 0.6 mM ;

▼-▼ ,0.45 mM ; ▽-▽ , 0.15 mM, where the concentrations refer to NTP

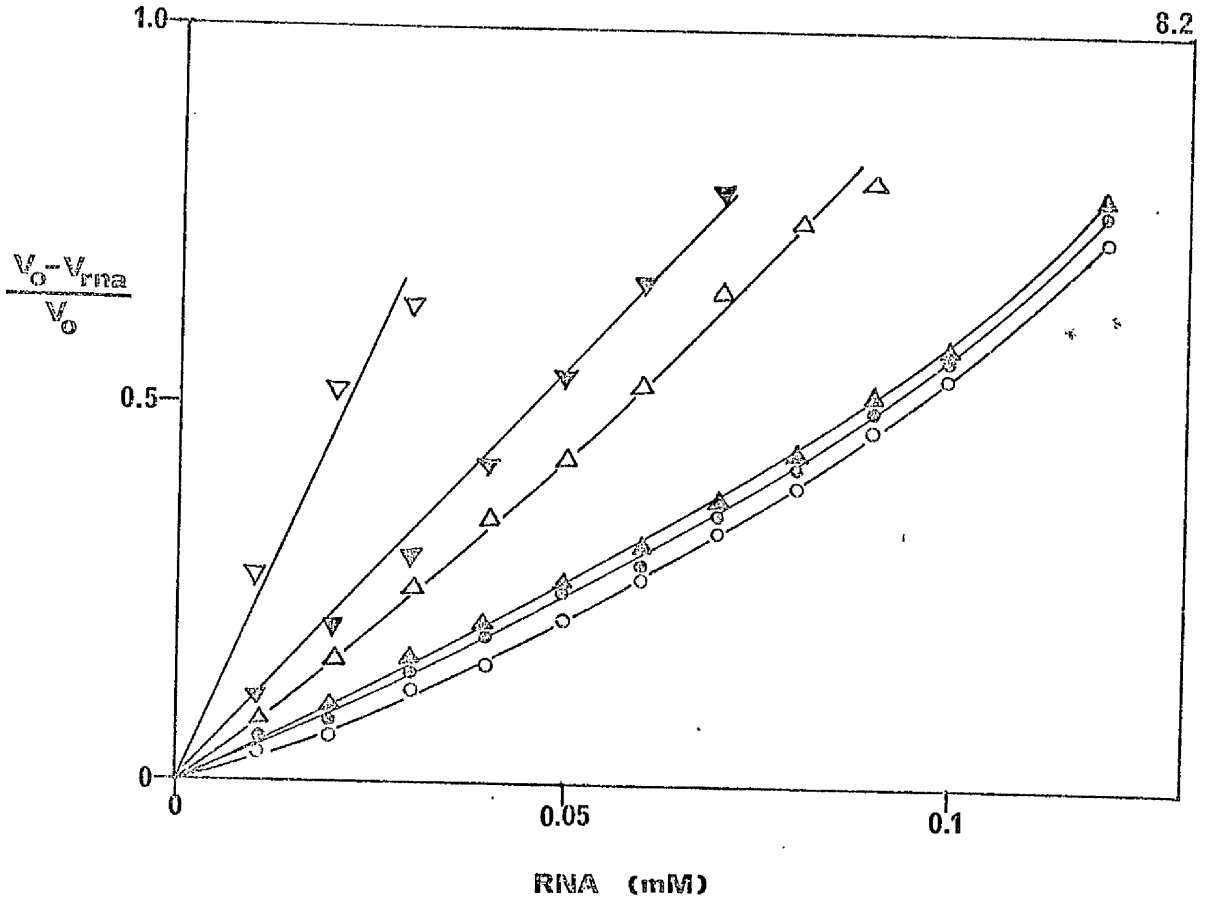

1.0
0.5
0
0
0.05
0.1
8.2
$\frac{V_o - V_{rna}}{V_o}$
RNA (mM)

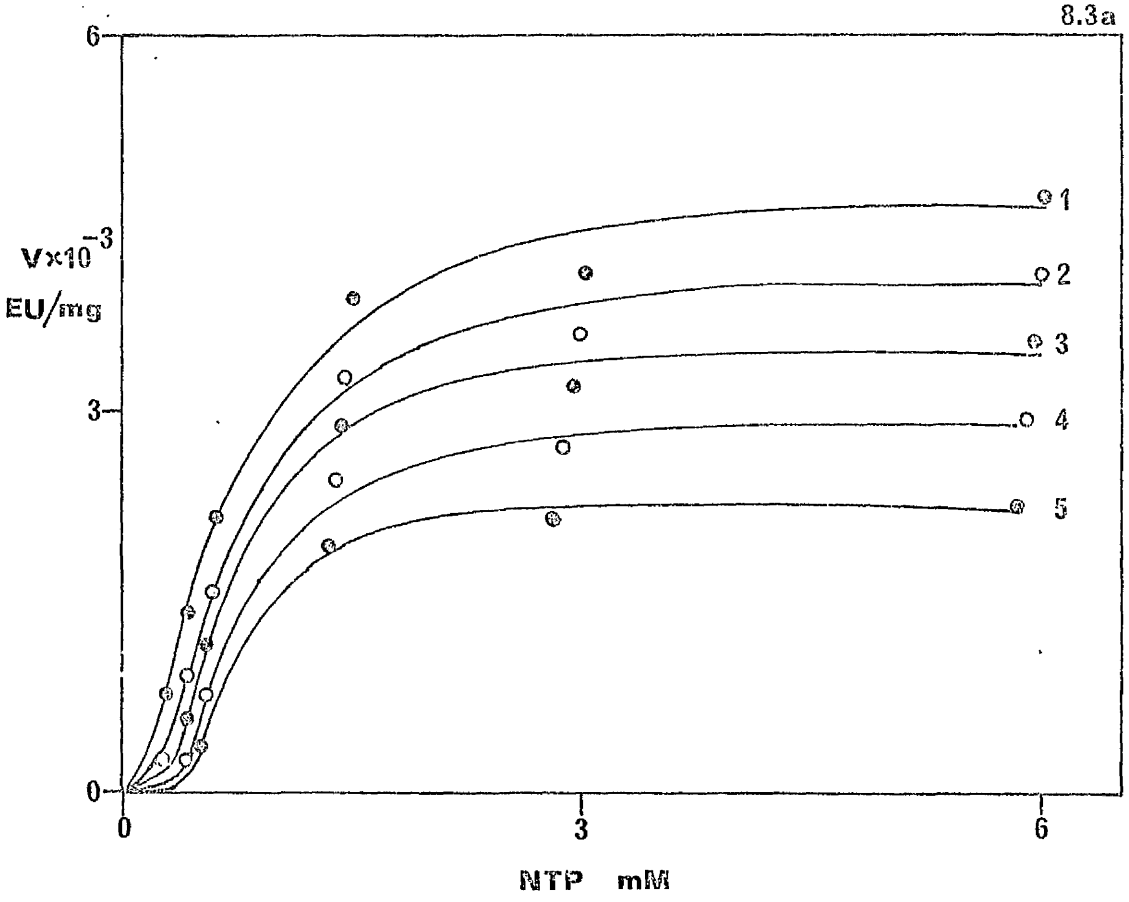
Fig. 8.3 Velocity-substrate curves and double reciprocal plots for RNA polymerase at various product RNA concentrations

Certain product RNA concentrations were chosen and, from the data of Fig. 8.1, the corresponding rates of RNA synthesis calculated for each of the reactions followed, i.e. at different initial NTP concentrations. At a given product RNA level, then, the reaction rate could be estimated for a range of substrate concentrations. The substrate concentration was calculated by subtracting the RNA concentration from the initial NTP concentration. Thus, velocity-substrate curves and double reciprocal plots were drawn, as shown in the diagram. The values of RNA concentration selected were:

1) 0 mM, 2) 0.03 mM, 3) 0.05 mM, 4) 0.07 mM, 5) 0.09 mM

where the concentrations are with respect to monomer.

8.3a


6
3
0
V×10⁻³
EU/mg
1
2
3
4
5
0
3
6
NTP mM

8.3b

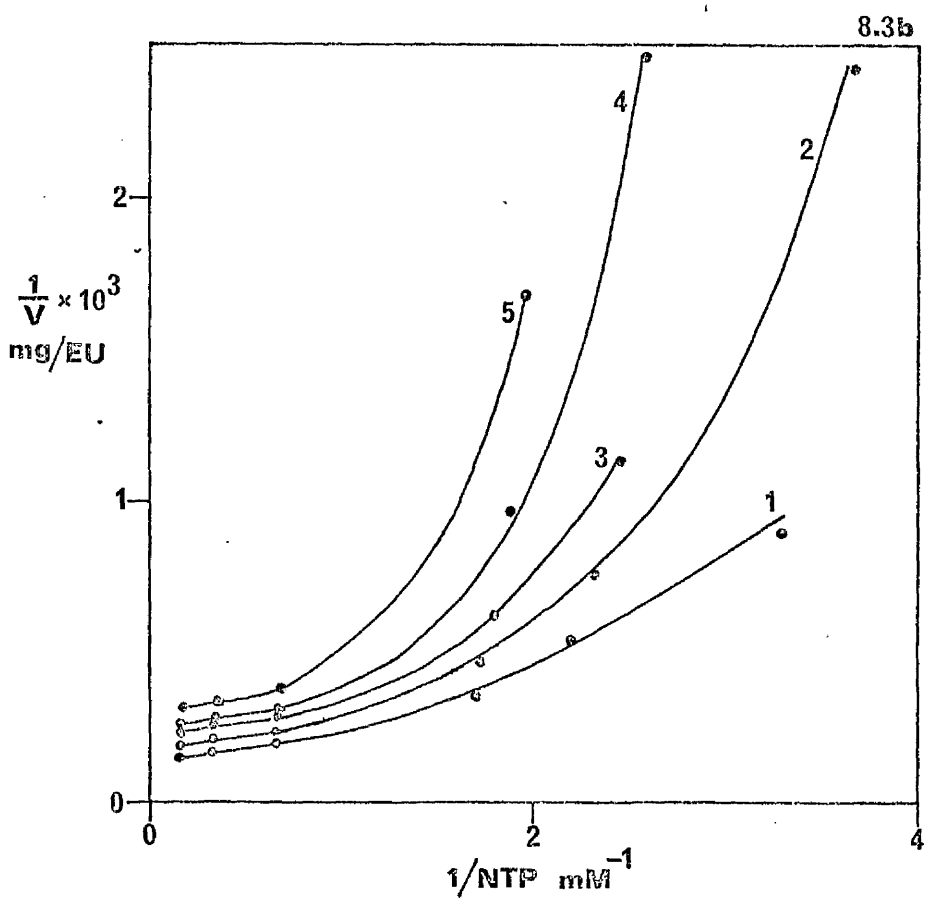
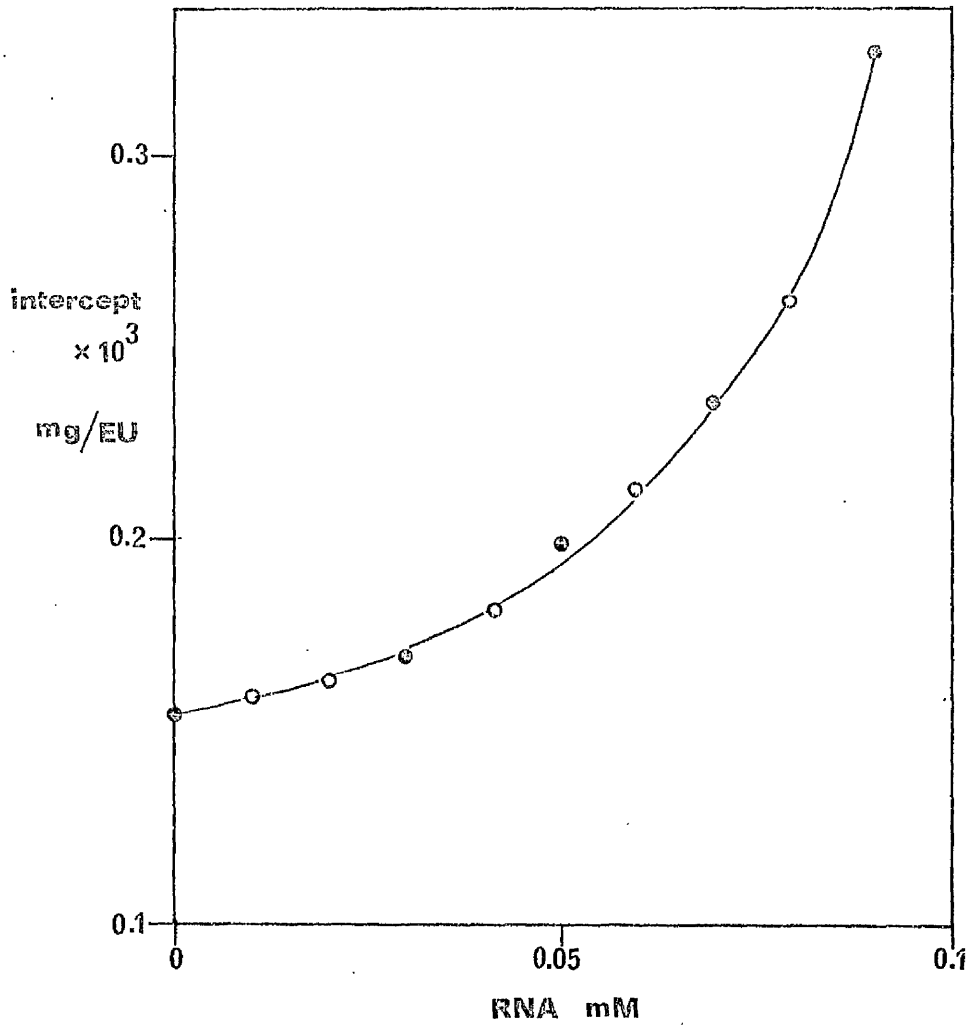

2
1
0
1/V × 10³
mg/EU
4
2
5
3
1
0
2
4
1/NTP mM⁻¹

Fig. 8.4 Slope and intercept replots for the data of Fig. 8.3b

For the double reciprocal plots in Fig. 8.3b, the slopes and intercepts were calculated, from the region of high NTP concentration, i.e. where the plots appeared most linear. These slopes and 1/v intercepts were plotted against RNA concentration, as seen in the figure. The filled circles represent the points from the data displayed in Fig. 8.3b. The open circles arose from similar analyses at other product RNA concentrations but which were not plotted in Figs. 8.3a & b for the sake of clarity.

8.4a


0.3
intercept
× 10^3
mg/EU
0.2
0.1
0
0.05
0.1
RNA mM

8.4b

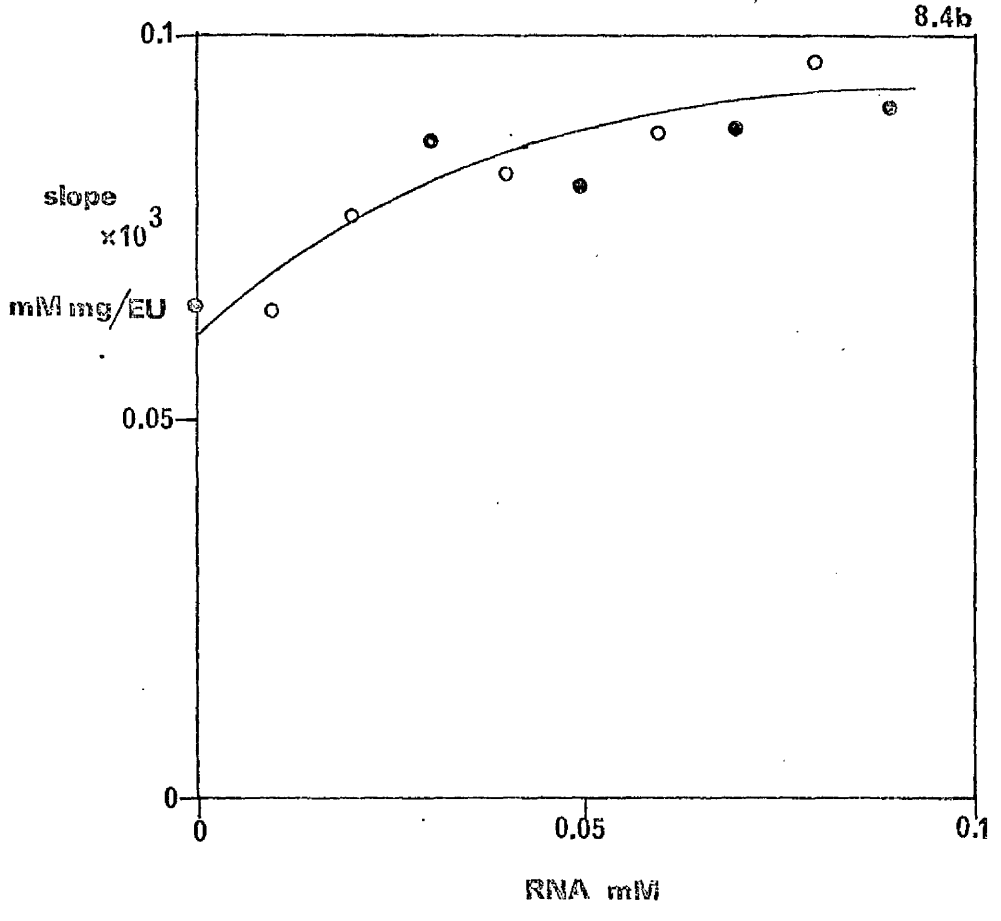

0.1
slope
×10^3
mM mg/EU
0.05
0
0
0.05
0.1
RNA mM

Fig. 8.5 Double reciprocal plot of 1/v against 1/NTP (1)

RNA polymerase activity was measured by the fluorimetric method described in Section 5D2. Assay conditions were standard (Section 5B) except that the substrate concentrations were varied and that the $MgCl_2$ was 0.04 M in one experiment (o-o). The concentrations of ATP, CTP, GTP and UTP were equal and were varied together - concentration units refer to each nucleotide. The reaction mixtures contained 30 μg/ml of $E_{\sigma\theta}$ and 50 μg/ml of T7 DNA. Details of the fluorescence measurements are as in the legend to Fig. 7.3.

•-• $MgCl_2$, 0.02 M : K_m = 0.52 mM ; S.E.M. = 0.05

o-o $MgCl_2$, 0.04 M : K_m = 0.51 mM ; S.E.M. = 0.15

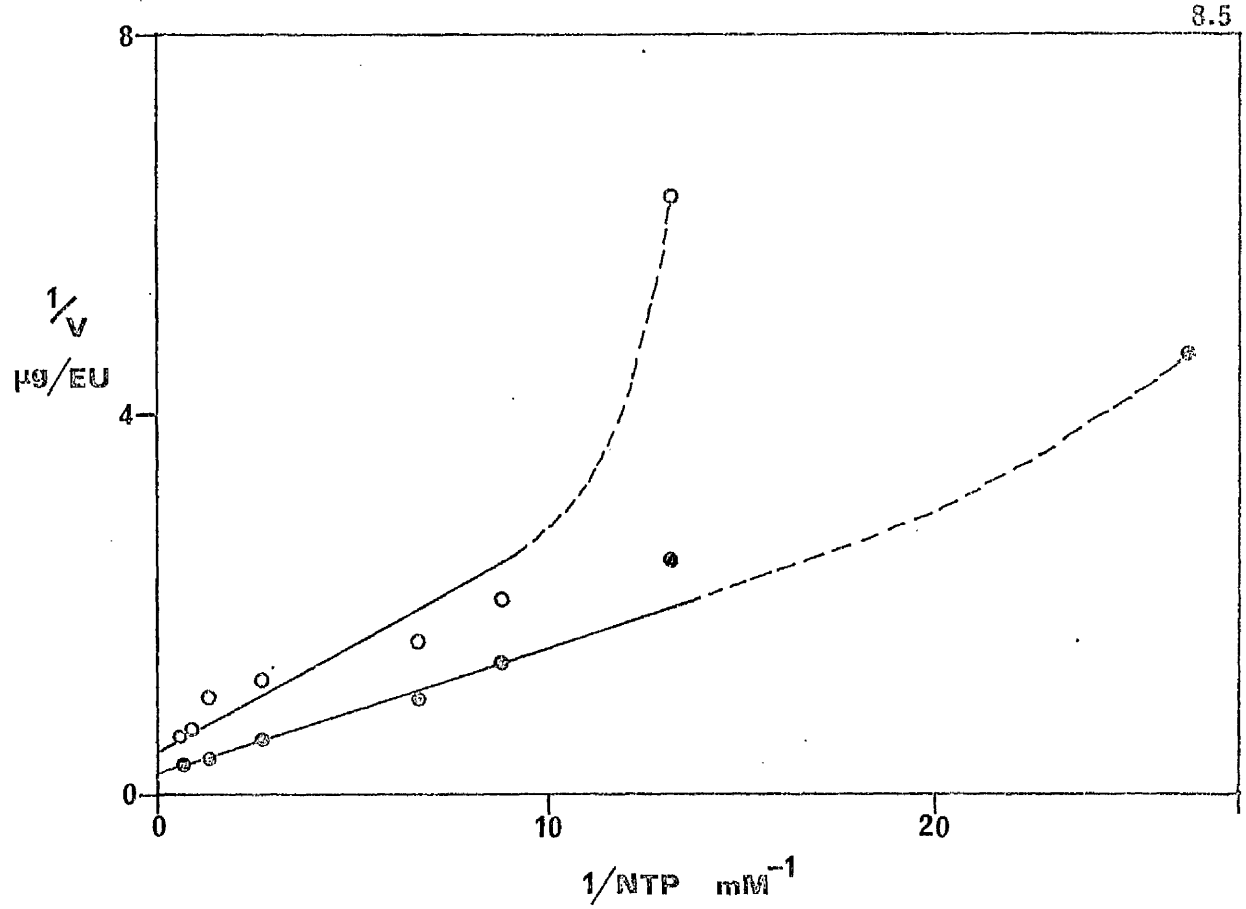
K_m's were calculated by the procedure of Wilkinson (Appendix 1) and the unbroken line in the figures is so computed.

Fig. 8.6 Double reciprocal plot of 1/v against 1/NTP (2)

Details were as for the above legend except that the enzyme activity was determined by the radioactive assay technique (Section 5C) using standard reaction conditions (Section 5B). The reaction mixtures contained 21.5 μg/ml of E_c and 70 μg/ml of calf thymus DNA and were for 5 min.

K_m = 0.47 mM, S.E.M. = 0.08

8.5



8.6

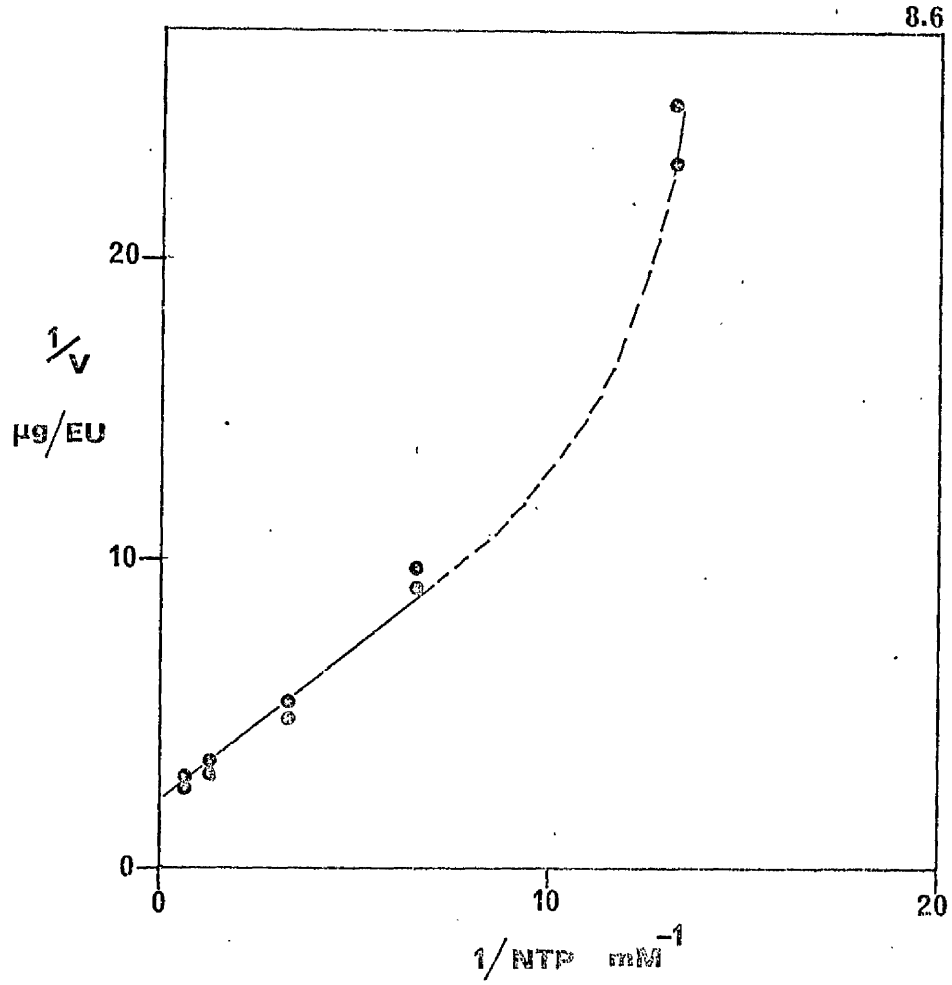


Fig. 8.7 Comparison of the response of the activity of a calf thymus DNA - E_c system to the variation of either PuTP or PyTP

The rate of RNA synthesis by RNA polymerase was measured by the radioactive technique (Section 5C) under standard reaction conditions (Section 5B) except that the substrate concentrations were varied. PuTP concentration refers to the concentration of each nucleotide in an equimolar mixture of ATP and GTP. PyTP is to be similarly interpreted. The experiments were carried out keeping either PuTP or PyTP fixed at 0.75 mM while the other substrate group (PyTP or PuTP) was varied. The reaction mixtures contained 20 μg/ml of E_c and 70 μg/ml of calf thymus DNA, and the duration of the reaction was 5 min, at 37°C.

Apparent K_m's were calculated by the procedure of Wilkinson (Appendix 1) and the unbroken region of the double reciprocal plot represents the computer fitted line.

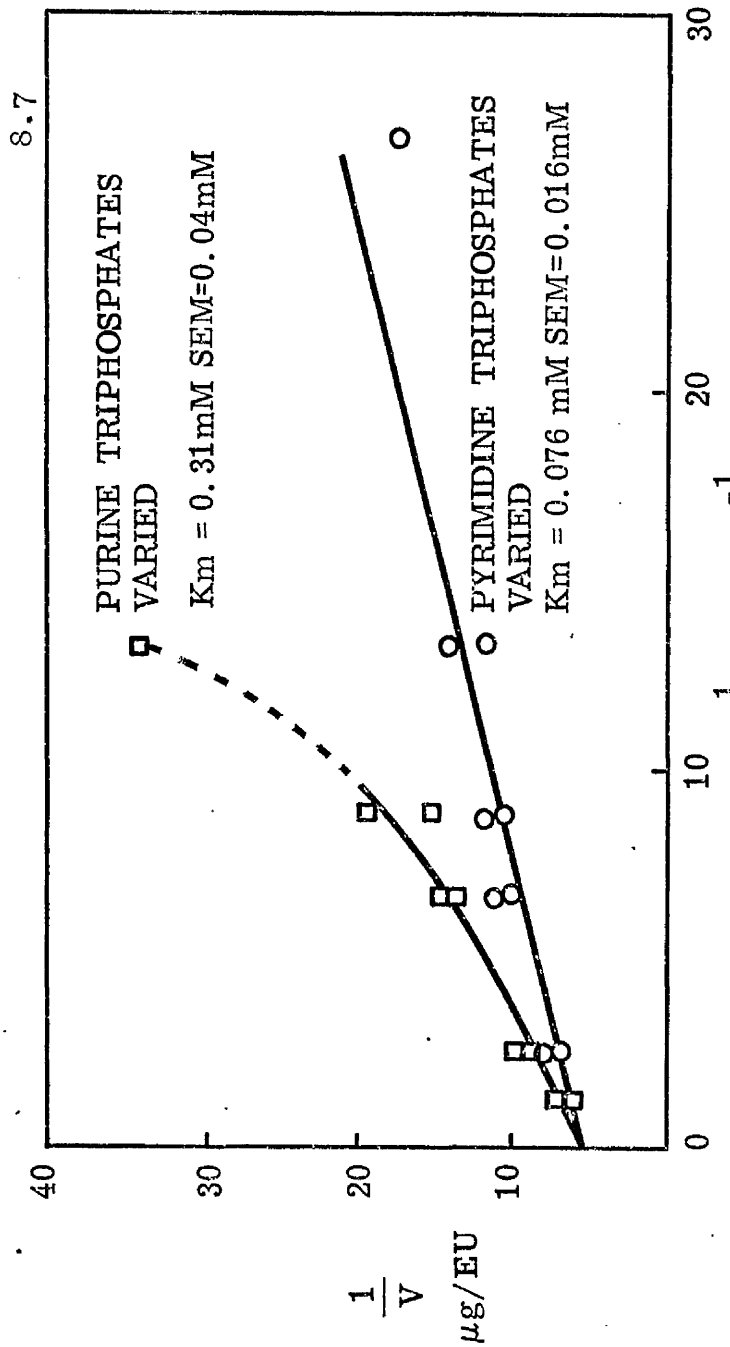
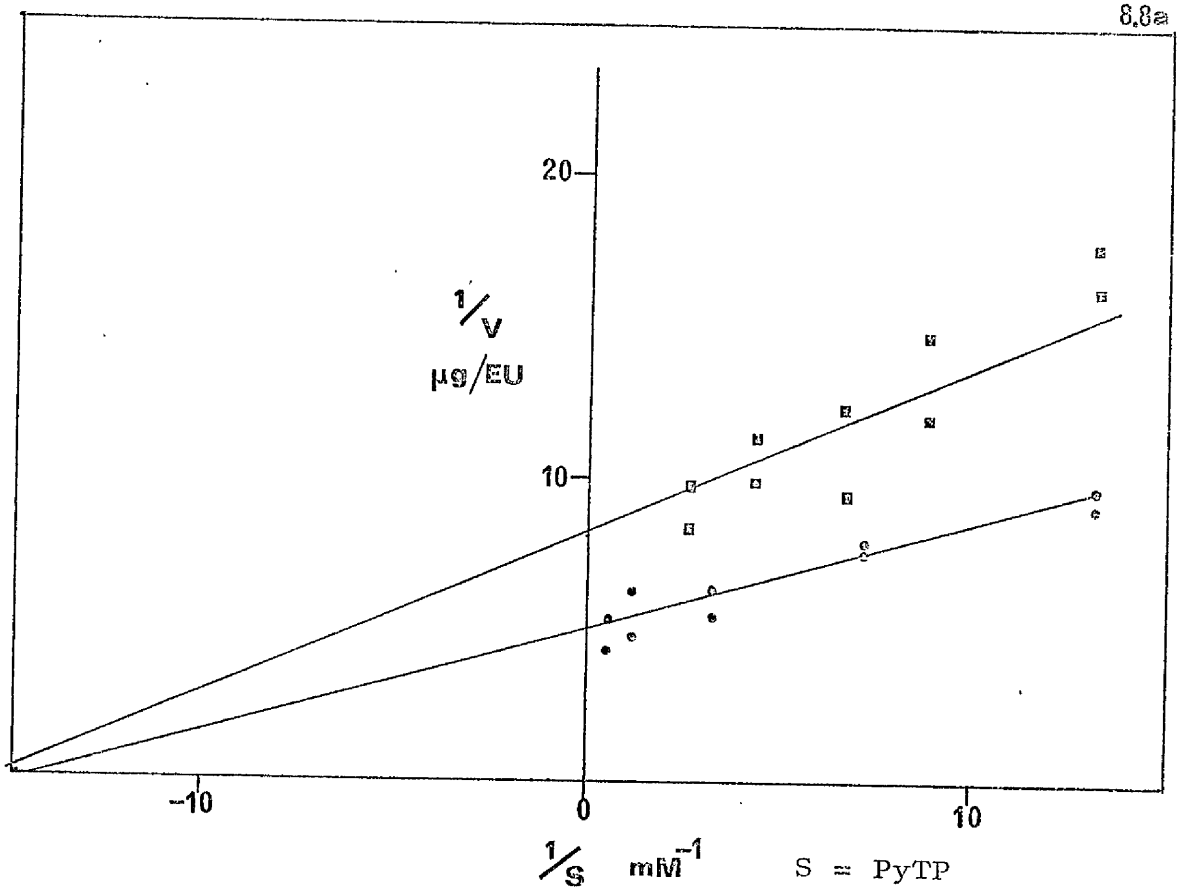

8.7
PURINE TRIPHOSPHATES
VARIED
Km = 0.31mM SEM=0.04mM
PYRIMIDINE TRIPHOSPHATES
VARIED
Km = 0.076 mM SEM=0.016mM
1/V
μg/EU
1/S mM-1
40
30
20
10
0
10
20
30

Fig. 8.8 a & b Effect of calf thymus DNA concentration on the kinetic behaviour of RNA polymerase

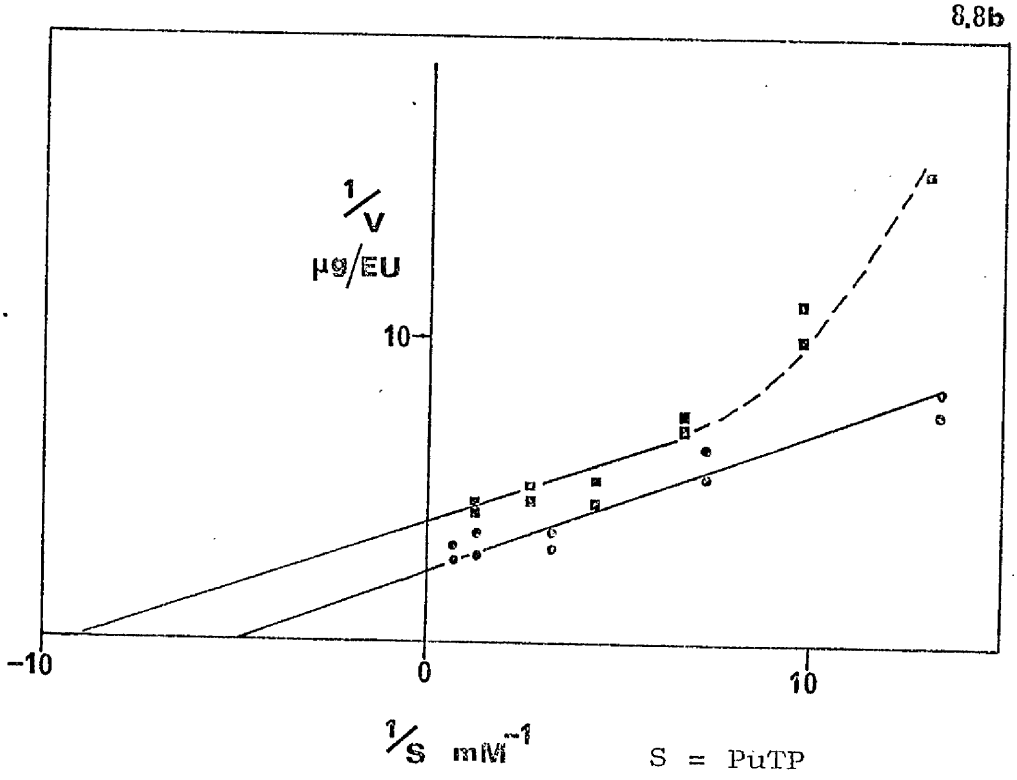
All experimental details were as in the legend to Fig. 8.7, with the following enzyme and template concentrations (all in μg/ml) : ●-● ; $E_{\sigma\theta}$ 23, calf thymus DNA, 74 : ■-■ ; $E_{\sigma\theta}$ 47, calf thymus DNA, 28. PyTP was the variable substrate in Fig. 8.8a ; PuTP was the variable substrate in Fig. 8.8b.

8.8a



S = PyTP

8.8b



S = PuTP

Figs. 8.9 a & b 1/v against 1/s plots for two different core enzyme preparations with a calf thymus DNA template

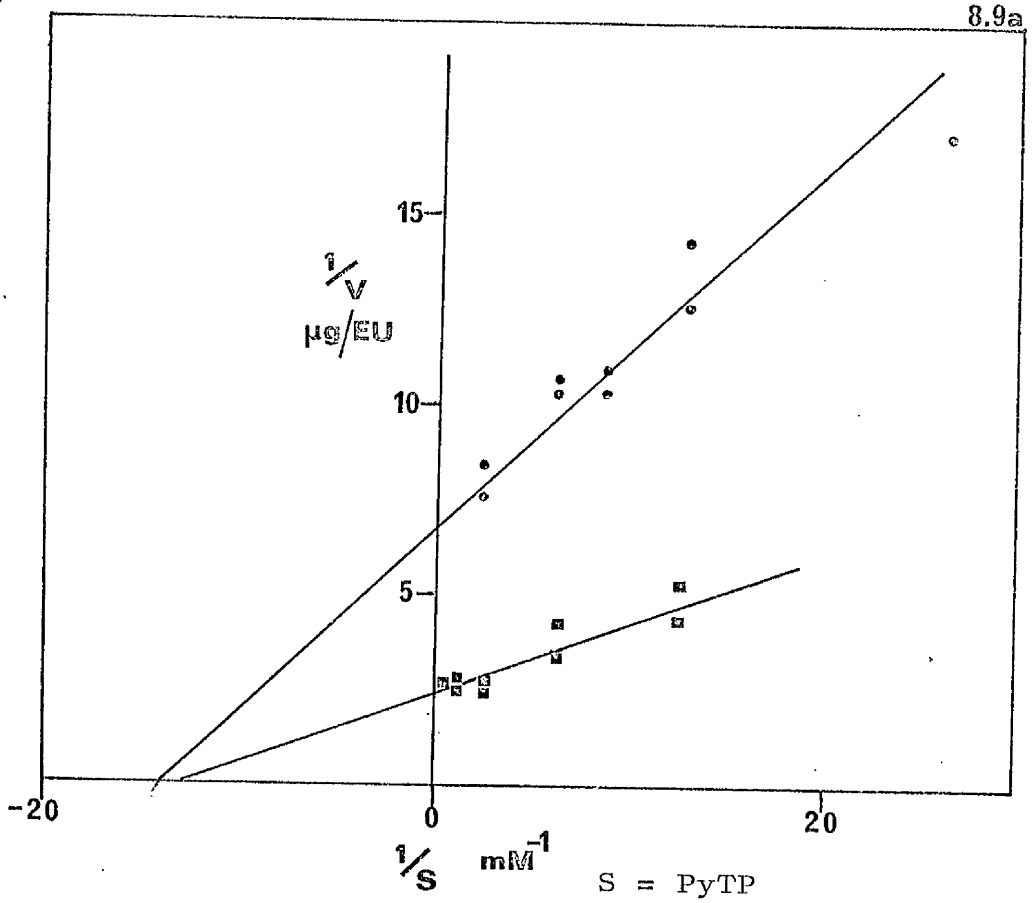
All experimental details were as in the legend to Fig. 8.7 with the following enzyme and template concentrations (all in μg/ml) :

o-o $E_c(1)$ 21.5 , DNA 70

▪-▪ $E_c(2)$ 20 , DNA 70

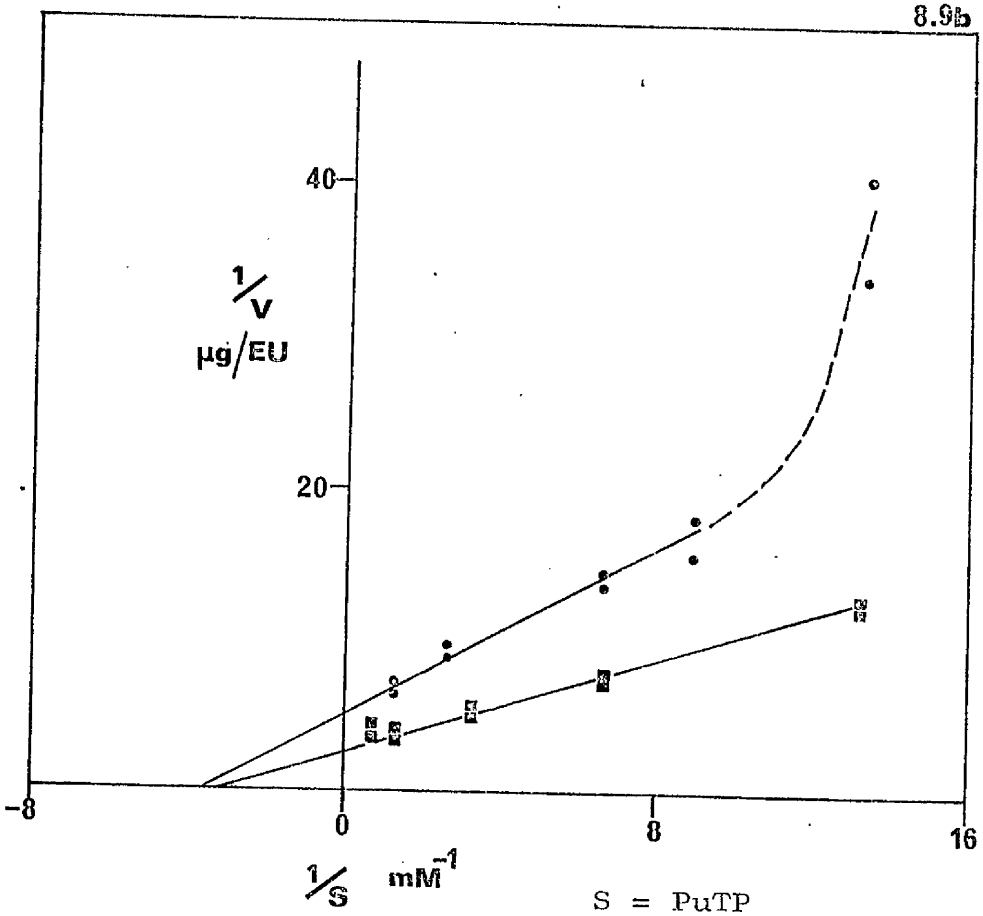
In Fig. 8.9a, PyTP was the variable substrate ; in Fig. 8.9b, PuTP was the variable substrate.

8.9a



S = PyTP

8.9b



S = PuTP

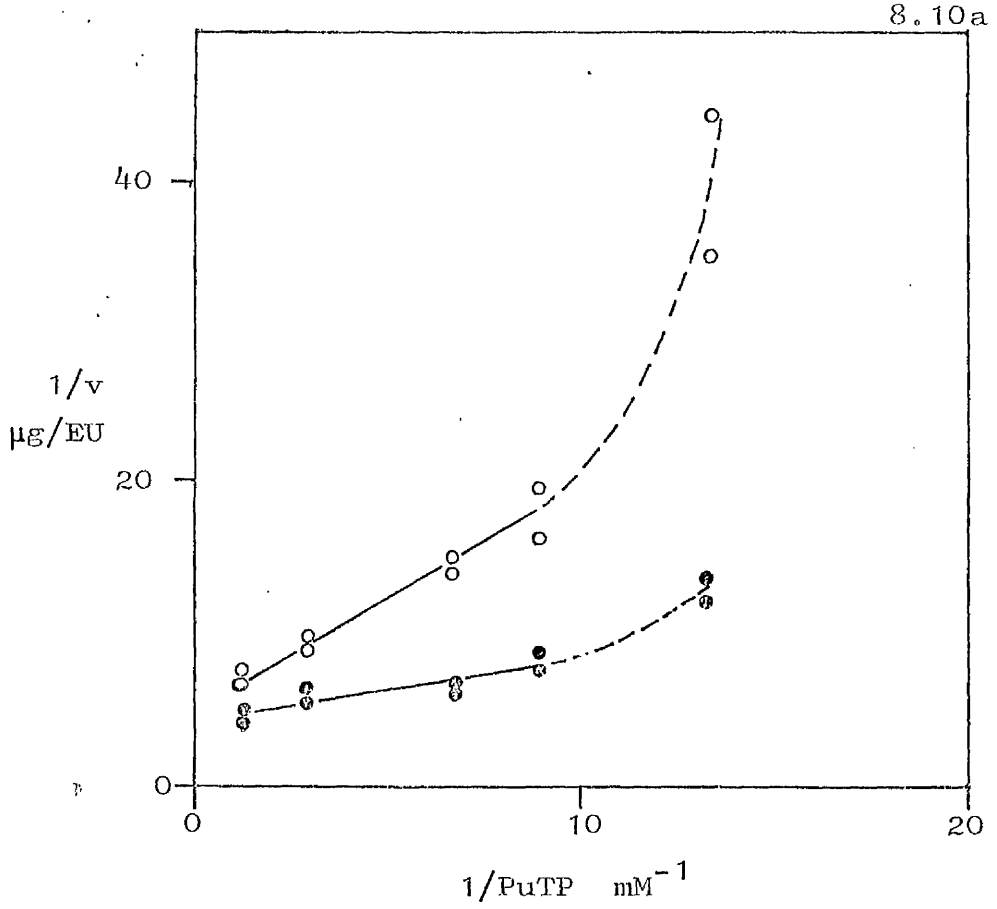
Figs. 8.10 a & b Comparison of the kinetic behaviour of E_c and $E_{\sigma\theta}$ with a calf thymus DNA template

All experimental details were as in the legend to Fig. 8.7 with the following enzyme and template concentrations (all in μg/ml) : o-o E_c 20 , DNA 70

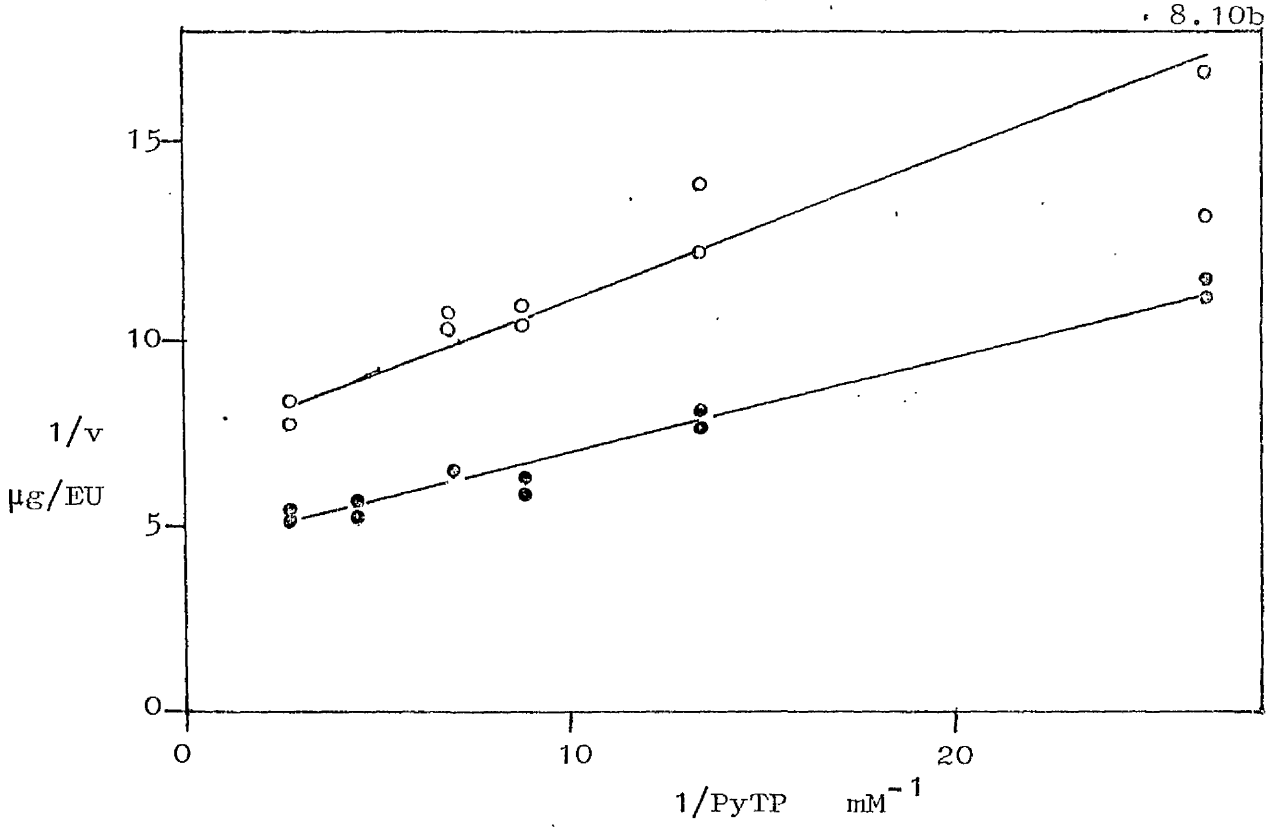
•-• E 25.4 , DNA 74

PuTP was the variable substrate in Fig. 8.10a ; PyTP was the variable substrate in Fig. 8.10b.

8.10a



8.10b



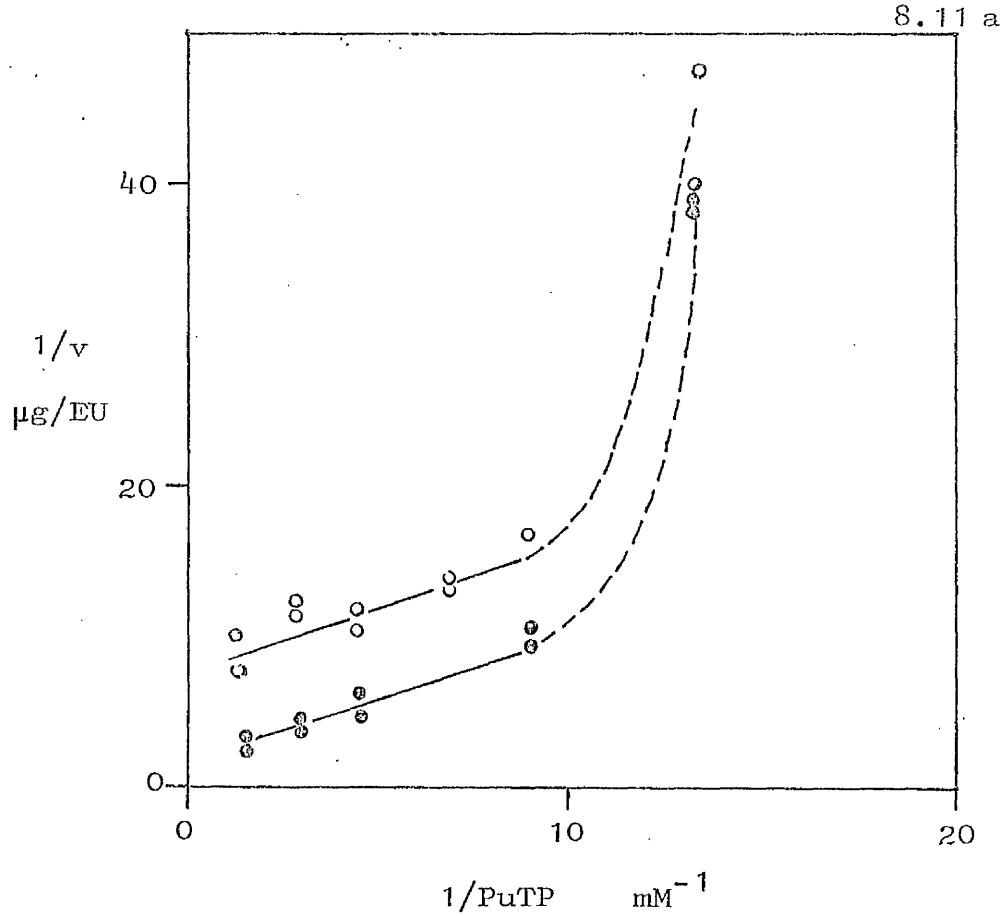
Figs. 8.11 a & b Comparison of the kinetic behaviour of E_c and $E_{\sigma\theta}$ with a T7 DNA template

All experimental details were as in the legend to Fig. 8.7 with the following enzyme and template concentrations (all in μg/ml) : o-o E_c 20 , DNA 40

●-● $E_{\sigma\theta}$ 24.6, DNA 40

PuTP was variable in Fig. 8.11a and PyTP was variable in Fig. 8.11b.

8.11 a


40
20
0
1/v
µg/EU
0
10
20
1/PuTP mM⁻¹

8.11b

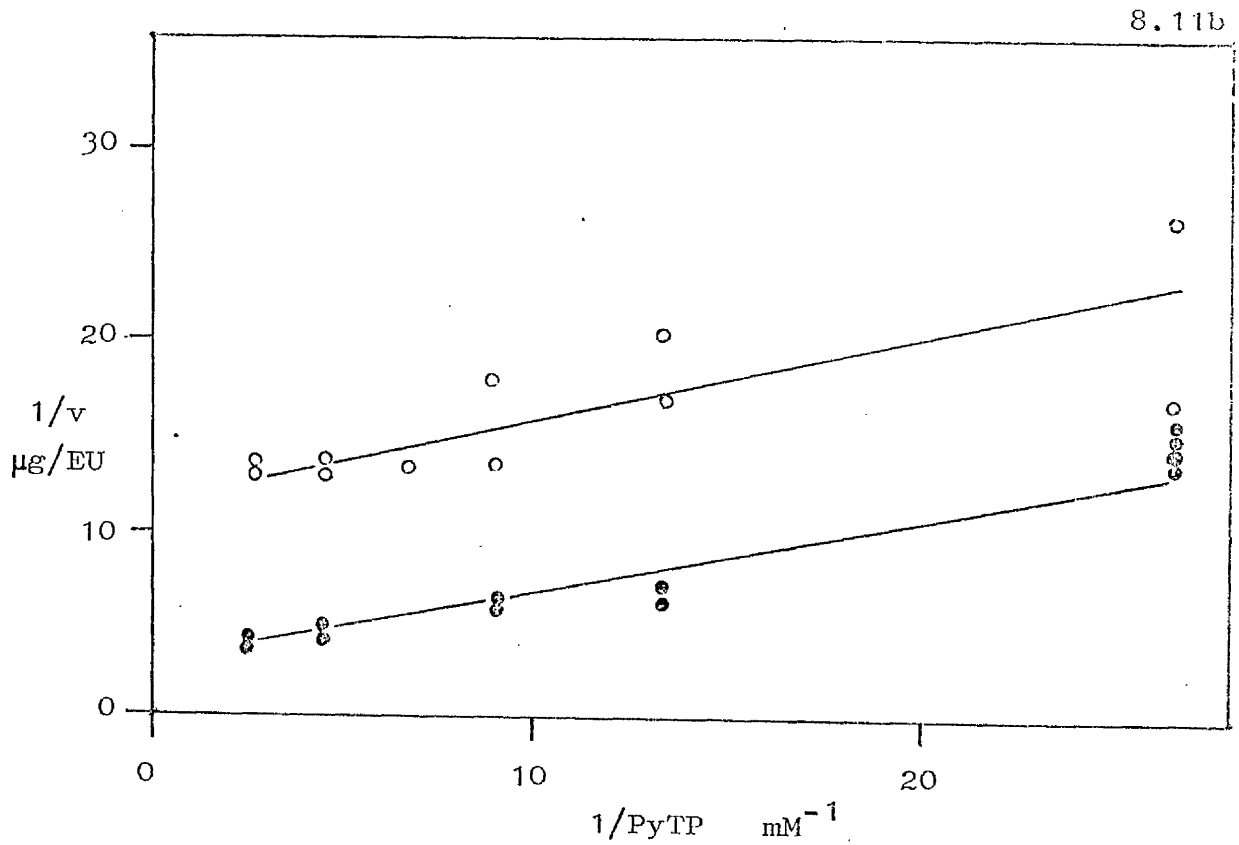
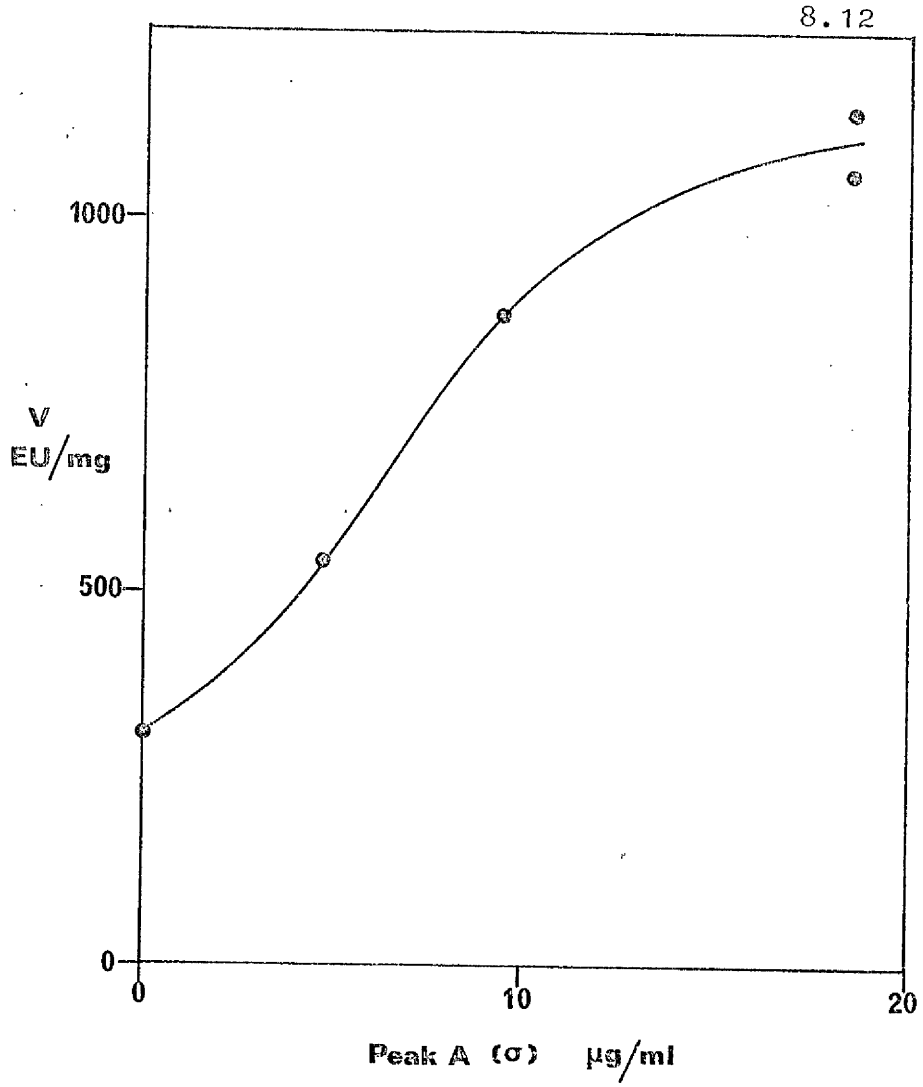

30
20
10
0
1/v
µg/EU
0
10
20
1/PyTP mM⁻¹

Fig. 8.12 The dependence of E_c activity on sigma concentration

RNA polymerase activity was measured by the fluorimetric technique described in Section 5D2. Reaction conditions were standard (Section 5B) with 50 μg/ml T7 DNA and 33 μg/ml of E_c. Peak A from phosphocellulose chromatography (which contained σ -Section 4B) was added to the reaction in the concentrations indicated on the horizontal axis of the figure. The details of the fluorescence measurements were as in the legend to Fig. 5.6.

8.12



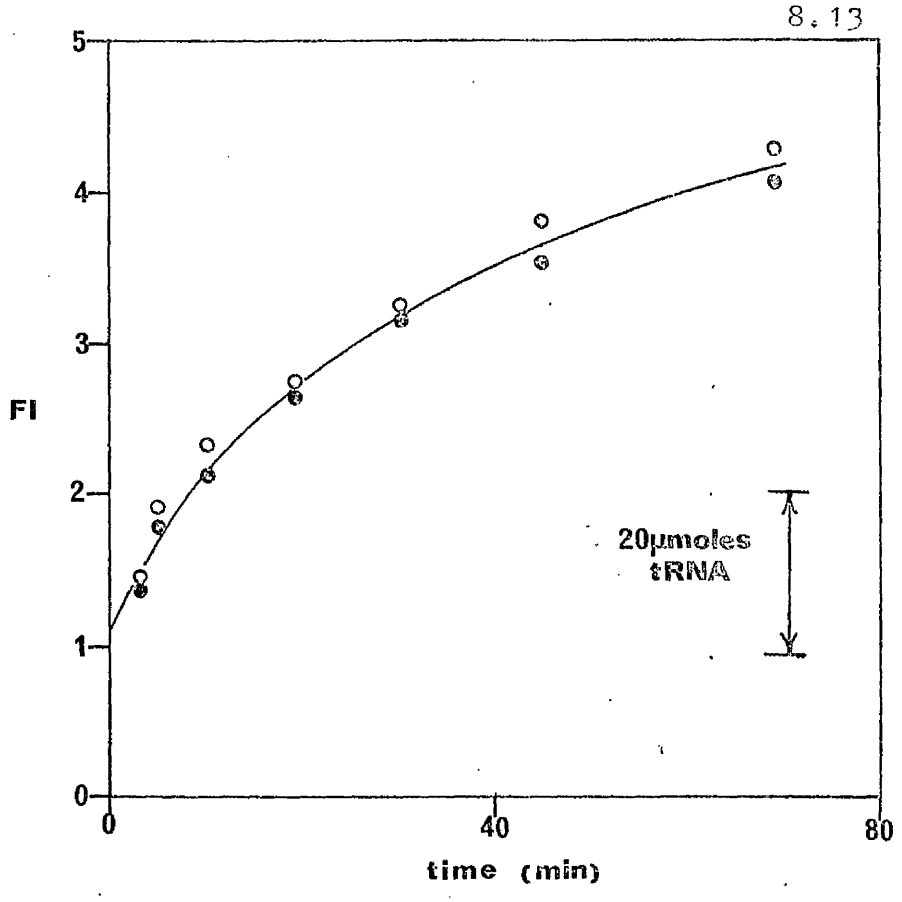


Fig. 8.13 Time course of RNA synthesis by a poly(dA-dT)-$E_{\sigma\theta}$ system

RNA polymerase activitywas measured by the fluorimetric method (Section 5D2) and the course of RNA production with time followed for an $E_{\sigma\theta}$ - poly(dA-dT) system. Reaction conditions were standard (o-o), but 1 mM PP_i was present in one reaction mixture (•-•). The assays contained also $E_{\sigma\theta}$, 108 µg/ml and poly(dA-dT), 75 µM(with respect to monomer). The details of the fluorescence measurements were as in the legend to Fig. 6.5, the vertical axis above being fluorescent intensity in arbitrary units.

Figs. 8.14 - 8.17 Velocity-substrate curves and double reciprocal plots for a poly(dA-dT)-E_c system

RNA polymerase E_c activity was measured, with poly(dA-dT) template, by the radioactivity method described in Section 5C. Reaction conditions were standard (Section 5B) except that ATP and UTP were the substrates (further purified as in Section 4D) and were present at various concentrations. Also, E_c was present at 13.4 µg/ml and poly(dA-dT) at 75 µM (with respect to monomer). The assay duration was 5 min and the temperature 37°C.

The experimental results, corrected for controls with no DNA, are shown in various ways.

Fig. 8.14 : velocity-ATP curves, at different UTP levels.

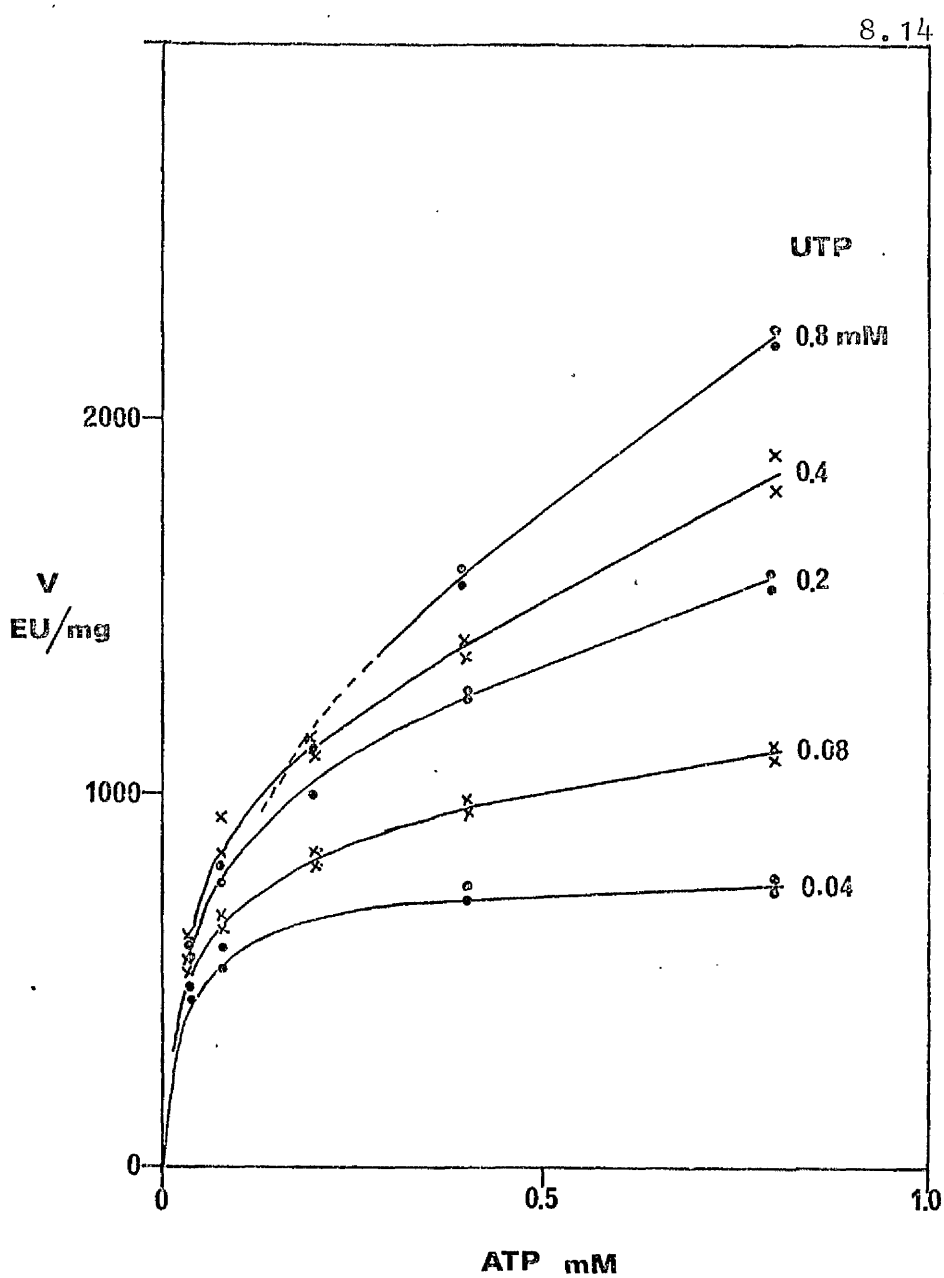
Fig. 8.15 : double reciprocal plots, 1/ATP variable at different UTP levels.

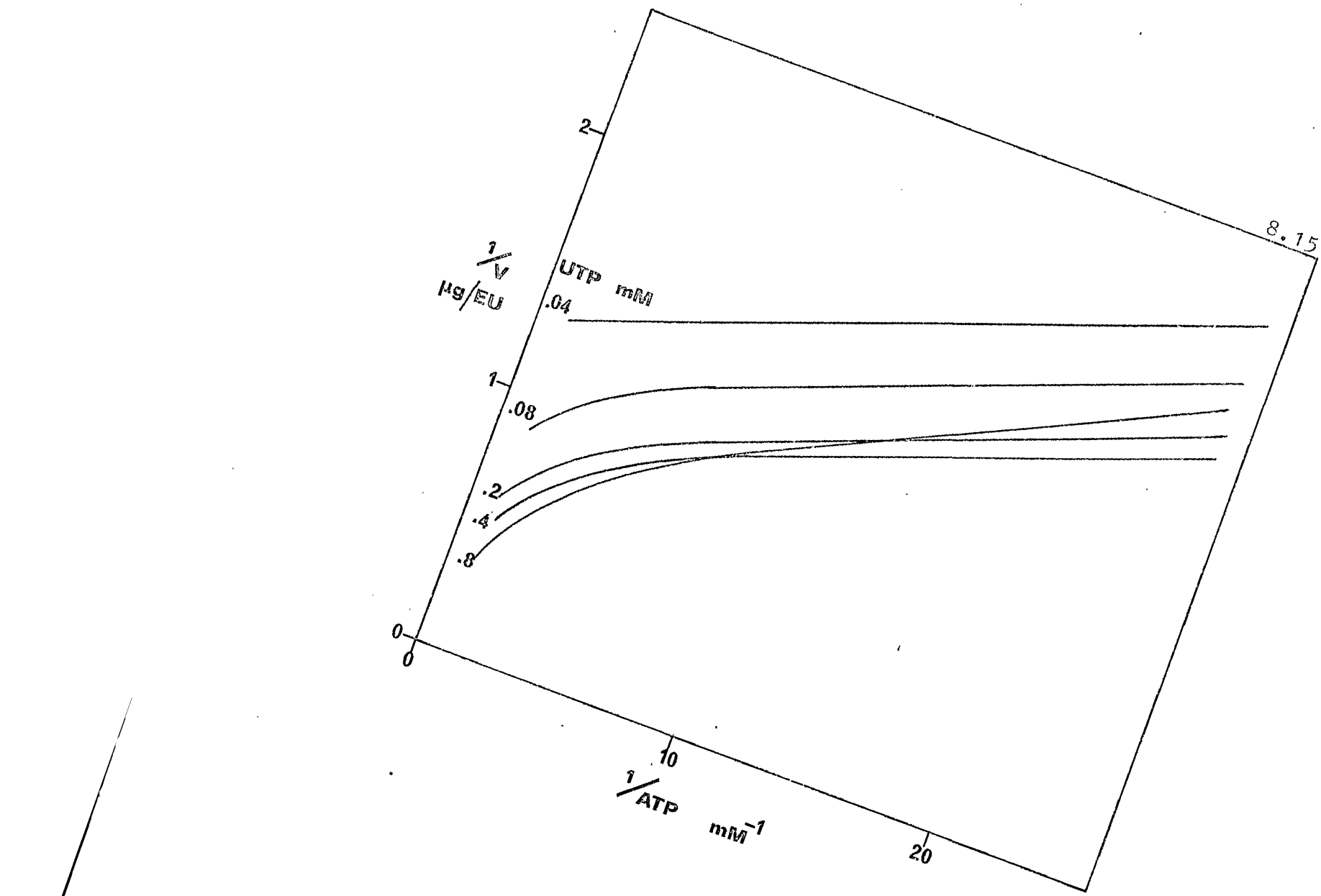
Fig. 8.16 : velocity-UTP curves, at different ATP levels.

Fig. 8.17 : double reciprocal plots, 1/UTP variable at different ATP levels.

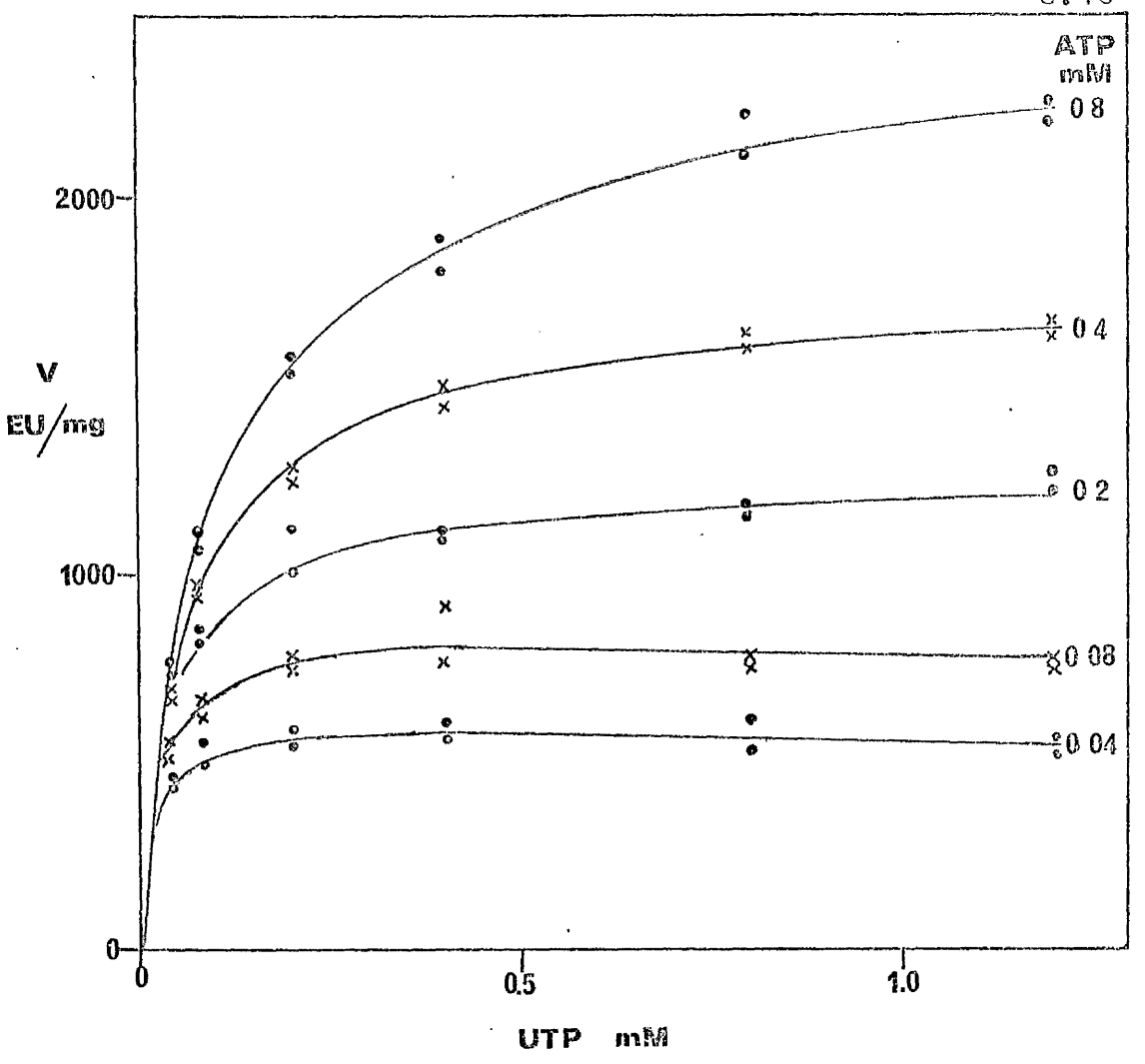
The solid curves are the best least squares fits to the velocity substrate curves (see Section 8C2.1) and the points experimental results ; in Fig. 8.15, the experimental points are not presented, for the sake of clarity, but can be effectively seen in Fig. 8.14.

8.14




8.15
1/V
µg/EU
UTP mM
.04
.08
.2
.4
.8
2
1
0
0
10
20
1/ATP mM⁻¹

8.16



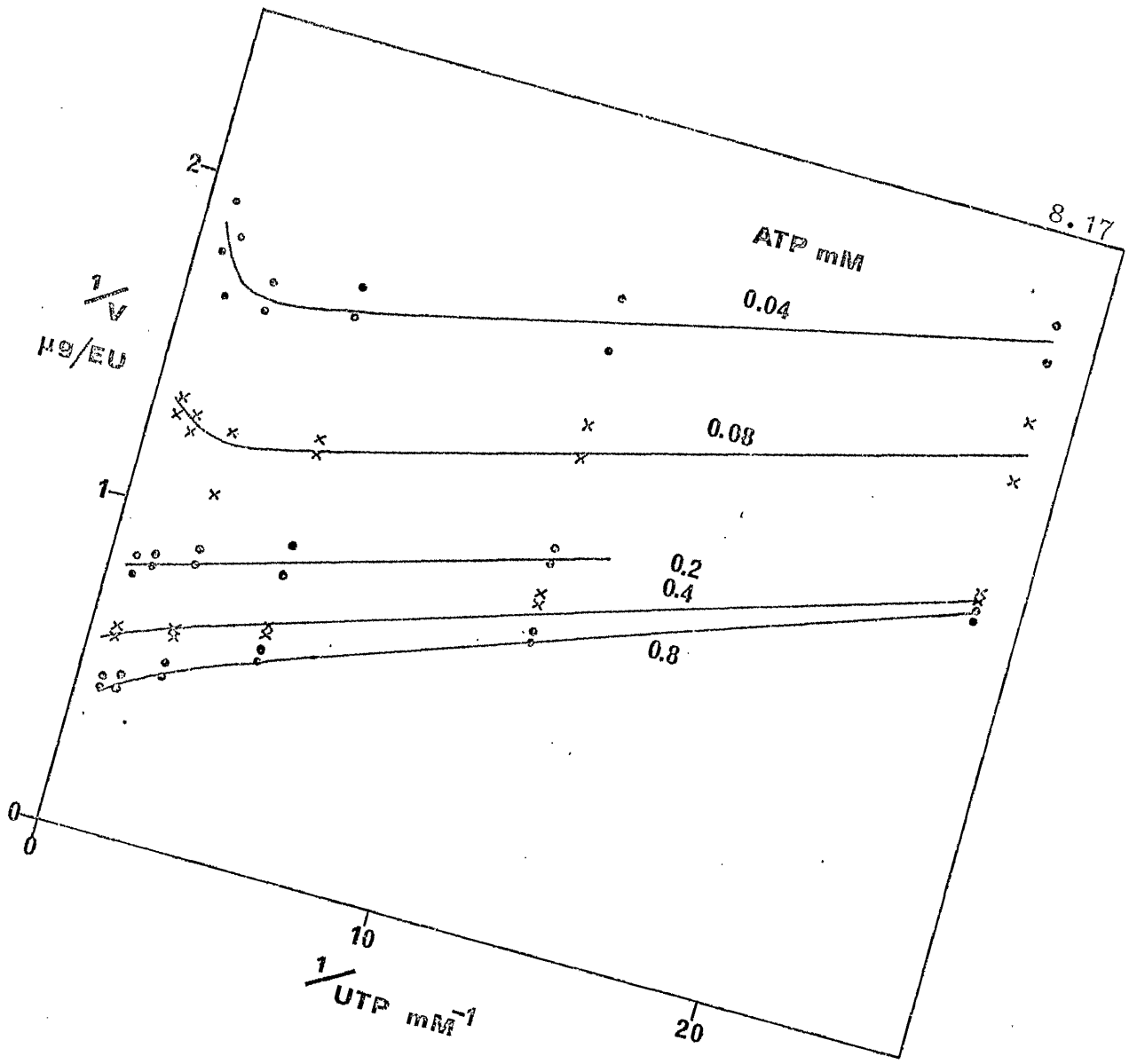
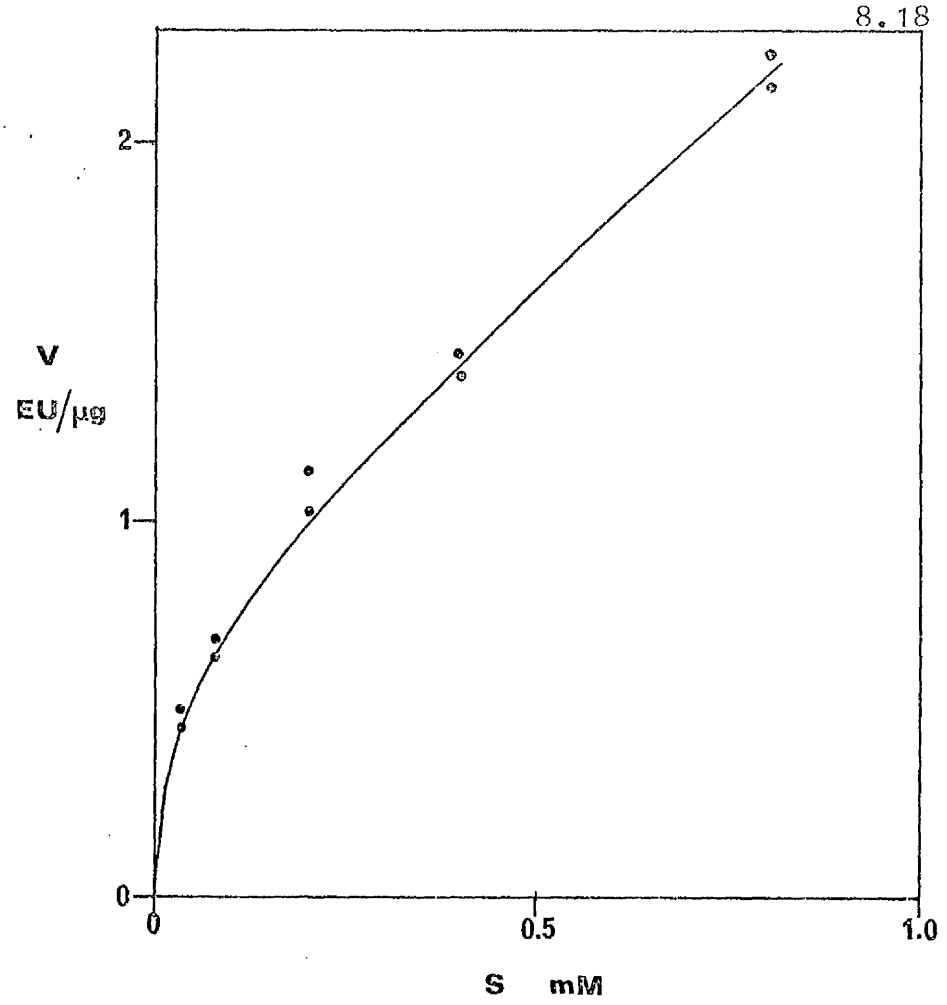

2
1
0
0
10
20
1/V
μg/EU
1/UTP mM⁻¹
ATP mM
8.17
0.04
0.08
0.2
0.4
0.8

Fig. 8.18 Velocity-substrate curve and double reciprocal plot for a poly(dA-dT)-E_C sytem when ATP and UTP (at equal concentrations) were varied together.

The figures show another representation of the results described in Figs. 8.14-8.17. S represents the concentration of either ATP or UTP as both substrate levels were varied together. The points again represent experimental values and the lines the best least squares fit of a model rate equation (see Section 8C2.1).

8.18



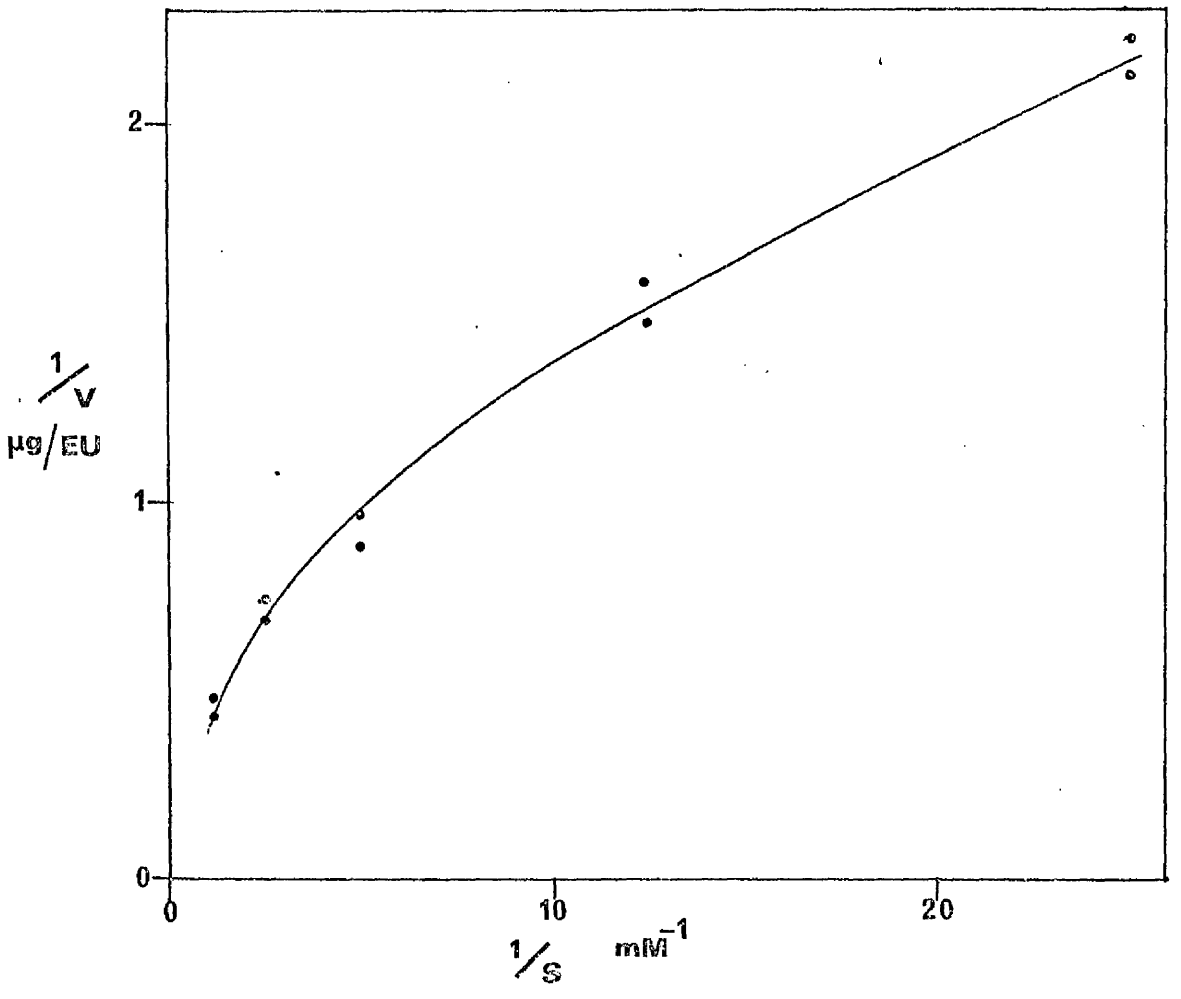
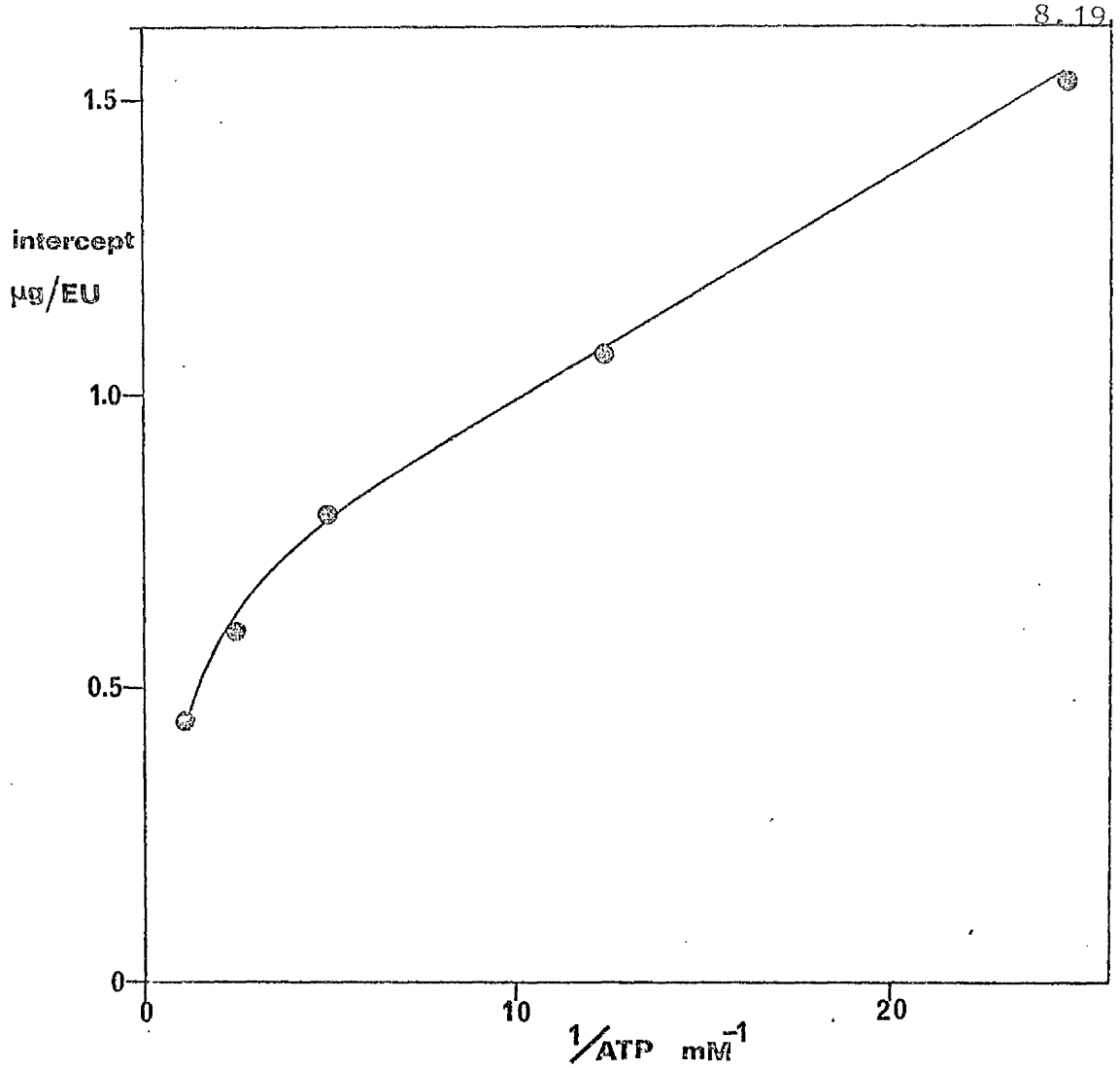


Fig. 8.19 Slope and intercept replots for the data of Fig. 8.17

From the region of Fig. 8.17 where the double reciprocal plots were linear, the slopes and 1/v intercepts were plotted against the fixed substrate concentration, ATP (for the slopes) or 1/ATP (for the intercepts). The intercepts were estimated by extrapolating the linear portion of the double reciprocal plots.

8.19


1.5
1.0
0.5
0
intercept
µg/EU
0
10
20
1/ATP mM⁻¹

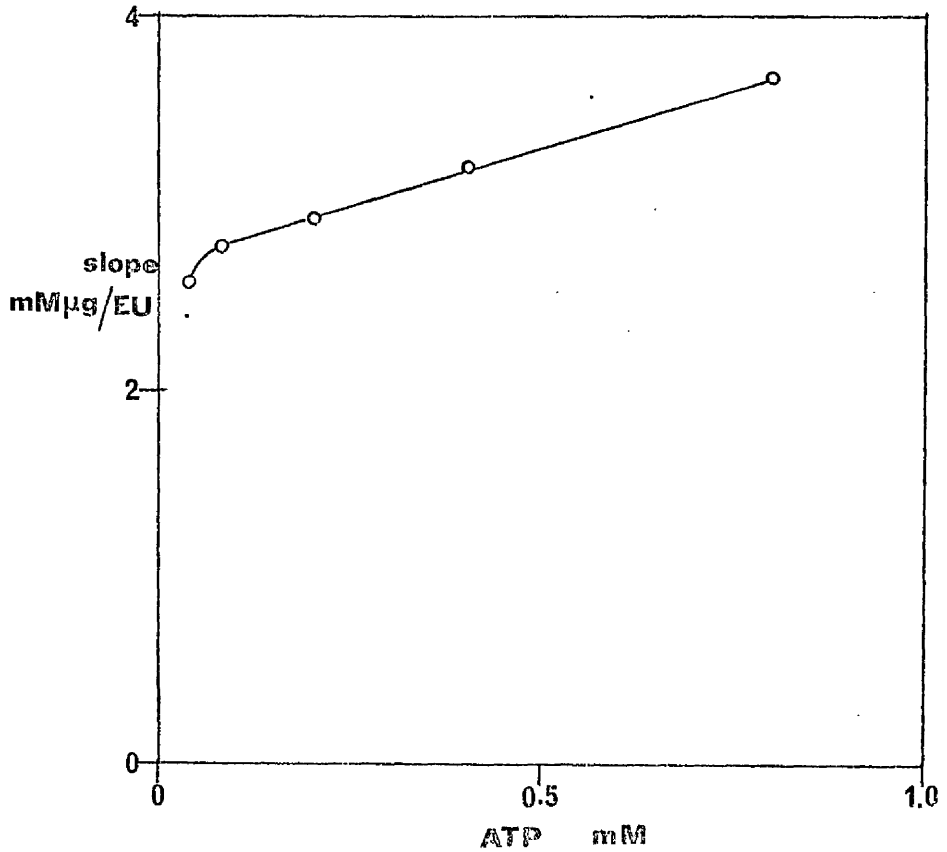

4
2
0
slope
mMµg/EU
0
0.5
1.0
ATP mM

Fig. 8.20 Slope and intercept replots for the data from Fig. 8.15

From the region of Fig. 8.15 where the double reciprocal plots were linear, the slopes and 1/v intercepts were plotted against the reciprocal of the fixed substrate concentration, UTP. The intercepts were estimated by extrapolating the linear portion of the double reciprocal plots.

8.20

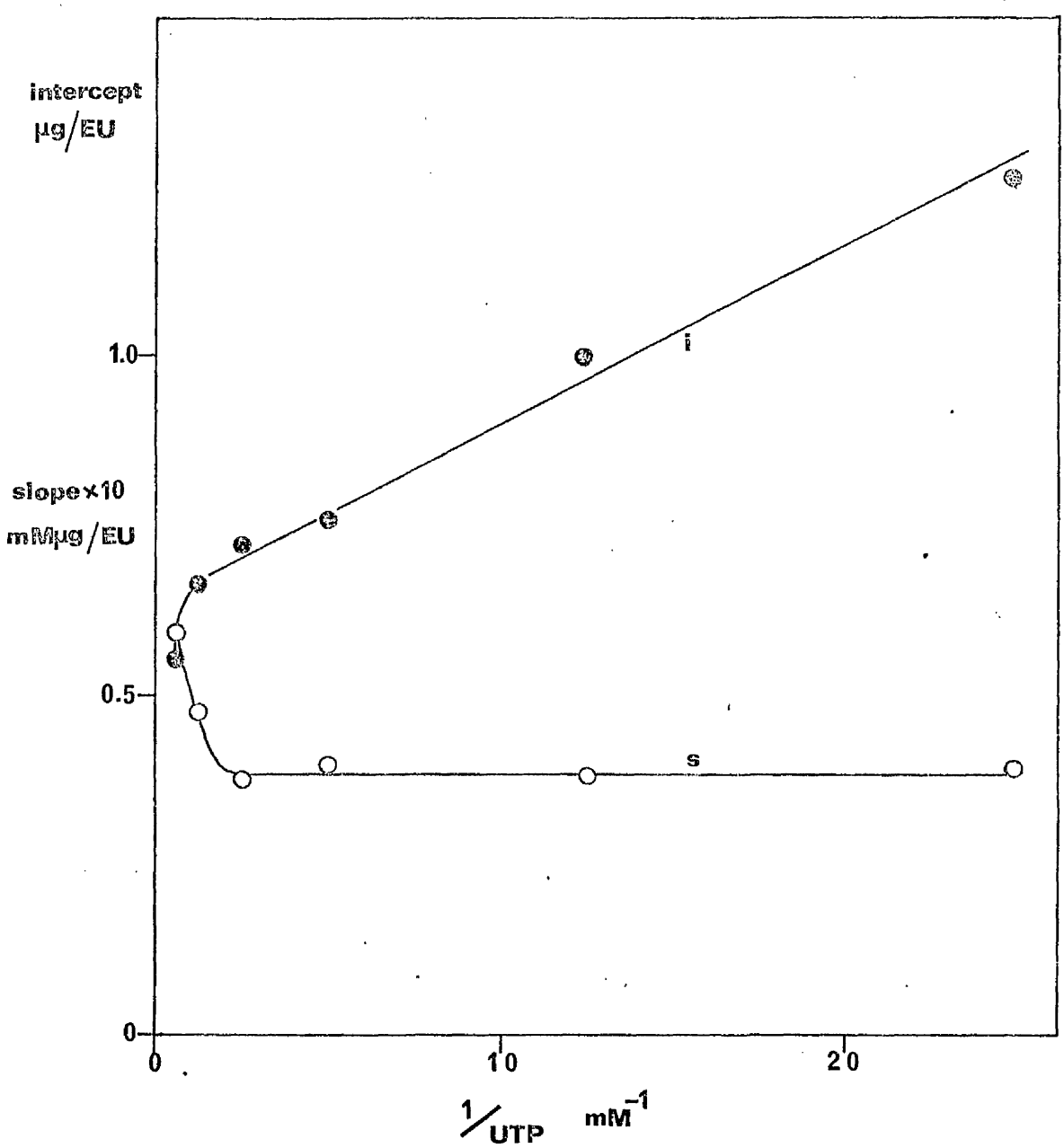


Fig. 8.21 Variation of the parameters of model rate equation 1 with changing fixed substrate concentration- data from Fig. 8.16.

The data of Fig. 8.16 were found to be fitted best by a model rate equation of the form

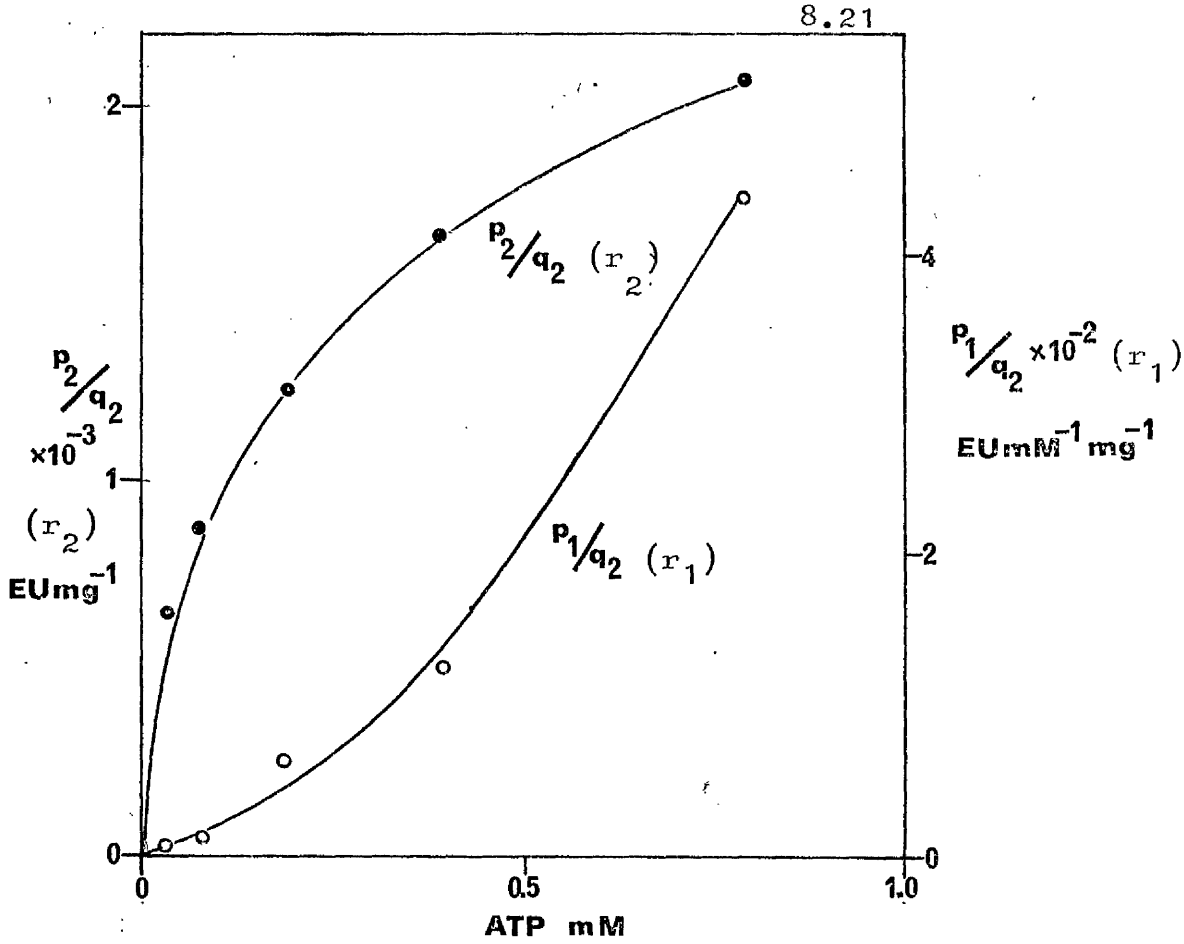
$$v = \frac{p_1 u^2 + p_2 u}{q_1 u^2 + q_2 u + q_3} \qquad \text{MODEL 1}$$

where u denotes UTP concentration (see Section 8C2.1). In fact, in the least squares analysis, model 1 was normalized by setting $q_3 = 1$. The figures show the dependence of parameters on the changing fixed substrate concentration i.e. ATP. For the figures, model equation 1 was normalized with respect to q_2 so that model 1 becomes

$$v = \frac{r_1 u^2 + r_2 u}{s_1 u^2 + u + s_3}$$

with $r_1 = p_1/q_2$, $r_2 = p_2/q_2$, $s_1 = q_1/q_2$, and $s_3 = q_3/q_2 = 1/q_2$ (since q_3 was 1).

8.21


2
1
0
0
0.5
1.0
ATP mM
p_2/q_2 (r_2)
p_2/q_2 $\times 10^{-3}$ (r_2) EUmg^{-1}
p_1/q_2 (r_1)
p_1/q_2 $\times 10^{-2}$ (r_1) EUmM^{-1} mg^{-1}
4
2

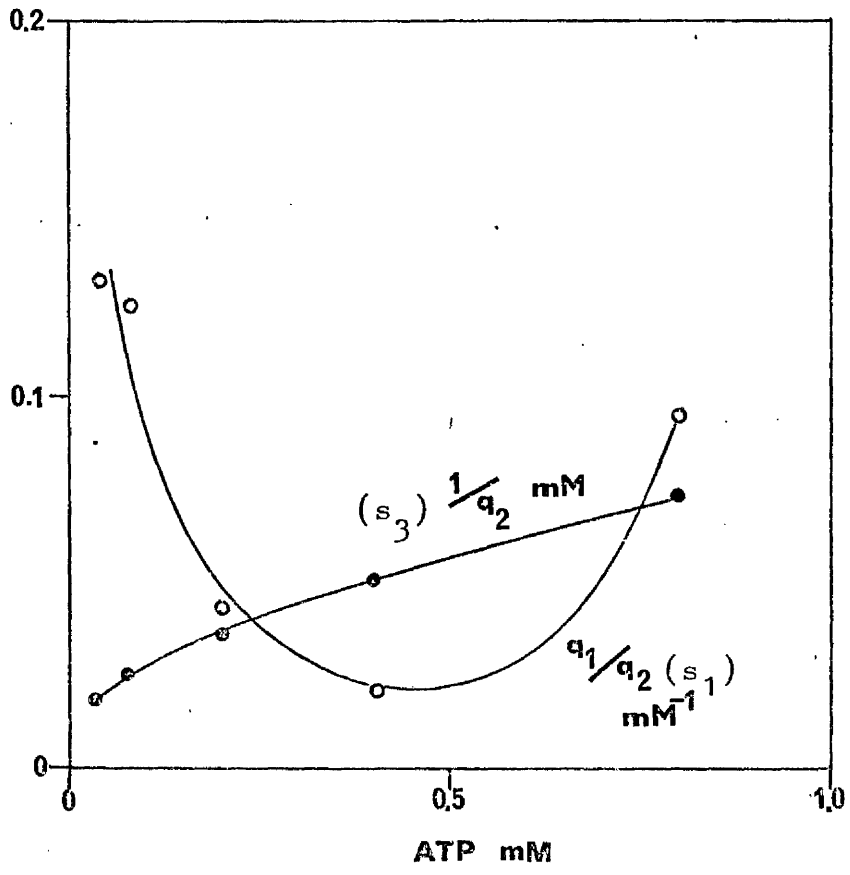
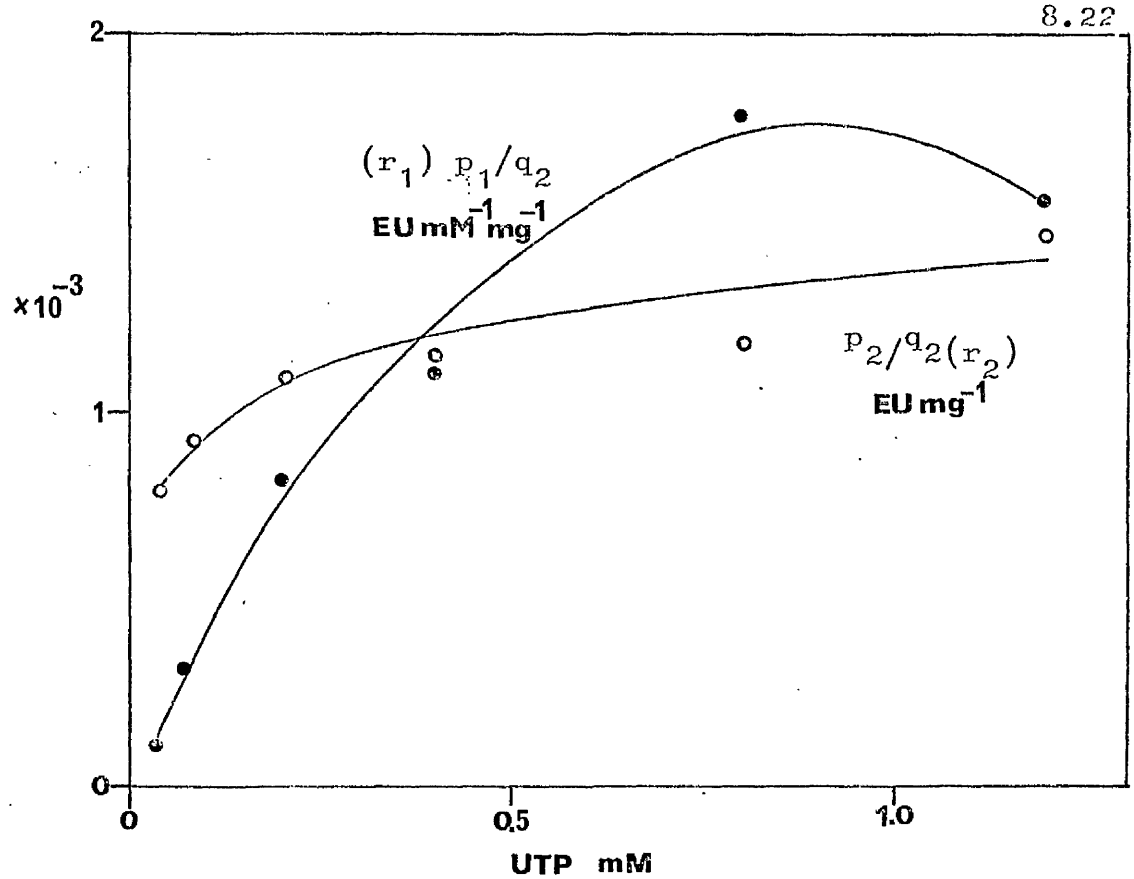

0.2
0.1
0
0
0.5
1.0
ATP mM
(s_3) $1/q_2$ mM
q_1/q_2 (s_1) mM^{-1}

Fig. 8.22 Variation of the parameters of model rate equation 1 with changing fixed substrate concentration - data from Fig. 8.14

The data of Fig. 8.14 were found to be fitted best, at any given fixed substrate (UTP) concentration by a model rate equation of the form

$$v = \frac{p_1 a^2 + p_2 a}{q_1 a^2 + q_2 a + q_3} \qquad \text{MODEL 1}$$

where a denotes ATP concentration (See Section 8C2.1). In the figures, the model equation was normalized by setting $q_2 = 1$ (see legend to Fig. 8.21 ; the constants there have the same meaning, except that ATP was the fixed substrate for Fig. 8.21)


2
1
0
$(r_1)\ p_1/q_2$
EU mM^{-1} mg^{-1}
x10^{-3}
$p_2/q_2(r_2)$
EU mg^{-1}
0
0.5
1.0
UTP mM

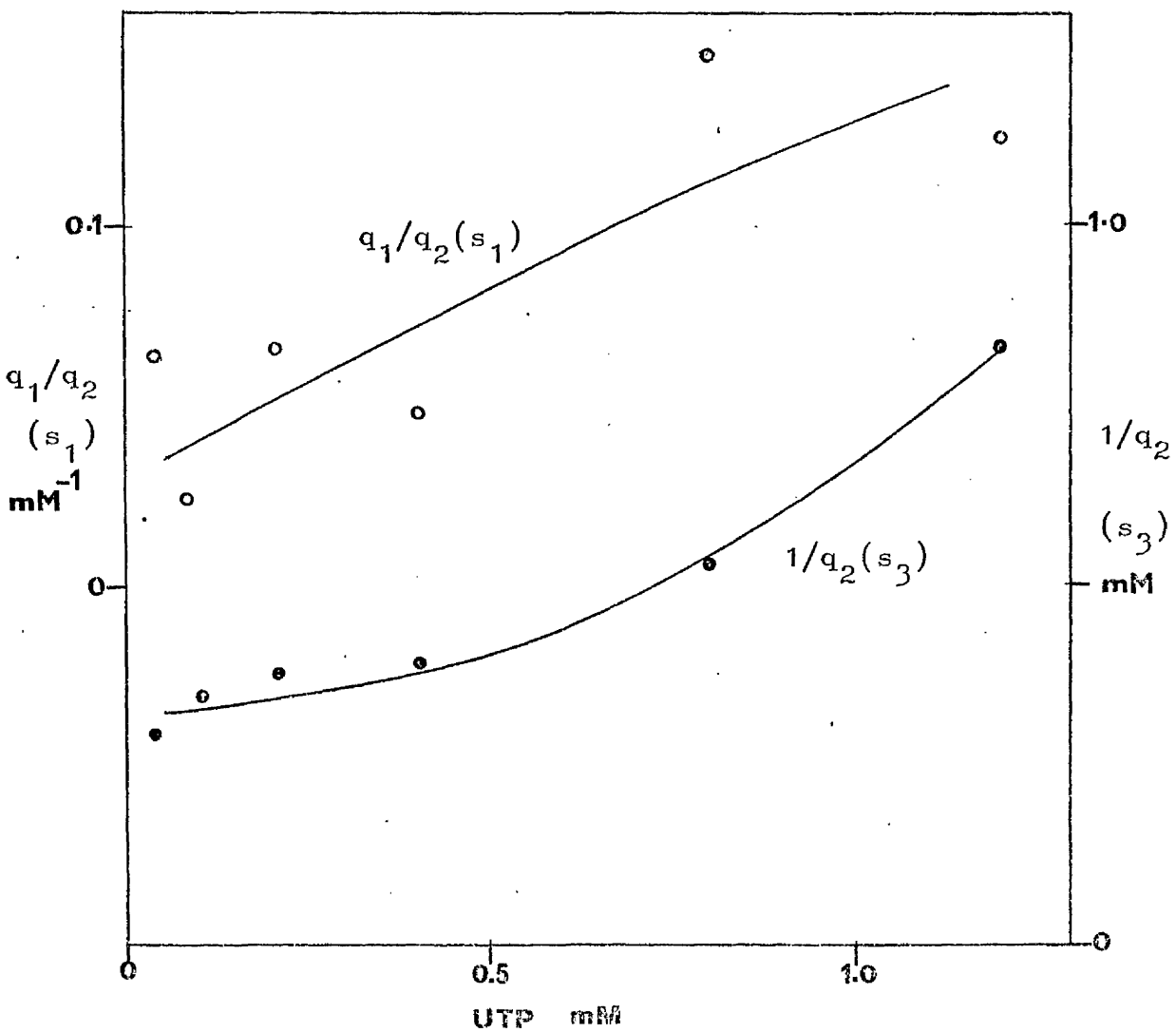

0·1
0
q_1/q_2
(s_1)
mM^{-1}
$q_1/q_2(s_1)$
1·0
$1/q_2$
(s_3)
mM
$1/q_2(s_3)$
0
0.5
1.0
UTP mM

Fig. 8.23 Stoichiometry of base residues in poly(A-U) synthesized by RNA polymerase (1)

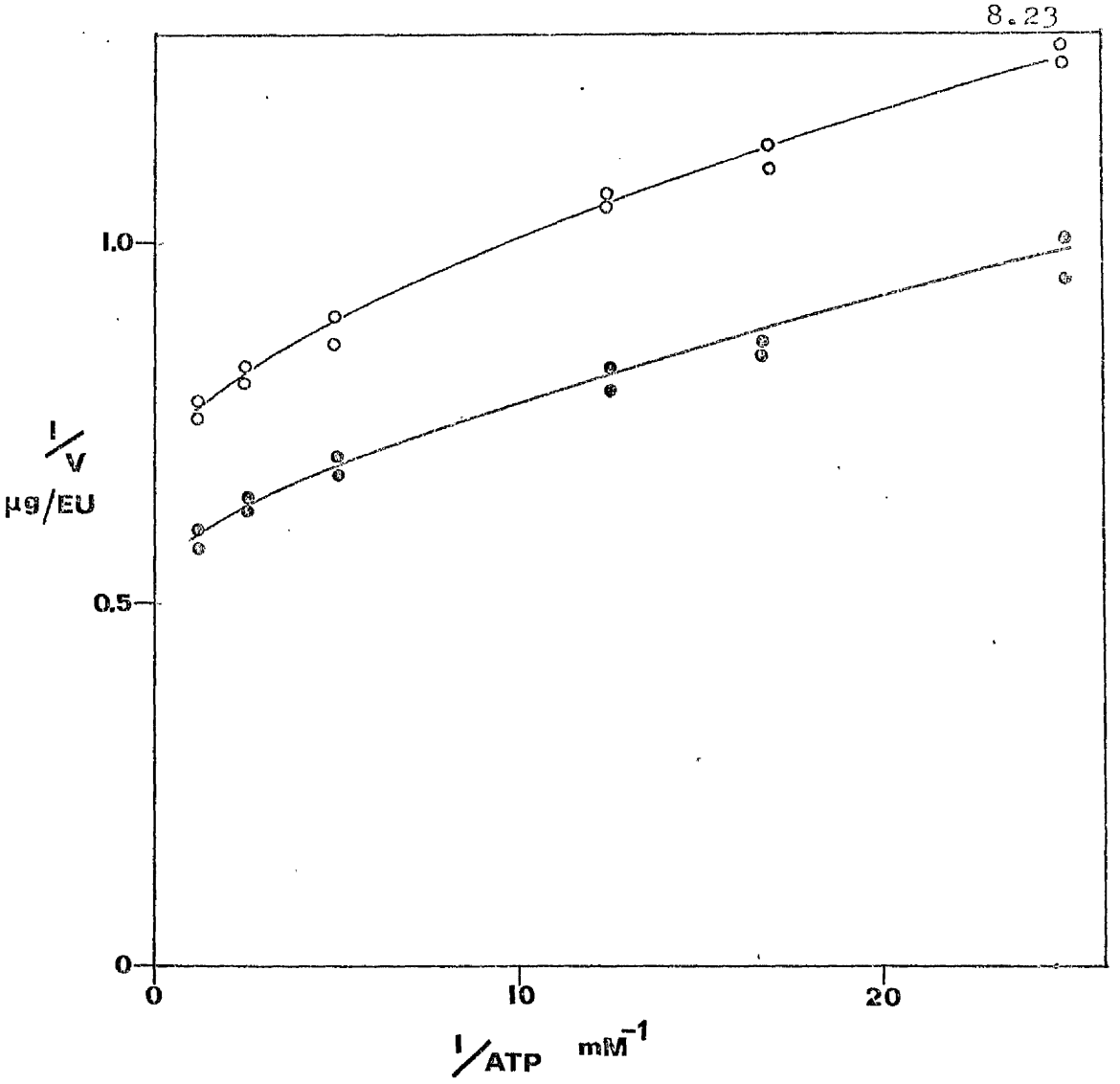
Assays for RNA polymerase activity were carried out by the standard radioactive method (Section 5C) with standard conditions (Section 5B) except that the substrate concentrations, ATP and UTP, were varied. Each reaction mixture contained 6.7 μg/ml E_c and 75 μM (with respect to monomer) poly(dA-dT). Two experiments were performed.

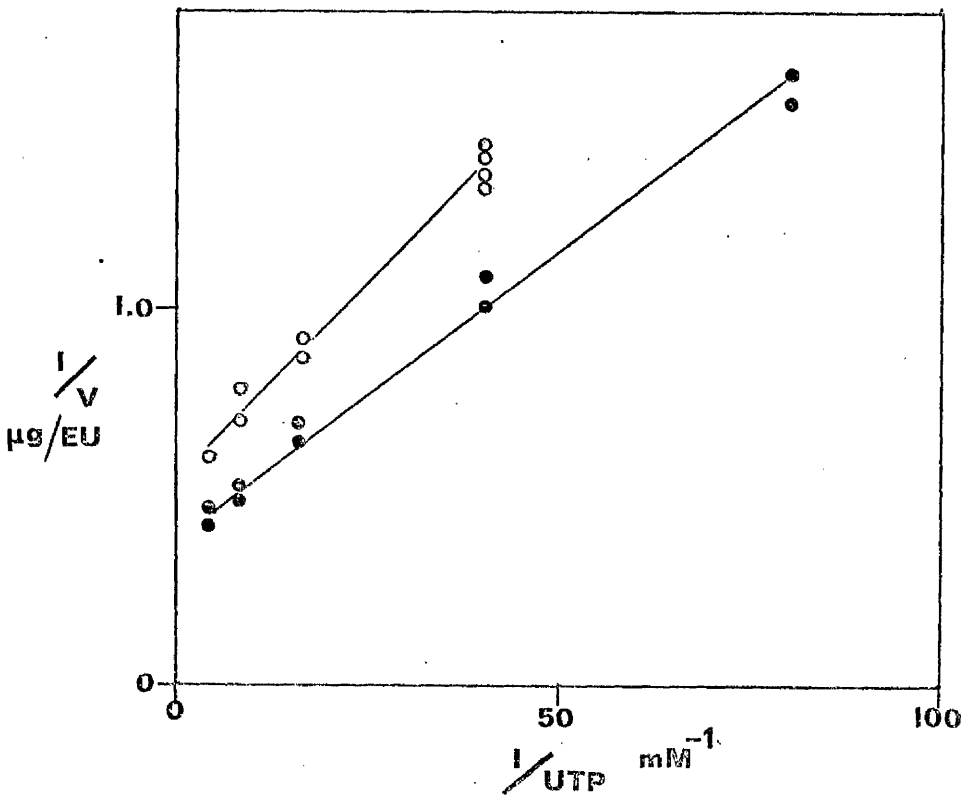
In one, UTP was held at 0.063 mM, whilst the concentration of ATP was varied. For each ATP level, the incorporation of both ^{3}H-ATP and ^{3}H-UTP was measured, and corrected for a zero template control containing the labelled substrate.

A similar experiment was carried out, except that ATP was fixed at 0.2 mM while UTP concentration was varied.

In the figures, the filled circles represent the reaction rate estimated by ^{3}H-ATP incorporation into polymer, and the open circles, the rate measured by ^{3}H-UTP incorporation.

8.23





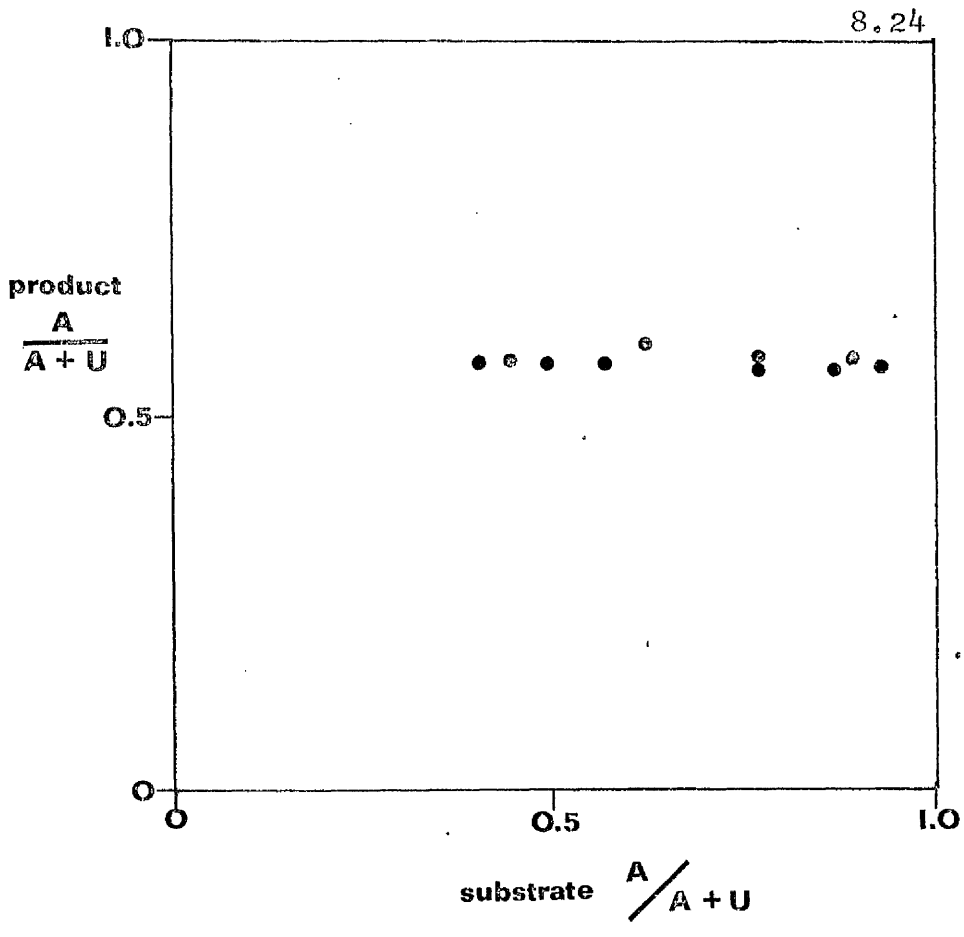


Fig. 8.24 Stoichiometry of base residues in poly(A-U) synthesized by RNA polymerase (2)

The data of Fig. 8.23 were expressed as the mole fraction of adenine residues in the poly(A-U) product at various values for the mole fraction of ATP in the substrate mixture (which was ATP and UTP). These two mole fractions were plotted against each other in the above diagram.

addition	concentration	specific activity (EU/mg)
-	-	211
tRNA	0.02 a	156
tRNA	0.1 a	108
PP_i	0.02 a	229
PP_i	0.1 a	233
A_2	5 b	196
A_2	25 b	189
A_3	5 b	236
A_3	25 b	255
A_4	5 b	222

Table 8.1 Effect of RNA, PP_i and oligoadenylates on RNA polymerase activity

The figure shows the effects of various compounds on the activity of RNA polymerase as measured by the radioactive assay method (Section 5C). The concentration units are : a) mM and b) µg/ml . Standard reaction conditions were used (Section 5B), with calf thymus DNA, 0.12 mg/ml and $E_{\sigma\theta}$, 60 µg/ml. A_n denotes an oligoadenylate of the form $A(pA)_{n-1}$. The assays were for 5 min at 37°C and the rates above are corrected for a control containing no DNA.

Table 8.2 Apparent K_m's for RNA polymerase with a T7 DNA or a calf thymus DNA template.

Template	Enzyme	K_m(PuTP) (mM)	S.E.M.	K_m(PyTP) (mM)	S.E.M.	Fig.
CT	$E_c(1)$	0.27	0.12	0.074	0.017	8.9
CT	$E_c(2)$	0.31	0.04	0.076	0.016	8.9
T7	$E_c(2)$	0.11	0.07	0.031	0.011	8.11
CT	$E_{\sigma\theta}(2)$	0.09	0.02	0.075	0.022	8.8*
CT	$E_{\sigma\theta}(2)$	0.17	0.08	0.083	0.019	8.8
CT	$E_{\sigma\theta}(1)$	0.11	0.02	0.052	0.012	8.10
T7	$E_{\sigma\theta}(1)$	0.36	0.10	0.11	0.014	8.11

T7 and CT refer to T7 DNA and calf thymus DNA respectively.

* - these results were from the experiment when the ratio of enzyme to DNA was higher than usual (●-● in Fig. 8.8)

The K_m's and their standard errors were calculated using the procedure of Wilkinson (Appendix 1). Data points obviously deviating from a hyperbolic model rate equation were not included in the statistical analysis.

Table 8.3 Least squares fits of velocity-substrate data (UTP changing fixed) to model rate equations.

model → / equation	1	2	3	4	5
UTP (mM) ↓	χ^2_ν				
1.2	0.751	10.739	26.671	1.756	0.715
0.8	0.675	11.018	12.593	0.727	0.565
0.4	0.487	4.152	6.896	1.192	1.150
0.2	0.510	3.423	3.913	1.251	1.448
0.08	0.472	1.081	1.282	1.132	0.535
0.04	0.695	0.460	0.609	0.552	0.925
*both	0.716	10.410	12.269	1.462	1.615

$$\text{eqn.} \quad : \quad v = \frac{p_1 a^2 + p_2 a + p_3}{q_1 a^2 + q_2 a + q_3}$$

The velocity-substrate data of Fig. 8.14 were fitted by the least squares method in Appendix 2 to various model rate equations of the above form. In all cases, q_3 was set equal to 1, and the other parameters constrained to be greater than zero. The χ^2_ν values for the different model equations are shown in the table. Different models were generated by setting different combinations of the constants equal to zero.

model 1 : $p_3 = 0$
model 2 : $p_3 = p_1 = q_1 = 0$, hyperbolic
model 3 : $p_3 = p_1 = 0$, simple substrate inhibition
model 4 : $p_1 = q_1 = 0$
model 5 : no further constraints.

* This relates to the data in Fig. 8.18 where ATP and UTP, at equal concentrations, were varied together.

Table 8.4 Least squares fits of velocity-substrate data (ATP changing fixed) to model rate equations

model equation	1	2	3
ATP (mM)	χ^2_ν		
0.8	1.017	1.798	11.632
0.4	2.279	2.743	4.227
0.2	0.606	1.311	1.148
0.08	0.464	0.948	0.410
0.04	0.463	0.586	0.373

general eqn. $$v = \frac{p_1u^2 + p_2u + p_3}{q_1u^2 + q_2u + q_3}$$

The velocity-substrate data of Fig. 8.16 were fitted by the least squares method in Appendix 2 to various model rate equations of the above form. In all cases, q_3 was set equal to 1 , and the other parameters constrained to be greater than zero. The values in the table are of χ^2_ν for different model equations, generated by setting different combinations of the parameters equal to zero.

model 1 : $p_3 = 0$
model 2 : $p_3 = p_1 = q_1 = 0$, hyperbolic
model 3 : $p_3 = p_1 = 0$, simple substrate inhibition.

Table 8.5a Analysis of 5'-terminal residues of poly(A-U) synthesized by a poly(dA-dT)-$E_{\sigma\theta}$ system

ATP (mM)	UTP (mM)	pppA (pmoles)	pppU (pmoles)
0.1*	0.2	7.3	-
0.1	0.2*	-	0
0.1*	0.4	7.8	-
0.1	0.4*	-	0

RNA polymerase assays were conducted as for the standard radioactive method (Section 5C) except that the substrate concentrations were as in the table, and that either γ^{32}P-ATP or γ^{32}P-UTP were the labelled substrates (indicated by * in the table). The specific activities were ^{32}P-ATP, 197 cpm/pmole and ^{32}P-UTP, 450 cpm/pmole. The other difference from the standard procedure was that the membrane filters, on which the labelled poly(A-U) was trapped, were washed with 50 ml of 5% (w/v) trichloracetic acid solution (1% (w/v) PP_i). Each reaction mixture contained 104 µg/ml $E_{\sigma\theta}$ and 75 µM (with respect to monomer) poly(dA-dT). The assays were for 5 min at 37°C, and controls, containing only the labelled substrate were also processed. The controls gave about 40% of the counts observed when both substrates were present, when ATP was labelled. With labelled UTP, there was no significant difference between the assays and the controls.

Table 8.5b Analysis of the 5'-terminal nucleotide residue of poly(A-U) synthesized by a poly(dA-dT)-E_c system

5'-terminal residues were estimated in this experiment by the method of Maitra et al. (1967). Standard RNA polymerase reaction conditions were used (Section 5C) except that the substrate concentrations were as in the table. Each reaction mixture (1ml in volume) contained 24 µg/ml E_c and 75 µM (with respect to monomer) poly(dA-dT). For determining the amounts of the 5'-terminal nucleotide, either γ^{32}P-ATP or γ^{32}P-UTP was also present (150 cpm/pmole and 290 cpm/pmole respectively). In some reaction mixtures, ^{3}H-ATP was present, to estimate the total poly(A-U) synthesized. Controls lacked poly(dA-dT) and the reaction lasted for 30 min at 37°C. The product poly(A-U) was collected by centrifugation as described by Maitra et al. (1967), except that the label was diluted with 0.2 ml of 60 mM-ATP or UTP, and that the final precipitate was dissolved in 5 ml of 0.2 M-NaOH, mixed with an equal volume of Unisolve and counted in a Nuclear Chicago liquid scintillation spectrometer. The asterisks denote which nucleotide had the ^{32}P label.

Table 8.5b Analysis of the 5'-terminal nucleotide residue of poly(A-U) synthesized by a poly(dA-dT)-E_c system

ATP (mM)	UTP (mM)	pppA (pmoles)	pppU (pmoles)
0.2*	0.2	6.9	-
0.2	0.2*	-	0

ATP* (mM)	UTP (mM)	pppA (pmoles)	poly(A-U) (nmoles)	chain length
0.04	0.08	0.8	20	25000
0.04	0.8	2.0	19	8500
0.2	0.2	6.9	35	5060
0.4	0.08	1.5	30	20000
0.4	0.8	11.1	41	3700

Tables 8.6 & 8.7 Nearest neighbour analysis and the determination of the base adjacent to the 5'-end of poly(A-U) synthesized by RNA polymerase

The results in both tables were obtained from the same experiment. The method followed that of Maitra et al. (1967) -see Section 5A6. Standard reaction conditions were used (Section 5C) except that the substrate concentrations were as in the tables and that either α^{32}P-ATP (98 cpm/pmole) or α^{32}P-UTP (66 cpm/pmole) was the labelled nucleotide. The reaction mixture (2ml volume) contained 24 µg/ml E_c and 75 µM (with respect to monomer) poly(dA-dT) and the reactions were for 15 min at 37°C.

After collecting the product poly(A-U) by centrifugation and hydrolysing with alkali, high voltage electrophoresis was used to separate pppAp, Ap and Up (Details as in Maitra et al. (1967) except that electrophoresis was at 3 kv for 1½ h). Analysis of the transfer of the α-P of the labelled substrate to Ap and Up gave the nearest neighbour analysis (Table 8.6). Analysis of the transfer of the α-P of the labelled substrate to pppAp gave estimates of the relative frequency of pppApA and pppApU (Table 8.7)

Table 8.6

ATP (mM)	UTP (mM)	dinucleotide frequencies (%)			
0.04	0.08	ApA, 0.2		UpA,	49.8
		ApU, 46.6		UpU,	3.4
0.4	0.8	ApA, 0.4		UpA,	49.6
		ApU, 46.8		UpU,	3.2

Table 8.7

ATP (mM)	UTP (mM)	^{32}P in pppA (pmoles)	pppApA (pmoles)	pppApU (pmoles)
0.04	0.08	23.0	4.9	18.1
0.4	0.8	42.6	2.4	40.1

SECTION 9

DISCUSSION

9A ENZYME DEFINITION

SDS-polyacrylamide gel electrophoresis of RNA polymerase preparations in a number of laboratories has clearly established α, β, β', and σ as subunits of the enzyme. Enzyme of composition $\alpha_2\beta\beta$ is capable of the basic catalytic function and is the simplest active form of the enzyme from E. coli. Much recent investigation of RNA polymerase has been directed at how the activity of this core enzyme is controlled. The most studied of the known controlling elements has been the initiation factor, σ, but there remain several points regarding σ that are unresolved. For instance, σ has a variable stoichiometry in RNA polymerase preparations. Other factors which modify the action of RNA polymerase core enzyme (CAP, ψ, ρ, M) are even less well-characterized. It is reasonable also to anticipate the discovery of more control elements in the future. It is clear, though, that the system responsible for the transcription process in vivo comprises RNA polymerase core enzyme and a number of functionally related control factors. The extent to which these factors are physically associated with RNA polymerase remains to be determined ; certainly, σ has an affinity for core enzyme.

Because the transcription machinery is, as yet, not totally characterized, the composition of RNA polymerase preparations should be viewed critically. The distinction between co-purifying contaminants and weakly associated components of the transcription system must be made carefully, a task made difficult when potential functions are unknown. The polypeptides ω and τ, for example, have been reported several times in

RNA polymerase preparations but it has not yet been possible to decide whether they are implicated in the synthesis of RNA. Interestingly, though, the aggregation of the enzyme appears to be affected by the presence of ω (Millette, R.L., cited in Burgess, 1971).

9A.1 THETA

The significance of the species θ, described in Section 6B should be assessed with the above comments in mind. Although θ has not been shown to have any effect on standard assays of RNA polymerase activity (Section 6B4), a clear implication in transcription may only await the performance of the appropriate experiments. From the glycerol gradient experiments described in Sections 6B1 and 6B2, it is evident that θ can be separated from enzyme containing α, β, β', and σ, at 1.0 M-KCl, and under these conditions, θ appeared to be present as a species of high mol.wt. (greater than that of RNA polymerase). Since the mol.wt. of θ in the presence of SDS was 58 000 (Lochhead, 1972), the aggregate of θ presumably contains more than about six monomers. In one glycerol gradient run, there was some evidence for the formation of a complex between θ and ββ' (Section 6B1). When RNA polymerase, at an earlier stage of purification, was chromatographed on DEAE-cellulose, the relative proportion of θ to β or β' in the fractions depended on the KCl concentration of the eluting buffer. Whether this was caused by a salt-dependent association of θ with RNA polymerase or an independent property of θ was not clear from this experiment.

These results provided suggestive evidence for the specific association of θ with RNA polymerase, but, considered alone, were not conclusive. Previous investigations of θ in this laboratory (Lochhead, 1972), however, gave other indications of a specific interaction of θ with RNA polymerase ;

1) Ultracentrifugation of enzyme containing about 15% (w/w) θ gave only one peak in Schlieren photographs during a sedimentation run.

2) DEAE-cellulose chromatography of purified RNA polymerase yielded two active RNA polymerase species, containing a) $\beta, \beta', \alpha, \sigma$, and θ; and b) β, β', α, and σ.

In summary, then, θ is a main component of RNA polymerase purified by the method described in Section 4B. The contention here is that θ is associated in some way with RNA polymerase, and that its properties are sensitive to KCl concentration.

9A2 THE PRESENCE OF THETA IN RNA POLYMERASE PREPARATIONS IN OTHER LABORATORIES

An obvious hypothesis regarding the origin of θ is that it results from the particular bacteria and methods used in this laboratory. Whilst this is in part true, examination of the literature shows that protein species of similar mol.wt. to θ are present in the RNA polymerase prepared in some other laboratories. Also, a commercial preparation of RNA polymerase, when analysed by SDS-polyacrylamide gel electrophoresis, contained θ (A.F.H.Anderson, unpublished result).

To standardize the results of various publications, gel electrophoretic patterns were analysed as described in Section 3F5, taking Lochhead's values for the mol.wt.'s of the RNA polymerase components β, β', α, and σ. In the list below, the values in brackets are mol.wt.'s so derived.

1) Krakow & von der Helm (1970) reported a polypeptide, ε, in preparations of the RNA polymerase from *A. vinelandii*, with a mol.wt. of about 65 000 (65 000).

2) Paetkau & Coy (1972) described a protein with ATPase activity in preparations of RNA polymerase from *E. coli* with mol.wt. of 68 000 (62 000). This protein resembled θ in that

it associated in a high salt glycerol gradient experiment to form an aggregate with a mol.wt. in excess of 800 000.

3) Examination of SDS-polyacrylamide gel electrophoresis patterns of Hirschbein et al. (1969) indicated the presence of a polypeptide of similar mobility to θ, (mol.wt. about 62 000)

4) Similar analysis of the results of Stonington & Pettijohn, (1971) also showed the presence of a polypeptide of about the same size as θ, (55 000).

5) Zillig et al. (1970a) reported the breakdown of σ to two polypeptide species, μ and ν, with mol.wt.'s of 60 000 and 15 000 respectively.

6) Burgess (1969b) described the breakdown of β and β' to polypeptides of mol.wt. from 60 000 to 130 000.

It seems probable that θ is identical to some, at least, of the polypeptides listed above.

9A3 DEPENDENCE OF THE COMPOSITION OF RNA POLYMERASE PREPARATIONS ON THE PURIFICATION PROCEDURE

The reason for the absence of θ in many RNA polymerase preparations is, in some cases, explicable from the results of Section 6B. From these experiments, it is obvious that any RNA polymerase purification using high salt glycerol gradient centrifugation will produce an enzyme containing very little θ. Also, the θ content of RNA polymerase was sensitive to the ionic conditions used at the stage of DEAE- cellulose chromatography (Section 6B3).

The θ content, then, of RNA polymerase samples is dependent on the method of their isolation. A similar effect has, of course, been encountered in the case of phosphocellulose chromatography which, if included as a preparative step, yields enzyme lacking σ (and θ). An analogous situation may obtain for other components of the RNA synthesizing system. In general,

the preparative procedure may be an important determinant of the exact composition of this complex and, as yet, ill-defined enzyme system. Polyacrylamide gel electrophoresis appears to be a promising means of determining the precise composition of the fractions containing RNA polymerase (or any other enzyme) through at least the latter stages of purification. With RNA polymerase, in fact, this may be possible with very impure samples of enzyme, since β and β' are thought to be two of the largest polypeptides present in E. coli and are hence readily identifiable.

9A4 OTHER COMPONENTS OF RNA POLYMERASE PREPARATIONS

In the course of several enzyme purifications, a number of trace components have been detected and these are compiled in Table 6.2. Of these, one (x1) appears to be the polypeptide T. None of these polypeptides have been ascribed specific functions and their appearance was variable. In fact, whether or not they were unrelated impurities is not clear at this time. As with σ and θ, the preparative procedure influenced the final enzyme composition with respect to these minor components. A prominent band (x7) of mol.wt. 52 000, for instance, was found only when the DEAE-cellulose chromatography was carried out using a KCl gradient to elute the enzyme.

The observation of polynucleotide phosphorylase activity in RNA polymerase preparations (Section 6C2) prompted an attempt to correlate this activity with one or more of the trace polypeptides. This proved difficult as the commercially available sample of polynucleotide phosphorylase from E. coli itself gave a complex SDS-polyacrylamide gel electrophoresis pattern. However, both RNA polymerase and polynucleotide phosphorylase appeared to have some polypeptide species in common, as judged by electrophoretic behaviour. This implies that certain

polypeptides co-purify with both RNA polymerase and polynucleotide phosphorylase activities and this possibility is in itself of some interest. The role of polynucleotide phosphorylase in metabolism is obscure and it is not unreasonable to suggest that it might have some functional and/or physical relationship with RNA polymerase in the cell. This suggestion is speculative and is evoked mainly by the conceptual connection between two of the few known enzymes in the cell involved in RNA metabolism.

9B EFFECT OF IONIC CONDITIONS ON RNA POLYMERASE ACTIVITY

Ionic conditions can affect RNA synthesis by RNA polymerase in a number of ways. The properties of the enzyme, substrates, products and template are all altered by the ionic environment, and the dependence of dynamic aspects of RNA synthesis on ionic conditions presumably arises from a combination of these individual effects. The experimental investigation of ion effects, described in Section 7C, was aimed mainly at characterizing the behaviour of the particular enzyme systems used. In the course of this work, several features of the response of RNA polymerase to ionic conditions were observed and these agreed largely with previous findings (Fuchs *et al.*, 1967 ; So *et al.*, 1967).

The two enzyme-template systems studied were RNA polymerase $E_{\sigma\theta}$ with T7 DNA, and core enzyme E_c with poly(dA-dT). These differed slightly in their response to the variation of KCl concentration, core enzyme with poly(dA-dT) having a broader peak of enzyme activity. Also, the two combinations of enzyme and template differed as to optimal $MgCl_2$ concentrations. The explanation of these findings is not easy, although they are not unexpected since the two systems differed greatly, especially as regards the specificity of the transcription.

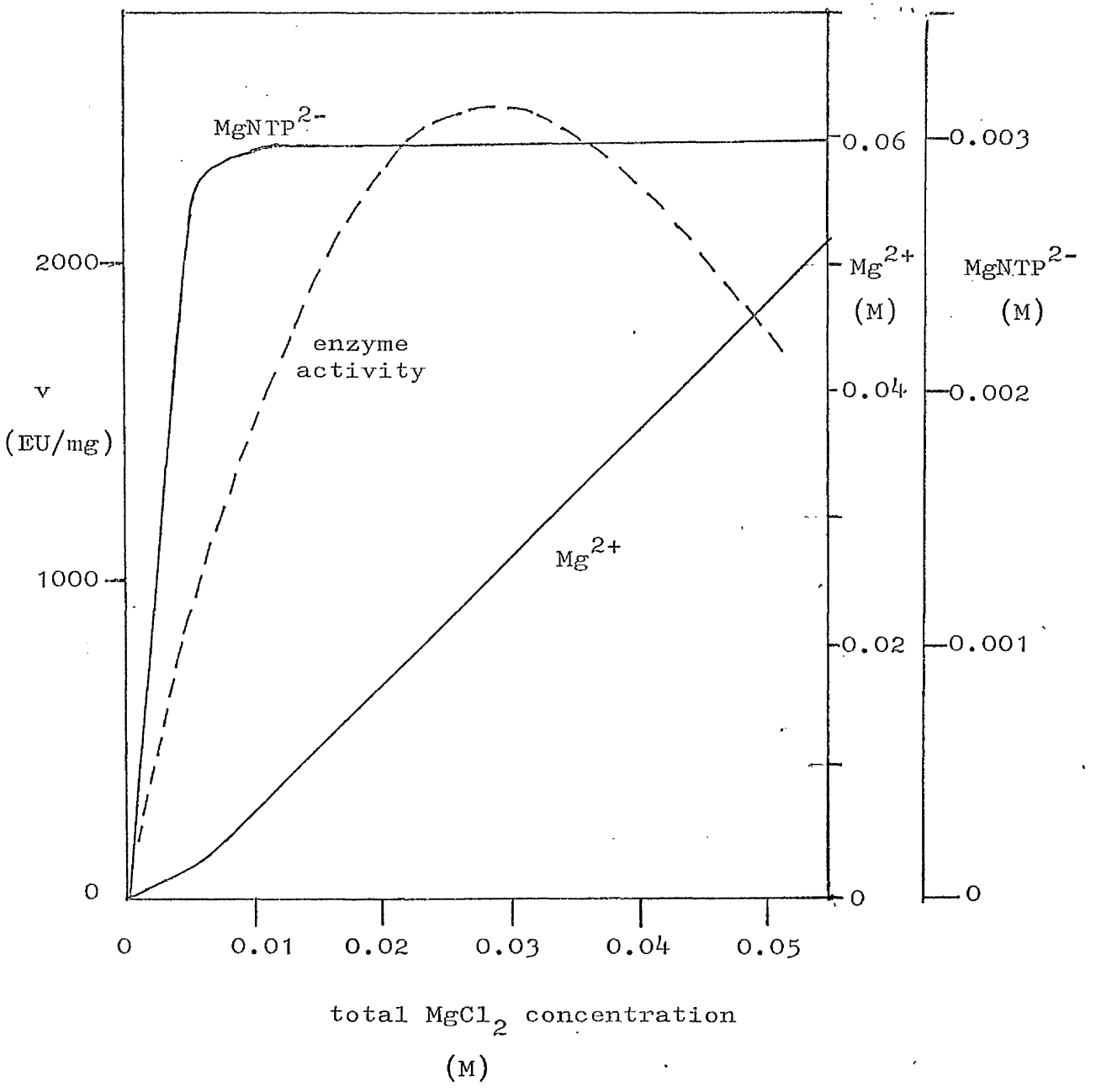


Fig. 9.1 Computed free Mg^{2+} and $MgNTP^{2-}$ concentrations in RNA polymerase assays

The experimental curve (broken line) for the variation of RNA polymerase activity with $MgCl_2$ concentration is taken from Fig. 7.6. Assuming the formation constants of $ATPMg^{2-}$ and $UTPMg^{2-}$ to be equal (= 40 000 $molar^{-1}$) and similarly for the K^+ complexes (formation constant 10 $molar^{-1}$), the Mg^{2+} and $NTPMg^{2-}$ concentrations were computed for the experimental conditions of Fig. 7.6 (Appendix 4), assuming no other influences on the equilibria involved.

The role of Mg^{2+} is not clear at this time. The requirement of Mg^{2+} for RNA polymerase activity is absolute, although the $MgCl_2$ concentration will, of course, contribute to the total ionic strength of the reaction mixture. In addition, Mg^{2+} can form complexes with nucleoside triphosphates and if the two-site model of the enzyme is correct (Section 1C4) also influences the binding of the substrates to RNA polymerase. High $MgCl_2$ concentration causes a lag in the onset of RNA synthesis (Section 7C2.2 ; Fuchs et al., 1967) and this, as well as similar lags induced by high ionic strength, have been related to local melting of the DNA template (Fuchs et al., 1967 ; Hinkle & Chamberlin, 1970).

The role of the complexes formed between Mg^{2+} and NTP^{4-} has not been extensively discussed. Taking literature values for the formation constants of $NTPMg^{2-}$ and $NTPK^{3-}$, the distribution of Mg^{2+} between $NTPMg^{2+}$ and free Mg^{2+} is shown superimposed on the results displayed in Fig. 7. (Fig. 9.1). The calculation is described in Appendix 4. An essentially constant value of $NTPMg^{2-}$ is attained long before the total Mg^{2+} concentration corresponding to maximal enzyme activity is reached. The free Mg^{2+} concentration varies almost in proportion to total Mg^{2+}. It is clear, then, that although $NTPMg^{2+}$ may indeed be the substrate for RNA polymerase, Mg^{2+} affects the reaction in other ways.

The influence of other divalent cations on RNA polymerase activity was not investigated in any detail. Mn^{2+} was able to substitute for Mg^{2+} with either $E_{\sigma\theta}$ or E_c with either a T7 DNA or a calf thymus DNA template (Section 6B3). Zn^{2+} was inhibitory irrespective of the presence of σ or θ. Scrutton et al. (1971) found that RNA polymerase contained about 2 atoms of Zn per RNA polymerase molecule. Presumably, then, RNA

polymerase samples used in the present study already contained a full complement of Zn, and the presence of further Zn in the system had only an inhibitory effect on the reaction.

9C TIME COURSE OF RNA POLYMERASE CATALYZED REACTION

The results of Section 8A illustrated the inhibition of RNA polymerase by the products of the reaction, and showed also that PP_i was much less effective than RNA in this respect. RNA inhibition of RNA polymerase has been described previously (Richardson, 1966b ; So et al., 1967). Evidence for the specific inhibition by RNA, rather than PP_i, has also come from experiments where the reaction was followed by pyrophosphate liberation (Maitra & Hurwitz, 1967). In the presence of RNase, the release of pyrophosphate continued long after an analogous reaction lacking RNase would have ceased. So et al. (1967) and Fuchs et al. (1967) also found that elevated ionic strength could alleviate the inhibition by RNA. High $MgCl_2$ concentration was found to have a similar effect in this work (Section 7C2.2).

A simple explanation of the inhibition by RNA would be that the reaction was approaching equilibrium of the products with respect to the substrates, and that the progressive retardation of RNA synthesis was caused by an increased rate for the back reaction. If the value of ΔG^o, of about 2 kcal./mol, for the formation of a 3'-5' phosphodiester linkage (Section 1C1) is accepted, then for an initial NTP concentration of 6 mM, equilibrium would represent 97% polymerization. Even allowing for the inaccuracy of the estimate of ΔG^o, the extent of the reactions described in Section 8A did not approach this value. This explanation therefore seems unlikely.

When the inhibition data was treated as in Section 8A, the pattern was complex, giving linear double reciprocal plots of 1/v against 1/NTP only at high substrate concentrations, where the inhibition was non-competitive. Also, with increasing RNA concentration, the velocity-substrate curves became more and more sigmoid in character.

In general, numerous interactions between the growing RNA chains, the RNA polymerase molecules and the DNA template are conceivable. Also, interactions of the type RNA-protein, RNA-DNA or DNA-protein are likely to be influenced by ionic conditions. In terms of molecular events, the inhibition by RNA is evidently complex and the results presented here and elsewhere are probably insufficient to explain the phenomenon in detail. However, if, as postulated by Anthony et al. (1969), sigmoid velocity-substrate curves result when initiation is rate limiting, the findings quoted above (Section 8A) suggest that RNA has the effect of inhibiting the initiation of new RNA chains. This assumes that initiation occurred throughout the course of the reaction, as has been reported (Maitra & Hurwitz, 1965 ; Millette,R.L. cited in Fuchs et al.,1967). It is of interest that the results of Section 8A also imply that the extent to which velocity-substrate curves are sigmoid will depend on the level of RNA contamination in the enzyme sample used. Other explanations for inhibition by RNA are of course possible. For example, the dissociation of RNA polymerase-RNA-DNA complexes may depend on RNA chain length, and also ionic conditions. In conclusion, much is still to be learned concerning the time course of the RNA polymerase reaction.

9D1 COMPARISON OF RESULTS WITH THOSE OF OTHER INVESTIGATIONS

A number of apparent K_m's were found for RNA polymerase under various experimental conditions. Quantitatively, these fell within the range of values quoted in the literature (Table 1.2), although the apparent K_m's obtained in the present study were mostly at the upper end of this range. As stated previously, comparison of the values obtained in different laboratories is difficult because of the many sources of variation in the experimental conditions.

9D2 COMPARISON OF KINETICS WITH PuTP AND PyTP VARIABLE

Most of the experiments in Section 8B were performed on the premise that purine nucleotides might influence the reaction rate differently from pyrimidine nucleotides, as had been originally proposed by Anthony et al. (1969). This was in fact found to be so. Firstly, the apparent K_m(PuTP) was consistently greater, by a factor of 2-4 than the corresponding K_m(PyTP). Secondly, although the reaction rate varied hyperbolically with PyTP, in several experiments, variation of PuTP gave a sigmoid velocity-substrate curve. These results agreed qualitatively with those of Anthony et al. (1969) who interpreted their findings on the basis that the first nucleotide to be incorporated into the RNA chain bound, with a high K_m, to an initiation site on the enzyme. Subsequent substrates bound at an elongation site, which exhibited a lower apparent K_m. As evidence of this special effect of initiation on the kinetic behaviour of the enzyme, they found :

1) A positive correlation between a substrate exhibiting non-hyperbolic kinetics, with a relatively high K_m, and the 5'-terminal residue of the RNA product.

2) Pre-initiation of RNA synthesis elliminated non-hyperbolic behaviour and reduced the apparent K_m.

Further indications that the apparent K_m with respect to a variation in PuTP was most sensitive to the initiation phase of the reaction was provided by the results of this investigation. It was found (Section 8B) that, in several experiments, K_m(PuTP) was more subject to fluctuation than K_m(PyTP). In these experiments, combinations of enzyme and template were used that undoubtedly differed widely in the nature of the initiation process (T7 DNA with and without σ : calf thymus DNA with and without σ). With calf thymus DNA, different enzyme to template ratios were also used. Thus, it seems reasonable to propose that this variability in K_m(PuTP) reflected the difference in the initiation process. Once initiated, however, RNA synthesis will be insensitive to the prevailing initiation conditions and the elongation steps will be less dependent on the enzyme and template used. This could explain the relative invariance of K_m(PyTP), although the particularly low apparent K_m(PyTP) for core enzyme with T7 DNA is not accommodated by this hypothesis.

In summary, the results reported here are consistent with the findings of Anthony et al.(1969) and supply some further evidence for the proposal that the different kinetic behaviour with respect to PuTP as compared with PyTP arises from a specific involvement of PuTP in initiation. The apparent K_m(PuTP) and K_m(PyTP) must be complex parameters, composed of many individual rate constants for the steps in RNA synthesis, and to relate them to the affinities of enzyme sites for substrates is a gross simplification, as recognized by Anthony et al. (1969). Whilst it is reasonable to infer that initiation had a greater effect on K_m(PuTP) than K_m(PyTP), the explanation of this phenomenon in terms of a more detailed kinetic model must be approached with caution.

 EFFECT OF SIGMA ON RNA POLYMERASE KINETICS

σ has commanded considerable attention in recent years in relation to its role in enhancing the initiation of RNA synthesis at specific sites on a DNA template. The experiments described in Section 8B3 analyse the influence of σ by the coarser measure of its effect on the total rate of RNA synthesis. With either T7 DNA or calf thymus DNA, the presence of σ was shown to have a marked effect on K_m(PuTP) and K_m(PyTP) as well as the rate of the reaction. In a general kinetic sense, σ could be described as an activator of core enzyme. The pattern of the activation differs between these DNA's, as might be expected for two DNA's from such different sources. Some general comments about the action of σ can be made on the grounds of these experiments.

1) In its capacity as an activator of core enzyme, σ modified both apparent K_m's and V_{max}'s, in a possibly complex way, but did not alter the difference in the kinetics with respect to PuTP as compared with PyTP. RNA polymerase binds to a DNA template to form a number of enzyme-template complexes whose properties will depend on the DNA sites involved. In the presence of σ, according to current views, enzyme-template complexes are more stable at certain locations on the DNA. In kinetic experiments, the enzyme behaviour will reflect which set of such complexes has been established, which in turn will be dependent on the presence and concentration of σ. It appears, then, that all enzyme-template complexes retain the property of responding differently to variation of PuTP compared with PyTP in kinetic experiments.

2) As judged by measurements of total RNA synthesis, σ acts as an activator of core enzyme, irrespective of any action it may have in causing specific transcription. Indeed, although σ is certainly necessary, it may not be sufficient, for

specific initiation on an E. coli template, as is exemplified by the requirement of further positive control factors (CAP and perhaps ψ) for the transcription of certain genes. σ might represent an 'enable' signal for RNA synthesis but the ultimate 'go' command may require the participation of additional control elements. In this way, the concentration of σ in vivo could regulate what proportion of cellular resources are channelled into RNA synthesis, and this might be as important as its role as a determinant of specificity. In fact, much of the experimental investigation of the action of σ has been with phage DNA's and the extrapolation to σ's role in the bacterium must be made with caution ; phages have evolved so as best to utilise available resources and not to provide illuminating examples of the action of those bacterial systems that they take over.

3) From a practical point of view, it is clear from the results in Section 8B3 that the kinetic properties of any RNA polymerase sample will be strongly dependent on the σ content. This may partly explain the variation in the observed K_m's for the enzyme (Table 1.2), as was pointed out by Burgess (1971). In few cases has the σ content of the RNA polymerase used in experiments been reported.

9D4 SIMPLE KINETIC ANALYSIS OF THE RNA POLYMERASE CATALYZED REACTION

RNA polymerase was found to respond differently to the variation of PuTP and PyTP concentrations and this difference in behaviour was related, in a non-specific way, to a special involvement of PuTP in the initiation reaction. A simple model would be

1) $E \xrightarrow{k_i} E(i)$ initiation

2) $E(i) \xrightarrow{k_e} E(i+1)$ elongation

E(i) represents an 'initiated' RNA polymerase molecule (and also the concentration of this species). k_i is a function of PuTP concentration, and possibly σ, while k_e is a function of all four substrate concentrations. The rate of initiation is

$$\frac{d(E(i))}{dt} = k_i E \qquad \text{eqn. 9.1}$$

where E is the concentration of un-initiated enzyme. The rate of RNA synthesis is then

$$\frac{dr}{dt} = k_e E(i) \qquad \text{eqn. 9.2}$$

where r is the concentration of RNA. Suppose, as an example, that initiation occurs at a constant rate, c, then integrating eqn. 9.1 gives

$$E(i) = ct$$

Substituting for E(i) in eqn. 9.2 gives

$$\frac{dr}{dt} = k_e ct$$

which integrates to

$$r = \tfrac{1}{2} k_e c t^2 \qquad \text{eqn. 9.3}$$

if k_e is time independent. A linear increase of E(i) with time then would imply that the rate of RNA synthesis should increase with time. Such behaviour has been observed, at high salt concentration, low substrate concentration or low temperature. However, under many experimental conditions, RNA synthesis is linear with respect to time (or, in fact, slows down) so that on the time scale of most experiments either

1) E(i) (the number of active polymerizing centres) does not change with time

or

2) as E(i) increases, k_e decreases and maintains dr/dt constant.

If the former is true, initiation either reaches a constant level (no more E(i) is formed) or else E(i) is in a steady state (polymerizing centres are deactivated as fast as they are

formed).

A simple model of the involvement of PuTP in initiation would be to consider that any given concentration of PuTP would sustain a certain fixed level of E(i), so that

$$E(i) = f(Pu) \qquad \text{eqn. 9.4}$$

Pu and Py will denote the concentrations of PuTP and PyTP respectively. A steady state rate equation for the elongation of a single RNA chain may derived following the formulations of Hyman & Davidson (1971). If the reaction is considered to have only two substrates, PuTP and PyTP, the following equation is obtained

$$v \quad (= k_e) = \frac{K_1 Pu.Py}{K_2 Pu + K_3 Py + K_4 Py.Pu} \qquad \text{eqn. 9.5}$$

The assumption is made that the RNA product contains equimolar amounts of PuTP and PyTP residues. The constants are composed of the K_m's for the formation of enzyme-substrate complexes with both substrates (K_u and K_y), and the rate constants for the breakdown of these complexes (k_u and k_y). The subscripts, u and y, refer to PuTP and PyTP respectively.

$$K_1 = k_u k_y \; ; \; K_2 = k_u K_y \; ; \; K_3 = k_y K_u \; ; \; K_4 = k_u + k_y$$

Eqn.'s 9.4 and 9.5 can be substituted in eqn. 9.2 to give

$$\frac{dr}{dt} = \frac{K_1 Py.Pu.f(Pu)}{K_2 Pu + K_3 Py + K_4 Py.Pu} \qquad \text{eqn. 9.6}$$

Whatever the form of f(Pu), this rate equation is obviously a more complex function of PuTP than PyTP, which has been found experimentally (Sections 1C7 and 8B).

As a specific example, E(i) might be considered to result from the equilibrium binding of PuTP to the enzyme. If the dissociation constant for this binding is K, then

$$E(i) = \frac{E'Pu}{K + Pu} \qquad \text{eqn. 9.7}$$

E' is the total enzyme concentration.

Using this expression for E(i) instead of eqn. 9.4, the rate equation becomes

$$\frac{dr}{dt} = \frac{E^{t}.L_1.Pu^2.Py}{L_2Pu + L_3Py + L_4Py.Pu + L_5Py.Pu^2 + L_6Pu^2}$$

where L_1, L_2, L_3, L_4, L_5, and L_6 are constants. eqn. 9.8

This equation is of degree 2 in PuTP but degree 1 in PyTP, and so is of the correct form to describe the results of Section 8B. However, this sort of analysis should not be taken too far, as it involves a number of assumptions. This specific model would in fact describe a system where a necessary activator must bind to an enzyme in order that the reaction occurs - in this example, the activator is also a substrate. This model is in some ways appropriate to RNA synthesis, since activated (in this case, initiated) enzyme can then catalyze the formation of many covalent bonds (elongation). The simple treatment above is of interest in that it illustrates how a fairly uncomplicated model of the specific involvement of PuTP in initiation can generate a rate equation which is a more complex function of PuTP concentration than PyTP concentration.

9E KINETICS OF RNA POLYMERASE CORE ENZYME WITH POLY(dA-dT)

9E1 GENERAL COMMENTS

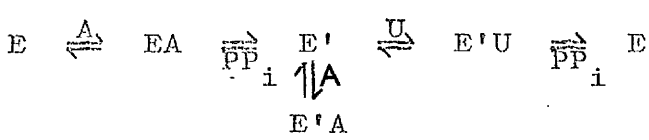
In the work described here, the kinetic behaviour of RNA polymerase core enzyme with a poly(dA-dT) template differed from that directed by T7 DNA or calf thymus DNA, notably in that sigmoid velocity-substrate curves were never observed with poly(dA-dT). In common with calf thymus DNA or T7 DNA, however, the kinetics deviated most from a hyperbolic rate law when a purine nucleotide was the variable substrate. This deviation from hyperbolic behaviour took the form of double reciprocal plots that were concave down (that is, concave to the 1/s axis). Neither Anthony *et al* (1969) nor Downey & So (1970) found such behaviour with a poly(dA-dT) template. This may be due to the higher substrate ranges used in this investigation, and other differences in reaction conditions such as ionic strength or the presence of Mn^{2+}.

9E2 INTERPRETATION OF KINETICS WITH POLY(dA-dT)

The kinetic experiments described in Section 8C2 indicated that the kinetics of core enzyme with a poly(dA-dT) template could be divided into zones of hyperbolic and non-hyperbolic behaviour, depending on substrate concentrations. Where hyperbolic behaviour was observed, the results conformed to a rate equation of the form

$$v = \frac{K_1 au}{K_2 a + K_3 u + au + K_{ia} a^2} \qquad \text{eqn. 9.9}$$

This is the equation for a ping-pong mechanism in a two substrate enzymic reaction, with competitive substrate inhibition by ATP. It would result from the following reaction scheme :



Details of the steady state analysis of this model are given in Appendix 3. This scheme includes no special initiation step and in effect describes the addition, by a ping-pong mechanism, of a pApU unit to a poly(A-U) chain. In other words, the results suggested that the synthesis of poly(A-U) was kinetically indistinguishable from the synthesis of pApU where eqn. 9.9 was applicable. Certainly, the initiation of polyribonucleotide synthesis on a poly(dA-dT) template is different than on a natural template ; firstly, there must be less dependence on template base sequence and secondly, stable enzyme-template complexes are readily formed with poly(dA-dT) (Hinkle & Chamberlin, 1970 ; Chamberlin, 1970). The applicability of the above reaction scheme may indicate that initiation of poly(A-U) synthesis differs little from the elongation steps, within the appropriate range of substrate concentrations.

That the above scheme is for a ping-pong mechanism is reasonable, since models for RNA synthesis usually imply consecutive substrate binding and pyrophosphate release (1C4). Further, the exchange of pyrophosphate into nucleoside triphosphates (Krakow & Fronk, 1969 ; So & Downey, 1970) supports a ping-pong mechanism.

A simple two substrate ping-pong reaction model appears to be applicable to poly(A-U) synthesis by core enzyme, within certain substrate ranges. From a practical point of view, it is evident that the apparent K_m and V_{max} for this system will depend on the concentration of the fixed substrate.

9E3 REGION OF NON-HYPERBOLIC KINETICS

At higher substrate concentrations, the simple hyperbolic rate equation was less successful in describing the experimental

data. One feature of the kinetic behaviour described in Section 8C2 was the presence of inhibition by substrates. ATP was shown to be inhibitory at lower concentrations than UTP (Section 8C2.2), but inhibition by both ATP and UTP is implied, at higher concentrations, by the analysis of the product chain length (Section 8C2.4). Thus, in the region of non-hyperbolic kinetics, substrate inhibition of the reaction will be operative. If this inhibition is due to to the formation of a 'dead-end' complex of enzyme and substrate, then a steady state rate equation should have a quadratic term in the inhibitory substrate in the denominator (as, in fact, in eqn. 9.9). This alone cannot explain the deviations from hyperbolic kinetics, although it does describe the result of varying UTP at low ATP levels (Fig. 8.17). To account for the concave down double reciprocal plots, particularly when ATP was variable, it was also necessary to introduce a quadratic term in the variable substrate in the numerator of the rate equation. If such a rate equation has physical significance, then possible explanations of its form may be sought.

1) Substrate effects on enzyme-enzyme and enzyme-template associations : RNA polymerase is known to form aggregates of the monomer form (Section 1A2.3 ; Berg & Chamberlin, 1970) and it is possible that the concentrations of ATP and UTP might alter the equilibrium position of this aggregation. This could in turn influence the concentration of the active enzyme species and hence the reaction rate. Likewise, nucleoside triphosphate concentrations could also affect the binding of the enzyme to the template, although enzyme-template complexes are not thought to be stabilized by nucleotides in the absence of RNA synthesis (Chamberlin, 1970). Krakow & von der Helm (1970) do propose, however, that ATP stabilizes E_c -poly(dA-dT) complexes for the enzyme from A. vinelandii. These possibilities

cannot be discounted as explanations of non-hyperbolic kinetics on the grounds of the work described here.

2) Negative cooperativity : Rate laws of the form of model 1 in Section 8C2.1 can describe the behaviour of enzymes with allosteric properties, and concave down double reciprocal plots can arise if the enzyme exhibits negative cooperativity (for example, Koshland, 1970). Certainly, there is evidence for more than one substrate binding site on RNA polymerase (Wu & Goldthwait, 1969,a & b ; Ishihama & Hurwitz, 1969) but whether these sites interact is not known at this time. Anthony et al. (1969) concluded that sigmoid velocity-substrate curves obtained when purine nucleotides were varied were not due to an allosteric effect, but the possibility of interacting sites cannot be dismissed at present.

3) Alternative reaction pathways : Where a reaction mechanism involves alternative pathways, non-hyperbolic kinetics can be predicted (Wong & Hanes, 1962 ; Pettersson, 1969 ; Cleland, 1970). Substrate inhibition has been observed for RNA polymerase in this work and may have reflected the friutless binding of a nucleoside triphosphate non-complementary to the template base in register with the elongation site of the enzyme. Indeed, the elongation site proposed by Goldthwait et al. (1970) is thought capable of binding all four nucleoside triphosphates. It seemed possible, then, that misreadings of the template could occur, and with poly(dA-dT), this might give rise to alternative reaction pathways. Some experiments were carried out to test this possibility.

The stoichiometry of the poly(A-U) synthesized by RNA polymerase, measured by comparing UTP and ATP incorporation, was independent of substrate concentrations (relative and absolute). Investigation of the poly(A-U) sequence by nearest

neighbour analysis, however, showed a small but significant departure from a strict alternating sequence ; there was a 3% frequency of UpU. It is possible that this deviation was due to RNA polymerase action and this would mean misreading of the template by the enzyme. In enzyme kinetics such a model includes alternative reaction pathways.

Since each initiated enzyme molecule proceeds to catalyze the incorporation of many nucleotides, any effects on the rate or extent of initiation will have an amplified influence on the rate of RNA synthesis. Consequently, alternative reaction pathways were considered at the initiation stage of the reaction by analysing the first two nucleotides at the 5'-terminal end of poly(A-U) products. The sequence pppApU was predominant, but a significant proportion of pppApA was also found. As with nearest neighbour analysis, it was not possible to decide whether this deviation from a strictly alternating sequence was caused by the template or the enzyme. However, at the stage of initiation, either possibility represents an alternative pathway for RNA synthesis. During elongation, the occurrence of the wrong base in the template does not represent a choice of pathways for the polymerization.

The occurrence of pppApA can be explained on the two site model of RNA polymerase action (Section 1C4) in several ways (Fig. 9.2). Diagram 1 in Fig. 9.2 shows faithful initiation at an alterning A-T site on the template.

In diagram 2, the enzyme binds to poly(dA-dT) so that the initiation site of the enzyme is in register with an 'A' residue of the template but the specificity of the initiation site for ATP overrides the template signal. The elongation site on the other hand obeys the template direction to bind ATP and the 3'-5' phosphodiester linkage is formed between two ATP molecules.

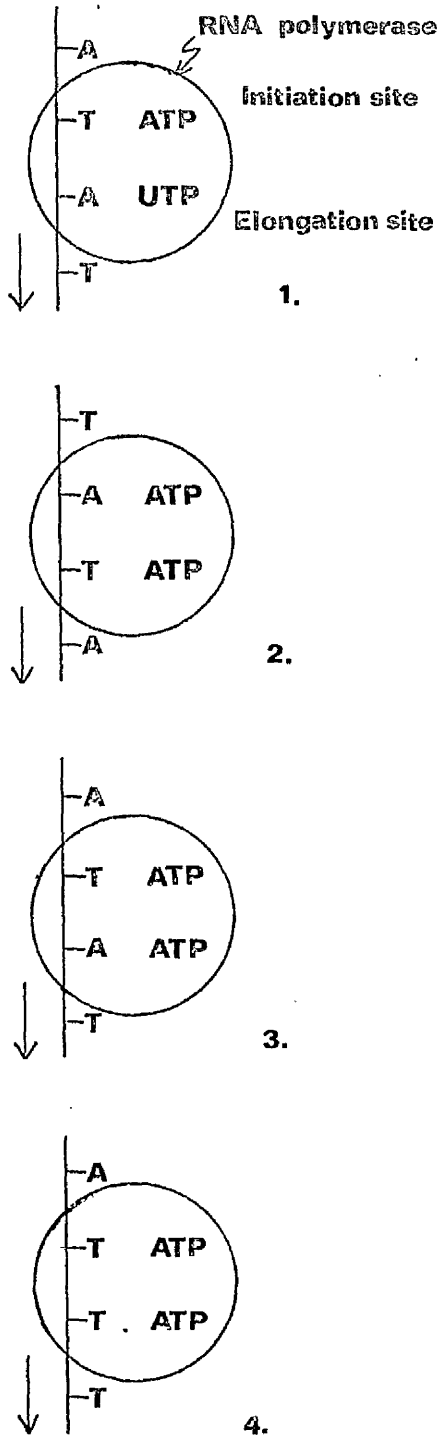


Fig. 9.2 Initiation of poly (A−U) synthesis

In diagram 3, the initiation site is adjacent to a 'T' residue of the template and binds ATP. In this case, the elongation site binds the non-complementary nucleotide, ATP.

Both of the two preceding suggestions assume that the poly(dA-dT) sequence at the point of enzyme binding is alternating. If the sequence was, in fact, a local departure from an alternating sequence, say -pTpTp- , then faithful transcription would yield pppApA at the 5'-terminal end of the poly(A-U) product (diagram 4 in Fig. 9.2)
The models in diags 2 & 3 could be distinguished by determining the third residue in the polyribonucleotide chain ; for diagram 2, it would be U but in diagram 3, A.

The results of the analysis of the stoichiometry and sequence properties of the poly(A-U) transcripts suggest that alternative reaction pathways may be operative, particularly at the stage of initiation. Further evidence for alternative pathways was the observation that, for ATP as the variable substrate, the kinetic behaviour became more hyperbolic as the UTP concentration was decreased. This behaviour is characteristic of alternate pathways (Wong & Endrenyi, 1971), and represents the diversion of the reaction through a particular path, of the alternatives, due to saturation of one of the enzyme forms by the fixed substrate.

Steady-state rate equations were derived for various simple models of poly(A-U) synthesis (Fig. 9.3).
It is impossible to analyse the complete synthesis of a long poly(A-U) chain since this involves a prohibitively large number of reaction steps. A truncated model must be written which takes the synthesis until, hypothetically, no further kinetically significant enzyme-containing species would be introduced by analysing the reaction any further. This still

leaves a large number of potential mechanisms. However, into such schemes, some of the possibilities for alternative pathways were introduced along the lines of Fig. 9.2 and these are shown in Fig. 9.3 . Rate equations of degree two in ATP or UTP were obtained, in concurrence with the kinetic results of Section 8C, but the distinction between kinetic models depends on a detailed knowledge of the complete rate equation, expressed for both ATP and UTP variable. This would require not only matching the behaviour with respect to the variable substrate, but also the nature of the dependence of the equation parameters on the fixed substrate concentration. The form of this dependence experimentally indicated rate equations more complex than any of the model equations in Fig. 9.3. It is of interest, though, that the terms causing deviation from hyperbolic kinetics (for example, in ATP^2 or UTP^2) generally increased with increasing fixed substrate (Fig. 8.21 & 8.22) and were least significant at a low fixed substrate concentration. Complete explanation of the experimental results was not possible by any of the models analysed. Possibly, more complicated reaction models would be needed, but, in any case, the final problem would be to distinguish between complex rate equations of similar general form. As such equations become more complex, many more experimental data are required for their resolution. Also, for RNA polymerase catalyzed reactions, even with a simple poly(dA-dT) template, there is evidently a large number of potential reaction models. There would seem to be limits then as to how far steady state analysis can be developed to give a greater understanding of the mechanism of this reaction. However, the results do indicate that in many ways RNA polymerase catalysis can be described by steady state rate equations.

To summarize, various aspects of the behaviour of a poly(dA-dT) $-E_c$ system could be explained by steady state rate equations

derived for various specific models. Ping-pong mechanisms held in certain substrate ranges. Substrate inhibition was explicable, in part, by the inclusion of a quadratic term in the denominator of the rate equation. Double reciprocal plots concave to the 1/s axis could be accounted for by the form of rate equation derived for simple models involving alternative reaction pathways. Non-hyperbolic kinetics resulting from alternative reaction pathways have not been well-documented to date (Cleland, 1970) and the observation of this phenomenon here could arise from the fact that any effect at initiation of poly(A-U) synthesis is amplified by the elongation reactions.

9E4 SUBSTRATE CONTROL OF RNA POLYMERASE ACTIVITY IN VIVO

The role of RNA polymerase in the bacterium appears to be the selective transcription of the genome in response to various control signals. To date, the concentration of the nucleoside triphosphates, ATP, CTP, GTP, and UTP have not been strongly implicated in this control process but the rate of RNA synthesis *in vivo*, if not its selection, may be a function of substrate concentrations. Estimates of nucleotide concentrations *in vivo* have exceeded many observed K_m's for RNA polymerase (Section 1A7) implying that the enzyme activity will be relatively insensitive to substrate concentration *in vivo*. However, concentrations may not reflect the effective activities in the environment of the bacterial cell's transcription complex and measurements of K_m *in vitro* may have little relation to the K_m *in vivo*. The work described here has shown the dependence of apparent K_m's for RNA polymerase on the fixed substrate concentrations and the presence of σ, and experimental values varied widely, depending on conditions. Thus, the activity of RNA polymerase in the cell may well be sensitive to substrate concentration.

It is of interest that any multi-substrate enzymic reaction is in some measure regulated not only by absolute substrate concentrations but also by the relative proportions of different substrates . If any one substrate drops to a relatively low level, the reaction rate may decrease. The ribonucleoside triphosphate pool, in terms of the relative proportions of ATP, CTP, GTP and UTP, could possibly regulate the rate of RNA synthesis by RNA polymerase. The possibility that this pool composition is under metabolic control is quite strong since these four nucleotides are metabolically related in many ways.

In conclusion, although the control of the rate of RNA synthesis by ribonucleoside triphosphate concentrations cannot be over-emphasized at this time, neither should it be dismissed as a feature of RNA polymerase control in vivo.

Fig. 9.3 Model reaction mechanisms and steady state rate equations for poly(A-U) synthesis

Only the algebraic form of rate equation only is given since each constant is composed of numerous individual rate constants. A shorthand notation is used for the reaction schemes, exemplified by the following :

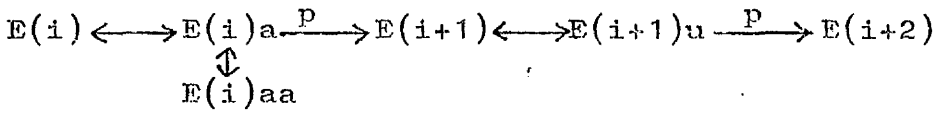
E(i) is initiated enzyme, with the product chain length being i. E(i) and E(i+2) are considered indistinguishable.

E(au) is enzyme which has synthesized an 'au' chain, where a and u represent ATP and UTP.

Ea is enzyme with bound ATP

Pyrophosphate release is denoted by p - irreversible for $PP_i = 0$.

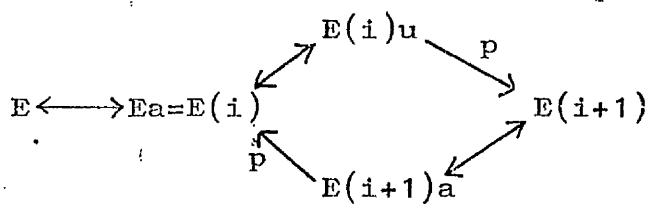
a)



Simple ping pong mechanism, with substrate inhibition by ATP, and no specific consideration of the initiation phase.

$$v = \frac{K_1 au}{K_2 a + K_3 u + au + K_{ia} a^2}$$

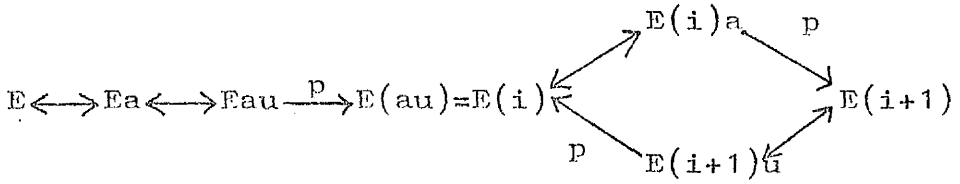
b)



Binding of ATP considered as initiation.

$$v = \frac{K_1 au}{K_2 + K_3 u + K_4 au + K_5 a}$$

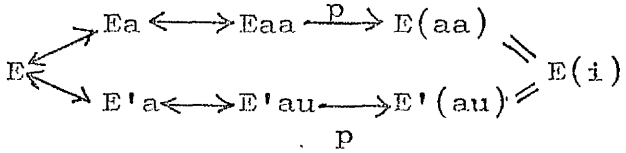
c) Fig. 9.3 continued



Formation of pppApU is considered as initiation.

$$v = \frac{K_1 a^2 u}{K_2 u + K_3 ua + K_4 a^2 u + K_5 a^2}$$

d)

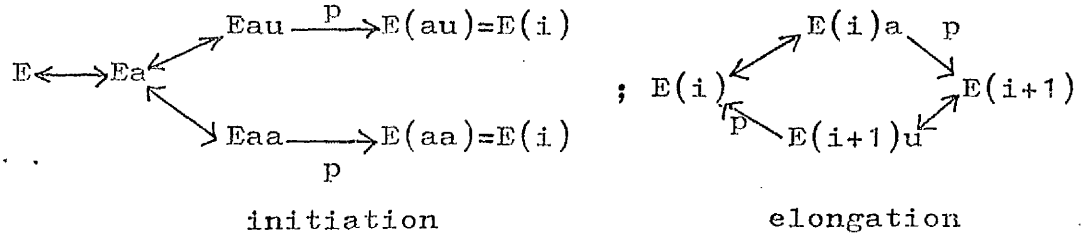


No special elongation step considered.

Alternatives at initiation stage (cf. Fig. 9.2).

$$v = \frac{K_1 a^2 u + K_2 au}{K_3 a + K_4 a^2 + K_5 au + K_6 a^2 u + K_7}$$

e)

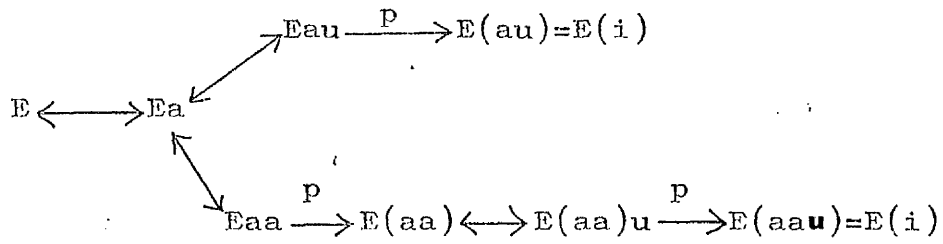


Separate initiation and elongation phases considered, with alternative pathways at initiation (diagrams 4 & 3 in Fig. 9.2 for example).

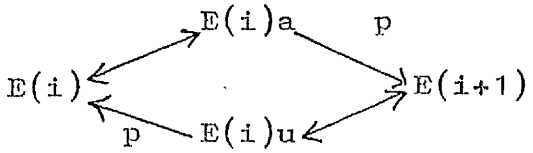
$$v = \frac{K_1 au^2 + K_2 a^2 u}{K_3 a^2 u + K_4 a^2 + K_5 au^2 + K_6 au + K_7 u^2}$$

Fig. 9.3 continued

f)



initiation



elongation

$$v = \frac{K_1 a^2 u + K_2 a u^2}{K_3 a^2 u + K_4 a^2 + K_5 u^2 + K_6 a u + K_7 u^2}$$

Separate initiation and elongation phases considered, with alternative pathways at initiation (diagram 2 in Fig. 9.2)

APPENDIX 1 Analysis of enzyme kinetic data by the procedure of Wilkinson

The procedure of Wilkinson (1961) fits measurements of initial rate and substrate concentration directly to a hyperbolic rate law by a least squares method. This obviates the problems associated with the fitting of data to linear forms of the hyperbolic rate equation (Section 1B4). The computer program described on the next page is a translation of one listed by Williams (1969) into a Fortran dialect compatible with a PDP 8L computer on which the program was run.

The input data are :

1) number of data points (twice)
2) data in the order, substrate$_1$, velocity$_1$, substrate$_2$, velocity$_2$, ...

The output is :

1) initial estimates of K_m and V_{max}
2) refined estimates of K_m and V_{max}
3) standard errors of K_m and V_{max}

The program listing and sample output are given in the following pages.

SAMPLE OUTPUT

```
 6 6

Ø.138  Ø.148
Ø.22Ø  Ø.171
Ø.291  Ø.234
Ø.56Ø  Ø.324
Ø.766  Ø.39Ø
1.46Ø  Ø.493
ESTIMATES
KM = +Ø.57Ø97ØE+Ø
 V = +Ø.679856E+Ø

COMPUTED VALUES
KM = +Ø.594779E+Ø
 V = +Ø.689753E+Ø


STANDARD ERROR OF KM = +Ø.637225E-1
STANDARD ERROR OF V = +Ø.358553E-1
!
```

Values from Wilkinson (1961) for treatment of the same input data.

0.595
0.690

0.064
0.036

4K FORTRAN PROGRAM FOR THE ANALYSIS OF ENZYME KINETIC DATA

BY THE PROCEDURE OF WILKINSON

```
C; MICHAELIS-MENTEN/WILKINSON
   DIMENSION S(20),V(20),F(20),F1(20)
   ACCEPT 15,PNUM
   ACCEPT 10,NUM
10; FORMAT (I)
   ALP=BET=GAM=DELT=EPS=VSQ=0.0
   DO 20 I=1,NUM
   ACCEPT 15,S(I)
   ACCEPT 15,V(I)
15; FORMAT (E)
   VSQ=VSQ+V(I)**2
   ALP=ALP+V(I)**3
   BET=BET+V(I)**4
   GAM=GAM+(V(I)**3)/S(I)
   DELT=DELT+(V(I)**4)/S(I)
EPS=EPS+(V(I)**4)/S(I)**2
20;CONTINUE
   THE=ALP*EPS-GAM*DELT
   AKM=(BET*GAM-ALP*DELT)/THE
   V1=(BET*EPS-DELT**2)/THE
   TYPE 25,AKM,V1
25; FORMAT("ESTIMATES",/,"KM = ",E,/," V = ",E,/,/)
   A=B=C=D=E=0.0
   DO 30 I=1,NUM
   F(I)=(V1*S(I))/(S(I)+AKM)
   F1(I)=(-V1*S(I))/((S(I)+AKM)**2)
   A=A+F(I)**2
   C=C+F(I)*F1(I)
   D=D+V(I)*F(I)
   B=B+(-F1(I))**2
   E=E+V(I)*F1(I)
30;CONTINUE
   S1=(A*B)-(-C)**2
   B1=(B*D-C*E)/S1
   B2=(A*E-C*D)/S1
   VO=B1*V1
   AKOM=AKM+B2/B1
   TYPE 35,AKOM,VO
35; FORMAT("COMPUTED VALUES",/,"KM = ",E,/," V = ",E,/)
   P2=(VSQ-B1*D-B2*E)/(PNUM-2.0)
   EKOM=(SQTF(P2)/B1)*SQTF(A/S1)
   EVO=VO*SQTF(P2)*SQTF(B/S1)
   TYPE 40,EKOM
40; FORMAT(/,/,"STANDARD ERROR OF KM = ",E)
   TYPE 45,EVO
45; FORMAT(/,"STANDARD ERROR OF V = ",E,/)
      END
```

APPENDIX 2. Description of computer program for non-linear least squares analysis

The computer program was used to fit data from enzyme kinetic experiments to model rate equations of the form

$$v = \frac{p_1x^2 + p_2x + p_3}{q_1x^2 + q_2x + q_3} \qquad \text{eqn.A2.1}$$

where p_1, p_2, p_3, q_1, q_2, and q_3 are the parameters of the model. Simpler equations could generated by setting any of the parameters to zero. The program computed estimates of the parameters, from the supplied data, that minimized X^2 (Section 1B4.1) within a given parameter space.

The computer program used was MINUIT (CERN 6600 computer program library D506 ; authors, James,F. & Roos,M.) and was run on the ERCC 360/50 computer through the Computing Service, Glasgow University. MINUIT minimizes a general function of several variables and calculates the true errors (the latter facility was not used in this study). The program includes a user written sub-routine so that the user can specify the function to be minimized (sub-routine FCN). In this instance, FCN reads in experimental data and calculates X^2 for an equation of the form of eqn.A2.1 . X^2 then is a function of p_1, p_2, p_3, q_1, q_2, and q_3 and this is the function minimized by MINUIT. Starting with initial estimates for the parameters, refinements are made until, within a selected parameter space, a minimum of X^2 is attained. The user written subroutine FCN is listed overleaf.

FORTRAN SUBROUTINE FCN

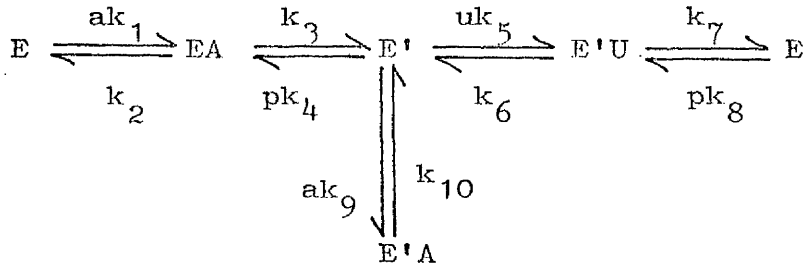
```
      SUBROUTINE FCN(NPAR,G,F,X,IFLAG)
      DIMENSION X(10),XAXIS(30),YAXIS(30),ERR(30),DIF(30),CHISQ(30),
     1  FMODEL(30),TITLE(20),ERRSQ(30)
      GO TO (10,20,30,20),IFLAG
C
10    CONTINUE
      WRITE(6,48)
      READ(5,3)TITLE
      READ(5,30)NDP,IPAR
C     NDP=NUMBER OF DATA POINTS
C     IPAR=NUMBER OF VARIABLES
      NDEGF=NDP-IPAR
      WRITE(6,53)NDP,IPAR,NDEGF
      WRITE(6,49)
C     READ DATA
      DO 60 NN=1,NDP
      READ(5,51)XAXIS(NN),YAXIS(NN),ERR(NN)
      WRITE(6,52)XAXIS(NN),YAXIS(NN),ERR(NN)
      ERRSQ(NN)=ERR(NN)*ERR(NN)
60    CONTINUE
      ISEK=0
C
20    F=0.0
      DO 21 KK=1,NDP
      A=XAXIS(KK)
      F1=X(3)*A*A + X(2)*A + X(1)
      F2=X(6)*A*A + X(5)*A + X(4)
      FMODEL(KK)=F1/F2
      DIF(KK)=FMODEL(KK)-YAXIS(KK)
      CHISQ(KK)=DIF(KK)*DIF(KK)/ERRSQ(KK)
      F=F + CHISQ(KK)
21    CONTINUE
      IF(ISEK.EQ.0)GO TO 30
15    ISEK=1
      RETURN
C
30    WRITE(6,5)
      WRITE(6,4)TITLE
      WRITE(6,6)
      DO 8 JK=1,NDP
      WRITE(6,9)XAXIS(JK),YAXIS(JK),ERR(JK),FMODEL(JK),DIF(JK),
     1  CHISQ(JK)
8     CONTINUE
      WRITE(6,13)
      WRITE(6,11)(X(J),J=1,6)
      VR=F/NDEGF
      WRITE(6,12)F,VR,NDEGF
      IF(ISEK.EQ.0)GO TO 15
      RETURN
C
C     FORMAT STATEMENTS
3     FORMAT(20A4)
4     FORMAT(1HO,20A4)
```

```
 5 FORMAT(1H1,'SUMMARY')
 6 FORMAT(//1HO,'       X-VALUE       Y-VALUE     ERROR     CALC VALUE
  1DIFFERENCE     CHISQUARED')
 9 FORMAT(1HO,6(F10.2,3X))
11 FORMAT(1HO,'P3=',F10.3,/1H ,'P2=',F10.3,/1H ,'P1=',F10.3,/1H ,
  1'Q3=',F10.3,/1H ,'Q2=',F10.3,/1H ,'Q1=',F10.3)
12 FORMAT(//1HO,'CHISQUARED FOR FIT=',F10.3,/1H ,'VARIANCE RATIO
  1=',F10.3,/1H ,'NUMBER OF DEGREES OF FREEDOM=',I5)
13 FORMAT(///1HO,'VALUE OF FITTED PARAMETERS')
48 FORMAT(///1HO,'INPUT DATA')
49 FORMAT(1HO,'      X-AXIS       Y-AXIS      ERROR')
50 FORMAT(10I10)
51 FORMAT(10F10.0)
52 FORMAT(1HO,3(F10.3,5X))
53 FORMAT(1HO,'NUMBER OF DATA POINTS=',I6,/1H ,'NUMBER OF
  1PARAMETERS=',I6,/1H ,'NUMBER OF DEGREES OF FREEDOM=',I6)
 C
    END
```

APPENDIX 3 Derivation of steady state rate equation using the King-Altman method

Various model reactions were analysed by the method of King & Altman (1956), but only a simple one is shown in any detail because of the complicated equations involved.

Ordered two substrate ping pong mechanism, with inhibition by one of the substrates :



Steady state equation :

$$v = \frac{K_1 au}{K_2 a + K_3 u + au + K_{ia} a^2}$$

where

$$K1 = k_3 k_7 e_o / (k_1 k_7 + k_1 k_5)$$

$$K_2 = (k_2 k_7 + k_3 k_7)/(k_1 k_7 + k_1 k_3)$$

$$K_3 = (k_3 k_6 + k_3 k_7)/(k_5 k_7 + k_3 k_5)$$

$$K_{ia} = (k_3 k_6 k_9 + k_3 k_7 k_9)/(k_5 k_7 k_{10} + k_3 k_5 k_{10})$$

$$= K_3 . k_9 / k_{10}$$

APPENDIX 4. Calculation of the equilibrium between NTP, Mg^{2+} and K^+

The following two equilibria are considered

$$NTP^{4-} + Mg^{2+} = NTPMg^{2-} \qquad \text{eqn.A4.1}$$

$$NTP^{4-} + K^{+} = NTPK^{3-} \qquad \text{eqn.A4.2}$$

The total concentrations of ATP^{4-}, Mg^{2+} and K^+ added to the system are a, m, and k, respectively. The concentrations of the complexes are c_m ($ATPMg^{2-}$) and c_k ($ATPK^{3-}$) and the formation constants of the complexes K_m ($ATPMg^{2-}$) and K_k ($ATPK^{3-}$). These are given by

$$K_m = \frac{c_m}{(a - c_m - c_k)(m - c_m)} \qquad \text{eqn.A4.3}$$

$$K_k = \frac{c_k}{(a - c_m - c_k)(k - c_k)} \qquad \text{eqn.A4.4}$$

Eliminating c_m between these equations gives a trinomial in c_k,

$$\xi_o + \xi_1 c_k + \xi_2 c_k^2 + \xi_3 c_k^3 = 0 \qquad \text{eqn.A4.5}$$

where

$$\xi_o = ak^2K_k/mK_m$$

$$\xi_1 = (akK_m - k - k^2K_k - 2kaK_k - mkK_m)/mK_m$$

$$\xi_2 = ((K_k - K_m)/K_k + (K_k - K_m)(a + k) + kK_k + mK_m)$$

$$\xi_3 = (K_m - K_k)/K_m m$$

Eqn.A4.5 was solved iteratively by the Newton-Raphson method on a PDP 8L computer for c_k. c_m can then be calculated from eqn.A4.4 . The uncomplexed ATP^{4-} is simply given by $(a - c_k - c_m)$.

REFERENCES

ALBERTY, R.A. (1953) J. Amer. Chem. Soc. 75, 1928-1932

ALBERTY, R.A. (1958) J. Amer. Chem. Soc. 80, 1777-1782

ALBERTY, R.A. (1969) J. Biol. Chem. 244, 3290-3302

ALBERTY, R.A. & Koerber, B.M. (1957) J. Amer. Chem. Soc. 79, 6379-6382

ANTHONY, D.D., Zeszotek, E. & Goldthwait, D.A. (1966) Proc. Nat. Acad. Sci. U.S. 56, 1026-1033

ANTHONY, D.D., Wu, C.W. & Goldthwait, D.A. (1969) Biochemistry 8, 246-256

ARDITTI, R., Eron, L., Zubay, G., Tocchini-Valentini, G., Connaway, S. & Beckwith, J. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 437-442

BARKER, R. (1971) Organic Chemistry of Biological Compounds in Foundations of Modern Biochemistry Series (Hager, L. & Wold, F. eds), Prentice-Hall Inc, New Jersey

BAUTZ, E.K.F. (1972) Progr. Nucl. Acid Res. Mol. Biol. 12, 129-160

BAUTZ, E.K.F. & Bautz, F.A. (1970) Nature (London) 226, 1219-1222

BAUTZ, E.K.F. & Hall, B.D. (1962) Proc. Nat. Acad. Sci. U.S. 48, 400-408

BAUTZ, E.K.F., Bautz, F.A. & Dunn, J.J. (1969) Nature(London) 223, 1022-1024

BERG, D. & Chamberlin, M. (1970) Biochemistry 9, 5055-5064

BERG, D., Barrett, K., Hinkle, D., McGrath, J. & Chamberlin, M. (1969) Federation Proceedings 28, 659

BERG, D., Barrett, K. & Chamberlin, M. (1970) in Methods in Enzymology vol. 20D (Grossman, L. & Moldave, K. eds) 506-519 Academic Press, New York & London

BEVINGTON, P.R. (1969) Data Reduction and Error Analysis for the Physical Sciences, McGraw-Hill, New York

BREMER, H. (1967) Molec. Gen. Genetic 99, 362-371

BREMER, H. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 109-120

BREMER, H. & Konrad, M.W. (1964) Proc. Nat. Acad. Sci. U.S. 51, 801-808

BREMER, H. & Yuan, D. (1968) J. Mol. Biol. 38, 163-180

BREMER, H., Konrad, M.W., Gaines, K. & Stent, G.S. (1965) J. Mol. Biol. 13, 540-553

BRIGGS, G.E. & Haldane, J.B.S. (1925) Biochem. J. 19, 338-339

BRINTZINGER, H. (1963) Biochim. Biophys. Acta 77, 343-345

BROWN, A.J. (1902) Trans. Chem. Soc. 81, 373 -388

BROWN, N.C. & Reichard, P. (1969) J. Mol. Biol. 46, 39-55

BURGESS, R.R. (1969a) J. Biol. Chem. 244, 6160-6167

BURGESS, R.R. (1969b) J. Biol. Chem. 244, 6168-6176

BURGESS, R.R. (1971) Annu. Rev. Biochem. 40, 711-740

BURGESS, R.R., Travers, A.A., Dunn, J.J. & Bautz, E.K.F. (1969) Nature(London) 221, 43-46

BURTON, K. (1959) Biochem. J. 71, 388-395

CANTOR, C.R., Jaskunas, S.R. & Tinoco, I. Jr. (1966) J. Mol. Biol. 20, 39-62

CASHEL, M. (1969) J. Biol. Chem. 244, 3133-3141

CASHEL, M. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 407-414

CASSANI, G., Burgess, R.R., & Goodman, H. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 59-63

CHAMBERLIN, M. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 851-873

CHAMBERLIN, M. & Berg, P. (1962) Proc. Nat. Acad. Sci. U.S. 48, 81-94

CHAMBERLIN, M., McGrath, J. & Waskell, L. (1970) Nature(London) 228, 227-231

CLELAND, W.W. (1963a) Biochim. Biophys. Acta 67, 104-137

CLELAND, W.W. (1963b) Biochim. Biophys. Acta 67, 173-187

CLELAND, W.W. (1963c) Biochim. Biophys. Acta 67, 188-196

CLELAND, W.W. (1967) in Advances in Enzymology, vol.29, pp 1-32 (Nord, F.F. ed) Interscience ; John Wiley, New York, London & Sidney

CLELAND, W.W. (1970) in The Enzymes, vol.2, 3rd. edn., (Boyer, P.D. ed), Academic Press, N.Y. & London

COHN, M. & Hughes, T.R. Jr. (1962) J. Biol. Chem. 237, 176-181

CROTHERS, D.M. & Zimm, B.H. (1965) J. Mol. Biol. 12, 525-536

DALZIEL, K. (1957) Acta Chem. Scand. 11, 1706-1722

DALZIEL, K. (1958) Trans. Faraday Soc. 54, 1247-1253

DALZIEL, K. (1962) Nature(London) 196, 1203-1205

DARLIX, J.L., Sentenac, A., Ruet, A. & Fromageot, P. (1969) Eur. J. Biochem. 11, 43-48

DAVIES, D.R. & Baldwin, R.L. (1963) J. Mol. Biol. 6, 251-255

DAVISON, J., Pilarski, L.M. & Echols, H. (1969) Proc. Nat. Acad. Sci. U.S. 63, 168-174

DAVISON, J., Brookman, K., Pilarski, L. & Echols, H. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 95-99

DICKINSON, L., Chantrill, D.H., Inkley, G.W. & Thompson, M.J. (1953) British Journal of Pharmacology 8, 139-142

DOVE, W.F. & Davidson, N. (1962) J. Mol. Biol. 5, 467-478

DOWNEY, K.M. & So, A.G. (1970) Biochemistry 9, 2520-2525

ELLIOTT, W.H. (1963) Biochem. J. 86, 562-567

ELSWORTH, R., Miller, G.A., Whitaker, A.R., Kitching, D. & Sayer, P.D. (1968) J. Applied. Chem. 18, 157-166

FLECK, A. & Munro, H.N. (1962) Biochim. Biophys. Acta 55, 571-583

FLORENTIEV, V.L. & Ivanov, V.I. (1970) Nature(London) 228, 519-522

FOSTER, R.J. & Niemann, C. (1953) Proc. Nat. Acad. Sci. U.S. 39, 999-1003

FOX, C.F. & Weiss, S.B. (1964) J. Biol. Chem. 239, 175-185

FRIEDEN, C. (1967) J. Biol. Chem. 242, 1705-1715

FUCHS, E., Millette, R.L., Zillig, W. & Walter, G. (1967) Eur. J. Biochem. 3, 183-193

FURTH, J.J., Hurwitz, J. & Anders, M. (1962) J. Biol. Chem. 237, 2611-2619

GALLANT, J. & Harada, B. (1969) J. Biol. Chem. 244, 3125-3132

GEIDUSCHEK, E.P. & Haselkorn, R. (1969) Annu. Rev. Biochem. 38, 647-676

GEIDUSCHEK, E.P., Nakamoto, T. & Weiss, S.B. (1961) Proc. Nat. Acad. Sci. U.S. 47, 1405-1415

GOLDSTEIN, A., Kirschbaum, J.B. & Roman, A. (1965) Proc. Nat. Acad. Sci. U.S. 54, 1669-1675

GOLDTHWAIT, D.A., Anthony, D.A. & Wu, C.W. (1970) in RNA Polymerase in Transcription pp10-27, (Silvestri, L. ed) North Holland Publishing Co.,Amsterdam

HALL, B.D., Hager, G, Fields, K.L. & Beguin, F. (1969) Bull. Soc. Chim. Biol. 51, 1397-1405

HAMMES, G.G., Maciel, G.E. & Waugh, J.S. (1961) J. Amer. Chem. Soc. 83, 2394-2395

HASELTINE, W. (1972) Nature New Biology 235, 329-333

HAYASHI,M., Hayashi, M.N. & Spiegelman, S. (1963) Proc. Nat. Acad. Sci. U.S. 50, 664-672

HEARON, J.Z., Bernhard, S.A., Friess, S.L., Botts, D.J. & Morales, M.F. (1959) in The Enzymes, vol.1, 2nd. edn., (Boyer, P.D., Lardy, H. & Myrback, K. eds.), Academic Press, N.Y. & London

HEIL,A. & Zillig, W. (1970) FEBS lett. 11, 165-168

HEINEKEN, F.G., Tsuchiya, H.M. & Aris, R. (1967) Mathematical Biosciences 1, 95-113

HENDERSON, J.F. (1963) Cancer Res. 23, 491-495

HENRI, V. (1901) C.R. Acad. Sci. 133, 891-894

HENRI, V. (1902) C.R. Acad. Sci. 135, 916-919

HILL, T.L. (1952) J. Amer. Chem. Soc. 74, 4710-4711

HINKLE, D.C. & Chamberlin, M. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 65-72

von HIPPEL, P.H. & Schleich, T. (1969) Accounts of Chemical Research 2, 257-265

HIRSCHBEIN, L., Dubert, J.M. & Babinet, Ch. (1969) FEBS Lett. 3, 260-262

HOFSTEE, B.H.J. (1952) Science 116, 329-331

HURWITZ, J., Furth, J.J., Anders, M., Ortiz, P.J. & August, J.T. (1961) Journal de Chimie Physique 58, 934-943

HURWITZ, J., Furth, J.J., Anders, M. & Evans, A. (1962) J. Biol. Chem. 237, 3752-3759

HYMAN, R.W. & Davidson, N (1970) J. Mol. Biol. 50, 421-438

INMAN, R.B. & Baldwin, R.L. (1962a) J. Mol. Biol. 5, 172-184

INMAN, R.B. & Baldwin, R.L. (1962b) J. Mol. Biol. 5, 185-200

INMAN, R.B. & Baldwin, R.L. (1964) J. Mol. Biol. 8, 452-469

ISHIHAMA, A. & Hurwitz, J. (1969) J. Biol. Chem. 244, 6680-6689

ISHIHAMA, A. & Kameyama, T. (1967) Biochim. Biophys. Acta 138, 480-498

JACOB, F. & Monod, J. (1961) J. Mol. Biol. 3, 318-356

JOHANSEN, G. & Lumry, R. (1961) C.R. Trav. Lab. Carlsberg 32, 185-214

JOHNSON, J.C., Debacker, M. & Boezi, J.A. (1971) J. Biol. Chem. 246, 1222-1232

KING, E.L. & Altman, C. (1956) J. Phys. Chem. 60, 1375-1378

KING, A.M.Q. & Nicholson, B.H. (1971) J. Mol. Biol. 62, 303-319

KISTIAKOWSKY, G.B. & Shaw, W.H.R. (1953) J. Amer. Chem. Soc. 75, 866-871

KOSHLAND, D.E. (1970) in The Enzymes, vol.1, 3rd. edn.,(Boyer, P.D. ed), Academic Press, N.Y. & London

KOSHLAND, D.E., Nemethy, G. & Filmer, D. (1966) Biochemistry 5, 365-386

KRAKOW, J.S. & Fronk, E. (1969) J. Biol. Chem. 244, 5988-5993

KRAKOW, J.S. & von der Helm, K. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 73-83

KRAKOW, J.S., Daley, K. & Karstadt, M. (1969) Proc. Nat. Acad. Sci. U.S. 62, 432-437

KREBS, H.A. & Hems, R. (1953) Biochim. Biophys. Acta 12, 172-180

LAIDLER, K.J. (1955) Can. J. Chem. 33, 1614-1624

LAIDLER, K.J. (1965) Chemical Kinetics, 2nd. edn., McGraw-Hill, London

LAMBERT, S.M. & Watters, J.I. (1957) J. Amer. Chem. Soc. 79, 5606-5608

LANGMUIR, I. (1916) J. Amer. Chem. Soc. 38, 2221-2295

LASKOWSKI, M. Sr. (1972) Progr. Nucl. Acid Res. Mol. Biol. 12, 161-188

LEPECQ, J.-B. (1971) Methods of Biochemical Analysis 20, 41-86

LEPECQ, J.-B. & Paoletti, C. (1966) Anal. Biochem. 17, 100-107

LEPECQ, J.-B. & Paoletti, C. (1967) J. Mol. Biol. 27, 87-106

LEPECQ, J.-B., Yot, P. & Paoletti, C. (1964) C.R.Acad. Sci. 259, 1786-1789

LINEWEAVER, H. & Burk, D. (1934) J. Amer. Chem. Soc. 56, 658-666

LOCHHEAD, D.S. (1972) PhD thesis, University of Glasgow

LOWRY, O.H., Rosebrough N.J., Farr, A.L. & Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

MACDONALD, C.T., Gibbs, J.H. & Pipkin, A.C. (1968) Biopolymers 6, 1-25

MAHLER, H.R. & Cordes, e.h. (1967) Biological Chemistry, Harper & Row, London.

MANGOLD, H.K., Schmid, H.H.O. & Stahl, E. (1964) Methods of Biochemical Analysis 12, 393-452

MAITRA, U. & Barash, F. (1969) Proc. Nat. Acad. Sci. U.S. 64, 779-786

MAITRA, U. & Hurwitz, J. (1965) Proc. Nat. Acad. Sci. U.S. 54, 815-822

MAITRA, U. & Hurwitz, J. (1967) J. Biol. Chem. 242, 4897-4907

MAITRA, U., Nakata, Y. & Hurwitz, J. (1967) J. Biol. Chem. 242, 4908-4918

MAITRA, U., Lockwood, A.H., Dubnoff, J.S. & Guha, A. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 143-156

MANILOFF, J. (1969) J. Theoret. Biol. 23, 441-454

MARTELL, A.E. & Schwartzenbach, G. (1956) Helv. Chim. Acta 39, 653-661

Di MAURO, E., Snyder, L., Marino, P., Lambertini, A., Coppa, A., Tocchini-Valentini, G.P. (1969) Nature(London) 222, 533-537

MARMUR, J. & Doty, P. (1962) J. Mol. Biol. 5, 109-118

McQUILLEN, K. (1968) in Biochemistry of Bacterial Growth 1st. edn. p303 (Mandelstam, J. & McQuillen, K. eds) Blackwell Scientific Publications, Oxford & Edinburgh

MELCHIOR, N.C. (1954) J. Biol. Chem. 208, 615-627

MICHAELIS, L. & Menten, M.L. (1913) Biochem. Z. 49, 333-369

MICHELSON, A.M. (1968) in Molecular Associations in Biology pp93-106 (Pullman, B. ed), Academic Press, N.Y. & London.

MONOD, J., Wyman, J. & Changeux, J.P. (1965) J. Mol. Biol. 12, 88-118

NANNINGA, L.B. (1961) Biochim. Biophys. Acta 54, 330-338

NAZAR, R.N. & Wong, J.T.-F. (1972) J. Biol. Chem. 247, 790-797

NAZAR, R.N., Tyfield, L.A. & Wong, J.T.-F. (1972) J. Biol. Chem. 247, 798-804

NEUHARD, J. & Munch-Peterson, A. (1966) Biochim. Biophys. Acta 114, 61-71

NICHOLS, L.W., Jackson, W.J.H. & Winzor, D.W. (1967) Biochemistry 6, 2449-2456

NORBY, J.G.R. (1970) Acta Chem. Scand. 24, 3276-3286

NIYOGI, S.K. & Stevens, A. (1965a) J. Biol. Chem. 240, 2587-2592

NIYOGI, S.K. & Stevens, A. (1965b) J. Biol. Chem. 240, 2593-2598

OCHOA, S., Burma, D.P., Kroger, H. & Weill, J.D. (1961) Proc. Nat. Acad. Sci. U.S. 47, 670-679

OKAMOTO, T., Sugiura, M. & Takanami, M. (1970) Biochemistry 9, 3533-3541

OLIVER, D. & Chalkley, R. (1971) Anal. Biochem. 44, 540-542

O'SULLIVAN, W.J. & Perrin, D.D. (1961) Biochim. Biophys. Acta 52, 612-614

PAETKAU, V. & Coy, G. (1972) Can. J. Biochem. 50, 142-150

PASTAN, I. & Perlman, R. (1970) Science 169, 339-344

PAULING, L. (1935) Proc. Nat. Acad. Sci. U.S. 21, 186-191

PETTERSSON, G. (1969) Acta Chem. Scand. 23, 2717-2726

PHILLIPS, R.C., George, S.J.P. & Rutman, R.J. (1966) J. Amer. Chem. Soc. 88, 2631-2640

PRIESS, H. & Zillig, W. (1967) Biochim. Biophys. Acta 140, 540-542

QASBA, P.K. & Zillig, W. (1969) Eur. J. Biochem. 7, 315-317

RICHARDSON, J.P. (1966a) Proc. Nat. Acad. Sci. U.S. 55, 1616-1623

RICHARDSON, J.P. (1966b) J. Mol. Biol. 21, 83-114

RICHARDSON, J.P. (1969) Progr. Nucl. Acid Res. Mol. Biol. 8, 75-116

RICHARDSON, J.P. (1970a) J. Mol. Biol. 49, 235-240

RICHARDSON, J.P. (1970b) Nature(london) 225, 1109-1112

ROBERTS, J.W. (1969) Nature(London) 224, 1168-1174

SCHACHMAN, H.K., Adler, J., Radding, C.M., Lehman, I.R. & Kornberg, A. (1960) J. Biol. Chem. 235, 3242-3249

SCHACHTER, D. (1959) J. Lab. Clin. Med. 54, 763-768

SCHRODINGER, E. (1944) What is Life, Cambridge University Press

SCHWERT, G.W. (1969a) J. Biol. Chem. 244, 1278-1284

SCHWERT, G.W. (1969b) J. Biol. Chem. 244, 1285-1290

SCRUTTON, M.C., Wu, C.W. & Goldthwait, D.A. (1971) Proc. Nat. Acad. Sci. U.S. 68, 2497-2501

SENTENAC, A., Simon, E.J. & Fromageot, P. (1968) Biochim. Biophys. Acta 161, 299-308

SHAPIRO, A.L. & Maizel, J.V. Jr. (1969) Anal. Biochem. 29, 505-514

SHAPIRO, A.L., Vinuela, E. & Maizel, J.V. Jr. (1967) Biochem. Biophys. Res. Commun. 28, 815-820

SHIGEURA, H.T. & Boxer, G.E. (1964) Biochem. Biophys. Res. Commun. 17, 758-763

SIPPEL, A.E. & Hartmann, G. (1970) Eur. J. Biochem. 16, 152-157

SMITH, R.M. & Alberty, R.A. (1956) J. Phys. Chem. 60, 180-184

SMITH, M. & Khorana, H.G. (1963) in Methods in Enzymology vol.6, (Colowick, S.P. & Kaplan, N.O.), Academic Press, N.Y. & London

SO, A.G. & Downey, K.M. (1970) Biochemistry 9, 4788-4793

SO, A.G., Davie, E.W., Epstein, R. & Tissieres, A. (1967) Proc. Nat. Acad. Sci. U.S. 58, 1739-1746

SPIRIN, A.S. (1963) Progr. Nucl. Acid Res. Mol. Biol. 1, 301-345

STECK, T.L., Caicuts, M.J. & Wilson, R.G. (1968) J. Biol. Chem. 243, 2769-2778

STEVENS, A. (1960) Biochem. Biophys. Res. Commun. 3, 92-96

STEVENS, A. & Henry, J. (1964) J. Biol. Chem. 239, 196-203

STEVENS, A., Emery, A.J. Jr. & Sternberger, N. (1966) Biochem. Biophys. Res. Commun. 24, 929-936

STONINGTON, O.G. & Pettijohn, D.E. (1971) Proc. Nat. Acad. Sci. U.S. 68, 6-9

STRAAT, P.A. & Ts'o, P.O.P. (1969) J. Biol. Chem. 244, 6263-6269

SUGIURA, M., Okamoto, T. & Takanami, M. (1970) Nature(London) 225, 598-600

SUMMERS, W.C. & Siegel, R.B. (1969) Nature(London) 223, 1111-1113

TAKANAMI, M., Okamoto, T. & Sugiura, M. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 179-188

TERZI, M., Cascino, A. & Urbani, C. (1970) Nature(London) 226, 1052-1054

TINOCO, I. Jr., Uhlenbeck, O.C. & Levine, M.D. (1971) Nature (London) 230, 362-367

TRAVERS, A.A. (1971) Nature New Biology 229, 69-74

TRAVERS, A.A., Kamen, R. & Cashel, M. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 415-418

TS'O, P.O.P., Helmkamp, G.K., Sander, C. & Studier, F.W. (1963) Biochim. Biophys. Acta 76, 54-67

VOGT, V. (1969) Nature(London) 223, 854-855

VOLENSTEIN, M.V. & Goldstein, B.N. (1966) Biochim. Biophys. Acta 115, 471-477

VOLKENSTEIN, M.V., Godzhayev, N.M., Gotlib, Yu. Ya. & Ptitsyn, O.B. (1963) Biofizika 8, 3-8

WALAAS, E. (1958) Acta Chem. Scand. 12, 528-536

WALKER, I.O.(1964) Biochim. Biophys. Acta 88, 407-414

WANG, J.C. (1969) J. Mol. Biol. 43, 25-39

WARING, M.J. (1964) Biochim. Biophys. Acta 87, 358-361

WARING, M.J. (1965) J. Mol. Biol. 13, 269-282

WATSON, J.D. & Crick, F.H.C. (1953) Nature(London) 171, 737-738

WEBB, J.L. (1963) Enzyme and Metabolic Inhibitors, vol.1, Academic Press, N.Y. & London

WEBER, K. & Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412

WEISS, S.B. & Gladstone, L. (1959) J. Amer. Chem. Soc. 81, 4118-4119

WILKINSON, G.N. (1961) Biochem. J. 80, 324-332

WILLIAMS, A. (1969) Introduction to the Chemistry of Enzyme Action, McGraw-Hill Publishing Co. Ltd., Maidenhead, England

WONG, J.T.-F. & Endrenyi, L. (1971) Can. J. Biochem. 49, 568-580

WONG, J.T.-F. & Hanes, C.S. (1962) Can. J. Biochem. Physiol. 40, 763-804

WU, C.W. & Goldthwait, D.A. (1969a) Biochemistry 8, 4450-4458

WU, C.W. & Goldthwait, D.A. (1969b) Biochemistry 8, 4459-4464

ZILLIG, W. & Holzel, H. (1958) Hoppe-Seylers Z. Physiol. Chem. 312, 140-143

ZILLIG, W., Zechel, K., Rabussay, D., Schachner, M., Sethi, V.S., Palm, P., Heil, A. & Seifert, W. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 47-58

ZILLIG, W., Fuchs, E., Palm, P. Rabussay, D. & Zechel,K. (1970a) RNA Polymerase in Transcription pp47-57 (Silvestri, L. ed), North Holland Publishing Co. , Amsterdam

ZIMMERMAN, J.M. & Simha,R. (1966) J. Theoret. Biol. 13, 106-130

Application by: Peter J. ROACH

A copy of the thesis has been sent to the Additional Examiner who is:

Dr. G.K. Radda,
Department of Biochemistry,
University of Oxford.

The Special Committee is: Professor Smellie (Convener)
Dr. Anderson
Dr. Vaughan

3