Studies on *Halobacterium cutirubrum*
DNA-dependent RNA polymerase

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ABBREVIATIONS

RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
RNase	Ribonuclease
DNase	Deoxyribonuclease
AMP, GMP, UMP, CMP	The 5' - phosphates of adenosine, guanosine, uridine, cytidine.
ADP etc.	The 5' - diphosphates of adenosine etc.
ATP etc.	The 5' - triphosphates of adenosine etc.
ApA.	Adenylyl - (3' -5' -) adenosine
Poly A	Linear 3' -5' polymer of adenylic acid
DMSO	Dimethyl sulfoxide.

I. INTRODUCTION

A. Extremely halophilic bacteria

Bacteria have been classified using their salt requirement for growth as a parameter and this classification is shown in Table I (Larsen, 1962). Organisms requiring salt concentrations of 3M or higher for growth are called extreme halophiles. The existence of these organisms was first reported by Klebahn (1919) and Harrison and Kennedy (1922). However serious studies on these organisms started only around 1951-1952. Even at present, the number of workers involved in the study of halophiles is relatively few.

The extremely halophilic bacteria are found in nature in brines containing very high salt concentrations, which is the condition of the salt lakes like the Great Salt Lake and the Dead Sea. They are specially conspicuous in the solar evaporation ponds of salt works, where they frequently occur in such large numbers that they give a bright red colour to the concentrated brine. They are also found in salted products such as bacon, fish and hides where they may cause spoilage.

Extreme halophiles are either cocci or rod-shaped. The taxonomy of these bacteria has been controversial because they change shape on handling and in different culture conditions and are difficult to stain. In Bergey's Manual of Determinative Bacteriology, Eighth Edition (1974) all the extreme halophiles are included in the family of HALOBACTERIACEAE. They are subdivided into two genera.

TABLE I

CLASSIFICATION OF BACTERIA BASED ON SALT CONCENTRATION FOR GROWTH

Salt strength	Class	Example
0 to 0.4 M	Non halophiles	*Escherichia coli* *Desulfovibrio desulfuricans*
0 to 1.7 M	Halotolerant bacteria	*Bacillus cereus*
0.5 to 3.5 M	Moderate halophiles	*Vibrio costicolus*
3.0 M to saturation	Extreme halophiles	*Halobacterium salinarium*

The rod shaped extreme halophiles are placed under the genus HALOBACTERIUM. The dimension of these rod shaped cells are 0.6 - 1.0 by 1 - 6 μm. They are Gram-negative, but Gram-staining is not satisfactory due to the presence of high concentrations of salt. They are best observed by phase-contrast microscopy. The genus Halobacterium has five species. They are H. Salinarium, H. halobium, H. marismortui, H. trapanicum and Amoebobacter morrhuae. In the previous edition of Bergey's Manual (Seventh Edition, 1967) the classification was different. The rod shaped species had another species with the name H. cutirubrum. In the latest edition H. cutirubrum is included with the species H. salinarium, because they were claimed to be very similar by various taxonomic and DNA-homology studies. H. cutirubrum had the proteolytic property of completely cleaning milk agar plates when first isolated, but after a while it lost this property. However, throughout this thesis H. cutirubrum will be treated as a different species because essentially all published work in this field has used the earlier classification.

Halophilic cocci are placed under the genus HALOCOCCUS. They are 0.6 - 1.5 μm in diameter. Halococcus cells retain their shape in lower concentration of salt and seem more resistant to osmotic damage although their cell walls are similar to that of Halobacteria in not containing diaminopimelic acid or muramic acid. These red coloured halophilic cocci have not been studied as extensively as their rod shaped counterparts. A different classification for them is based on their Gram reaction, high salt requirement for growth, the presence of diether linkages and dihydrophytol groups in their lipids and cell wall composition. The genus has only one species, Halococcus morrhuae.

<u>Properties of the cell envelope of halophilic bacteria</u>

The cell envelope of gram-negative bacteria consists of a multi-layered cell wall sometimes referred to as a compound membrane or an external membrane. Halophilic bacteria have an unusual cell wall. The external layers of the halophilic cells have been found to have remarkable physio chemical and metabolic properties and therefore they have been subjected to extensive studies. Most studies on the cell envelope have been done on the halobacteria only. Relatively very little has been done on the external layers of extremely halophilic cocci.

Abram and Gibbons (1960, 1961) first showed that a high salt concentration is necessary not only for growth, but also for maintenance of the cell envelope in <u>H. cutirubrum</u>. When the salt concentration is lowered step by step, initially the rods become spheres, and eventually the cells lyse completely at a NaCl concentration of about 1.0 to 1.5 M. NaCl and LiCl are much more effective than KCl or NH_4Cl in maintaining the integrity of the cell envelope.

The morphology of <u>H. cutirubrum</u> cells has also been shown to be affected by pH. At low pH, in the absence of salt, the cells lost their rod shape, but maintained their rigidity. In the presence of salt, even at low pH, the cells remained as rods (Kushner and Bayley, 1963).

The effect of the cations Li^+, Na^+, K^+, NH_4^+ and Mg^{++} on the morphology of the different strains of extreme halophiles has been tested by different investigators. NaCl is more effective than NH_4Cl or KCl

in maintaining the cell structure of H. cutirubrum (Kushner 1964). This shows that the external surface of the cell envelope specifically requires Na^+ to maintain its integrity, whereas the internal surface of the envelope is supported at least as well by K^+ and NH_4^+ as by Na^+. $MgCl_2$ prevents lysis of intact cells and cell envelopes at a concentration much lower than required in the presence of NaCl. 1.0 M NaCl was less effective than 0.5 $MgCl_2$ in preventing lysis of intact H. cutirubrum cells (Soo Hoo and Brown, 1967). The polyamine spermine at a concentration of 10^{-3} M made H. cutirubrum cells more rather than less sensitive to lower concentrations of NaCl, KCl, NH_4Cl and $MgCl_2$ (Kushner, 1964). These experiments indicate that electrostatic forces and osmotic pressure are very critical factors in maintaining the integrity of halophilic bacteria.

Steensland and Larsen (1969) have shown by electron microscopic and chemical studies on three different extreme halophiles that their cell envelopes are similar in general structure. On lowering of NaCl concentration to 2.2 M the outer membrane of H. salinarium became frayed and a release of material from the outer layer appeared to have taken place. Amino acid analysis done on the envelope protein which was about 66% of the salt free dry weight of the envelopes showed aspartic and glutamic acids formed 25% of the protein. Based on these observations it has been concluded that cell walls of halophilic bacteria are negatively charged and the presence of cations prevents their disintegration by neutralising the negative charges.

The protein and the carbohydrate composition may also affect

the morphology of the cell envelopes. The carbohydrate containing proteins of H. salinarium cell envelopes are different from that of E. coli and Serratia marcescens. A large amount of high molecular weight protein is present in the cell envelope of H. salinarium and it is likely that it plays a very important role in the structure of the cell envelope; e.g. in the maintenance of its shape. Unlike the majority of the envelope proteins, it remains bound to the lipid-enriched cell membrane at low salt concentrations and may serve as a bridge between the membrane and the less firmly bound membrane proteins (Mescher, et. al., 1974).

Intracellular salt concentration of extreme halophilic

It is clear that extremely halophilic bacteria live under conditions very unusual for life in general. So one of the very first obvious questions asked about extremely halophilic bacteria concerned their intracellular salt concentration. Do they have a very active pumping mechanism to maintain a "normal" internal salt concentration or is the latter the same as that of the growth medium? In fact, they do have an active pumping system, but its function is not to keep the internal salt concentration low; instead, it just keeps the sodium concentration relatively low and increases K^+ concentration inside the cell enormously. The intracellular ionic strengths of four different bacteria have been determined by Christian and Waltho (1962) and the data is shown in the Table II. It is very interesting to note that in H. salinarium K^+ is concentrated against a steep gradient and the intracellular concentration of KCl reaches a value close to

the solubility limit of this salt. Evidently water activity inside these cells must be exceptionally low.

TABLE II

Intracellular ionic concentrations in different bacteria

Intracellular concentrations are expressed as moles per Kg cell water

	Staphylococcus aureus	*Vibrio costicolus*	*Halobacterium salinarium*	*Sarcina morrhuae*
NaCl in medium (M)	0.150	1.0000	4.000	4.000
KCl in medium (M)	0.025	0.0004	0.032	0.032
Na^+ in cells	0.098	0.6840	1.370	3.170
K^+ in cells	0.680	0.2210	4.570	2.030
Cl^- in cells	0.008	0.1390	3.610	3.660

B. Halophilic enzymes:

Halophilic bacteria require a very salty external as well as internal environment for growth and survival. This is seen from enzymological studies. In general, halophilic enzymes reported so far can function only in high salt concentrations and most of them are inactive in the absence of salt. The presence of higher concentration of salts makes some of the standard techniques of protein purification like ammonium sulfate fractionation and column chromatography rather difficult. The high salt requirement for stability and activity makes these enzymes very interesting. However, the problems experienced in obtaining pure, active preparations in a reasonable yield has impeded detailed studies of halophilic enzymes. The enzymes isolated from these organisms so far are listed in Table III and are mostly involved in (1) proteolysis, (2) electron transport, (3) the citric acid cycle and (4) RNA metabolism.

Various methods have been used in the isolation and purification of halophilic enzymes. The procedures used to break the cells in order to release the enzymes include sonication, freeze-thawing, rupture with a French press, alumina grinding and homogenisation. Purification has been achieved by making use of methods like ammonium sulfate fractionation, column chromatography and Amicon ultrafiltration, all suitably modified for use in conditions of high ionic strength.

Some properties of a few halophilic enzymes.

In a number of halobacterium strains, three proteolytic activities were observed. They were an extracellular casinolytic

TABLE III

	Enzyme	Source	Reference
1)	Isocitrate dehydrogenase	*H. salinarium*	Aitken and Brown (1969)
2)	Fumerate hydratase	"	" " (1969)
3)	Malate dehydrogenase	"	" " (1969)
4)	Aconitate hydratase	"	" " (1969)
5)	Glutamate dehydrogenase	"	" " (1969)
6)	Glucose-6-Phosphate dehydrogenase	"	" " (1969)
7)	α-Ketoglutarate dehydrogenase	"	" " (1969)
8)	Citrate synthetase	"	" " (1969)
9)	Succinyl CoA synthetase	"	" " (1969)
10)	Isocitrate lyase	"	" " (1969)
11)	Succinic dehydrogenase	"	" " (1969)
12)	Lactic dehydrogenase	"	Baxter (1959)
13)	NADP specific Isocitrate dehydrogenase	"	Aitken *et al.*, (1970)
14)	Ornithine carbamoyltransferase	"	Dundas (1972)
15)	Proteolytic enzymes	"	Norberg and Hofsten (1969)
16)	Proteolytic enzymes	"	Norberg and Hofsten (1970)

TABLE III (cont'd)

	Enzyme	Source	Reference
17)	Polynucleotide Phosphorylase	H. cutirubrum	Peterkin and Fitt (1971)
18)	Cytochrome oxidase	"	Cheah (1970)
19)	Fatty acid synthetase	"	Pugh et al., (1971)
20)	DNA-dependent RNA polymerase	"	Louis and Fitt (1972)
21)	RNA-dependent RNA polymerase	"	Louis and Fitt (1972)
22)	Menadione reductase	"	Lanyi (1972)
23)	Alkaline phosphatase	"	Fitt and Peterkin (1976)
24)	Aminoacyl transcarbamylase	"	Griffiths and Bailey (1969)
25)	Adenosine Aminohydrolase	"	Bauer and Carlberg (1973)
26)	Asparatate transcarbamylase	"	Liebel et al.,(1969)
27)	Threonine deaminase	"	Lieberman and Lanyi (1972)
28)	Catalase	"	Lanyi and Stevenson (1969)
29)	Malic enzyme	"	Cazzulo and Vidal (1972)
30)	Isocitrate dehydrogenase	"	Hubbard and Miller (1969)
31)	L- Alanine dehydrogenase	"	Kim and Fitt (1977)
		H. salinarium	Holmes et al., (1965)
		"	Keradjopoulos and Wulf (1974)
32)	Amylase	H. halobium	Good and Hartman (1970)
33)	Ascorbate oxidase	"	Cheah (1969)

activity, a cell bound caseinolytic activity and a peptidase. All these enzymes showed no activity below 1M NaCl or KCl with optima between 2 and 4 M of each of the salt (Norberg and Hofsten, 1969).

Malate dehydrogenase purified 760-fold, from H. salinarium, but in vanishingly small yield, required at least 1M NaCl or KCl for optimum activity (Holmes and Halvorson, 1965).

Alkaline phosphatase from H. cutirubrum has been purified about 20-fold. High concentrations of salt, 0.5 M or above, are required for stability and activity. However, it still has appreciable activity in the absence of NaCl (or KCl) when there is enough Mn^{++} present (Fitt and Peterkin, 1976). (This enzyme has now been purified over 100-fold; private communication from Dr. P.S. Fitt.)

The effect of some salts and a few organic solvents on the activity of crude (five fold purified) H. cutirubrum catalase has been examined by Lanyi and Stevenson (1969). Monovalent salt stimulated enzyme activity threefold at concentrations between 0.5 and 1.5 M, whereas a 0.1 M concentration of divalent salts was required to cause similar effects. There was inhibition at higher concentration with all the salts. The effectiveness of a salt in inhibiting the enzyme at higher concentrations followed the pattern: $MgCl_2$ > LiCl > NaCl > KCl > NH_4Cl and LiCl > $LiNO_3$ > Li_2SO_4. Ethylene glycol, glycerol and dimethyl sulfoxide had optimum effects in the concentration range 3 - 5 M, but higher concentrations were inhibitory. The order of effect of the solvents depending upon concentration, stimulation at low concentration and inhibition at higher concentration was DMSO > glycerol > ethylene glycol. Therefore, the ability of these compounds

in inhibition and activation depends directly on their dipole moments.

L-alanine dehydrogenase from H. cutirubrum has been purified 100-fold and has an absolute requirement for K^+ for oxidative deamination, but the reverse reaction, reductive amination, proceeds equally well in the presence of NH_4^+, Na^+ or K^+. Another interesting property of this enzyme is that its activity in the standard reductive amination assay increases with temperature up to 70°C, but the enzyme itself is not thermostable (Kim and Fitt, 1977).

Adenosine aminohydrolase from H. cutirubrum has been purified 72-fold by introducing an aqueous polymer two phase fractionation step. It requires 4M NaCl at pH 7.5 for maximal activity (Bauer and Carlberg, 1973).

Citrate synthase from H. cutirubrum has been purified 400-fold using ammonium sulfate fractionation, chromatography on DEAE cellulose and hydroxyapatite and gel filtration on Sephadex G-200; but percentage recovery was rather low. Only 6% of the original activity was recovered in the final step of purification. It required 3M KCl for activity and stability. Other salts of potassium apart from KCl tested as activators show effectiveness in the following order $Cl^- > Br^- > No^- >$ SCN^-. Similar experiments done with different cations showed that Na and K were the most effective; NH_4Cl and LiCl were less effective and caused a relative inhibition at high concentrations. $CaCl_2$ and $MgCl_2$ were effective activators at concentrations upto 0.05 M but caused relative inhibition at higher concentrations. It was protected against inactivation in the presence of low salt concentrations, by its substrate oxaloacetate. The half life of the enzyme in the presence of

0.5 M - NaCl at 30°C, was increased from 0.55 min in the absence of oxaloacetate to 42.5 min in the presence of 108μm - oxaloacetate (Higa and Cazzulo, 1975).

Crude threonine deaminase from H. cutirubrum has allosteric properties. It has a sigmoidal substrate kinetics in the absence of ADP but in the presence of ADP it becomes hyperbolic, a property typical of an allosteric enzyme. In the absence of ADP enzyme activity increases with increasing concentration of NaCl and it is 2 - 2.5 times more active in 3.4M NaCl than in 0.05M NaCl. Low concentrations of $NaNO_3$ increase the activity while higher concentrations are inhibitory. $NaClO_4$ stimulates activity very slightly at low concentrations, but this salt is strongly inhibitory at higher concentrations. When ADP is present, all the three salts are inhibitory at high concentration. The rate of inactivation at low salt concentration is dependent upon pH. These properties show that threonine deaminase undergoes subtle, but significant changes in physical conformation as a result of its allosteric nature (Lieberman and Lanyi, 1972).

Bacterial aspartate transcarbamylase (ATCase) is a classical example of a regulatory enzyme. Liebel et al., (1969) reported results on a crude (3-fold purified) preparation of H. cutirubrum aspartate transcarbamylase. It required 1.5M NaCl plus 2.9M KCl for optimum activity and was highly sensitive to feedback inhibition by cytidine triphosphate. This inhibition itself was salt dependent, showing the largest percentage inhibition at 4M salt with no inhibition at 2 M or lower. Aspartate and carbamyl phosphate stabilised this enzyme. The most suitable temperature for its stability is 0°C. The rate of

inactivation accelerates with increase or decrease of this temperature. When carbamyl phosphate is used as the substrate, the substrate saturation curve is hyperbolic, but with aspartate it is sigmoidal. Polyethelene glycol, a precipitating agent causes separation of the subunits and the polyethylene glycol-treated enzyme is not inhibited by CTP. Therefore it appears that association of the subunits is essential for regulation of activity by the end product. On polyethylene glycol treatment the molecular weight of the enzyme is reduced to 34,000 which is about one fifth of the molecular weight of the crude enzyme (Norberg P. et al., 1973).

From the above examples, it can beeseen that extreme halophilic enzymes have many properties in common with the corresponding proteins from non halophiles but they require high ionic strength for their activity and stability. There are suggestions to explain salt-dependent properties of halophilic enzymes.

Some explanation on salt-dependent properties of halophilic enzymes.

Baxter (1969) first suggested that the function of salt is to reduce repulsive forces of electrostatic nature which causes the enzyme molecule to assume the catalytically active conformation. When salt is removed the enzyme will expand and will lose its catalytic ability. The salt dependent properties of these enzymes can be separated into three categories: (i) enzyme activity, (ii) stability and (iii) subunit association and allosteric effects.

The salt response of the enzyme activity of halophilic enzymes is highly variable. In initial reports activity was observed only in the presence of high concentrations of salt (Baxter, 1959); Baxter and Gibbons, 1956; Holmes et al., 1965; Larsen, 1967). However in many of these studies time dependent inactivation at lower salt concentrations before or during the enzyme assay was not taken into account. In more recent reports a number of enzymes have been shown

to be active with salt concentration as much as 0.1 M (Lieberman and Lanyi, 1971; Liebel et al., 1969; Hochstein and Dalton 1968, 1968a). Three enzymes (fatty acid synthetase and RNA-dependent RNA polymerase from H. cutirubrum; amylase from H. halobium) have been shown to be active in the absence of salt (Good and Hartman, 1970; Louis and Fitt, 1971; Pugh et al., 1971).

Although the salt requirement for enzyme activity is variable in halophilic systems, all enzymes investigated showed a time dependent inactivation in the absence of salt. The process of inactivation in a number of cases took place according to the first order kinetics (Lieberman and Lanyi, 1972; Norberg et al., 1973). In a few cases the salt dependence of enzyme activity is clearly separable from that of stability. For example the enzyme activities of isocitrate dehydrogenase and threonine deaminase of H. cutirubrum and malic dehydrogenase of H. salinarium are optimal at an intermediate NaCl concentration, but stabilities are increased with higher salt concentrations up to saturation (Lanyi, 1969). It is possible that at high salt concentrations the polypeptide chains of these enzymes can undergo a reversible tighter folding, which results in increase of activation energy of unfolding. Isocitrate dehydrogenase, malic dehydrogenase and four more halophilic enzymes which lose activity on lowering salt concentration could be partially reactivated by dialysis against 25% NaCl, although not by directly adding salt (Holmes and Halvorson, 1963, 1965; Hubbard and Miller, 1969). If is quite possible that inactive forms of these enzymes obtained after incubation at low salt concentrations are extensively unfolded. Holmes and Halverson (1965) showed that sedimentation constant of inactive malic

dehydrogenase is much lower than the active form: the $S_{20,w}$ value was reduced to 2.4 from 9.5. In the case of isocitrate dehydrogenase the same value went down to 2 from 5.3. Sedimentation velocity of complex proteins decreases on treatment, with guanidinium hydrochloride, a compound which is thought to cause extensive unfolding of polypeptide chains. The Stokes radius of the inactive form of isocitrate dehydrogenase is greater than that of its active form (Hubbard and Miller, 1969). These observations indicate that the process of inactivation results in expansion of the enzyme to a less folded state.

The proteins of the cell envelope and the ribosome of the halophilic bacteria are highly acidic. (Brown, 1963; Kushner and Onishi, 1966; Stoeckenius and Kunau, 1968; Steensland and Larsen, 1969; Bayley, 1966) Amino acid analysis of only the following two halophilic enzymes have been reported in the literature so far: DNA-dependent RNA polymerase and RNA-dependent RNA polymerase. Neither of them is particularly basic in nature and they both have a relatively low content of hydrophobic amino acid residue. (Louis and Fitt, 1972). Therefore, it has been suggested by Lanyi (1974) that the role of salt in promoting the stability and activity of halophilic enzymes is not necessarily to diminish ionic interactions, but also to increase hydrophobic interactions within the protein molecules.

In addition, Ingram (1947) suggested that the enzymes of extreme halophiles might have to be unusually small in order for them to remain in solution at the high ionic strengths found within these bacteria. Evidence in favour of this hypothesis has recently been provided by Fitt and his collaborators who have reported the unusually low molecular weights of five enzymes from <u>H. cutirubrum</u>

(Louis *et al.*, 1971; Fitt and Peterkin, 1976; Kim and Fitt, 1977).

Lanyi (1974) has reviewed the literature on the salt requirement of halophilic enzymes taking into consideration the available information about the interactions between salts and macromolecules in an attempt to provide a unified picture of the halophilic system.

C. DNA-dependent RNA synthesis

DNA-directed synthesis of RNA yielding RNA with a base sequence complementary to that of the one strand of the DNA template is called transcription. In this synthesis, RNA polymerase catalyzes the formation of internucleotide 3' to 5' phosphodiester bonds and thus plays a vital role in the transfer of information from DNA to RNA.

In 1959, Weiss and Gladstone first reported the existence of an enzyme in rat liver nuclei which requires all four ribonucleoside triphosphates and produced radioactively labelled product when radioactive substrate was supplied. Within a short while reports appeared showing the presence of a similar enzyme in bacteria and it was shown to be DNA-dependent (Hurwitz *et al*., 1960; Stevens 1960, 1961; Ochoa *et al*., 1961; Weiss 1960).

Ever since its discovery, RNA polymerase has been subjected to extensive studies both in prokaryotes and eukaryotes. The eukaryotic enzyme is much more complicated and it exists in different forms depending on its subcellular location. The different forms of the eukaryotic enzyme are much less stable than the bacterial ones. Therefore studies on these enzymes have not advanced as much as in the bacterial enzyme. Bacterial RNA polymerase has been purified from a number of bacteria. They include species of *Escherichia coli*, *Micrococus*, *Azoobacter*, *Bacillus*, *Pseudomonas* and *Caulobacter*.

RNA polymerase from *E. coli* has been studied most extensively and is an extremely complex enzyme system. This discussion will be

limited to those aspects of the structural and properties and of the mechanism of the *in vitro* synthetic reaction catalysed by the bacterial enzyme that are relevent to my work.

I. Molecular Properties:

a) Purification.

Purification of prokaryotic DNA-dependent RNA polymerase has been achieved by a number of procedures. However they can be grouped into three main categories.

The first method makes use of protamine sulphate precipitation of the crude enzyme as the major step, which is then followed by differential elution of the enzyme during DEAE-cellulose ion exchange chromatography. Chamberlin and Berg (1962) achieved a 150-fold purification with a yield of 45% in this way. The method was modified subsequently by introducing an ammonium sulphate fractionation step prior to protamine sulphate precipitation in order to lower the DNA content of the crude extract (Berg, *et al.*, 1971). This modification results in higher yields.

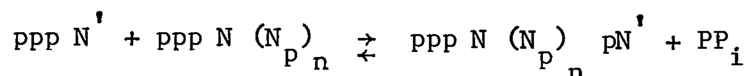
In another method, Zillig and his collaborators (1970) prepared RNA polymerase using polymin P, a quaternary amine-containing synthetic polymer, for initial precipitation of the crude enzyme followed by ion-exchange chromatography. Use of polymin P was advantageous because it binds to RNA polymerase much more strongly than protamine. However, it has the disadvantage that it inhibits RNA polymerase so strongly that the initial steps of purification cannot be followed by

enzymatic assays.

To avoid initial precipitation of contaminating nucleic acids by polymers as the first step, as in the first two methods, Burgess (1969) purified RNA polymerase by a different procedure which is now used more frequently than the other two in the purification of the bacterial RNA polymerases. In this method, the crude extract was treated with DNase, centrifuged, and fractionated with ammonium sulphate; it was than chromatographed successively on DEAE-cellulose, phospho-cellulose and agarose gel to give the core enzyme ($\alpha_2\beta\beta'$) with a yield of 56%. In their more recent publications, the same group modofied its procedure by introducing a polymin P precipitation of the crude extract as the first step. The polymin P precipitate is subjected to ammonium sulphate fractionation followed by chromatography on DNA-cellulose and Bio-gel A 5m (Burgess and Jendrisak, 1975). This method gave a yield of 45% and relative purification of 810-fold with a high sigma factor content. It is also very rapid. RNA polymerase from 500 gms of cells was purified to electrophoretic homogeneity yielding 250 mg of the holoenzyme.

b) Assay conditions.

The reaction catalysed by DNA-dependent RNA polymerase can be represented as follows:



$$\text{ppp N}' + \text{ppp N } (\text{N}_\text{p})_n \rightleftarrows \text{ppp N } (\text{N}_\text{p})_n \text{ pN}' + \text{PP}_i$$

where ppp N' and ppp N denote ATP, GTP, CTP, UTP. and PP_i inorganic pyrophosphate. The most common in vitro assay for this enzyme is the measurement of the amount of labelled nucleoside monophosphate (AMP,

GMP, UMP or CMP) incorporated into an acid insoluble product. A standard _in vitro_ reaction mixture contains all four nucleoside triphosphates, a template which is either DNA or some form of polydeoxynucleotide polymer and in certain cases a sulfhydryl agent. The pH at which the assay is done is of the order of 7.5 - 8.0. An enzyme unit is defined as the amount of enzyme needed to give a rate of incorporation of 1 nmole of the labelled nucleotide in 20 minutes or 1 h at 37°C. The catalytic reaction involving RNA polymerase consists of more than one individual step and this assay measures the total amount of RNA formed in the overall reaction. Various factors influence the rates of the different individual steps involved in this complex enzymic reaction. These include the ionic strength, and the concentrations of the nucleoside triphosphates, the divalent cation and the template. During regular enzyme purification, the activity is measured using a particular combination of reagents in the same conditions.

c) _Structure of the enzyme_.

Bacterial RNA polymerase is not a single polypeptide chain. However, irrespective of the source all well characterised prokaryote RNA polymerase isolated from non-halophiles resemble each other in subunit structure, although the molecular weights of the different subunits show variations from one bacterial species to another.

The enzyme from _Escherichia coli_ is composed of at least four different polypeptide chains α, β, β' and σ (Burgess 1969) and preparations frequently contain a minor component ω (Berg, _et al_.,

1971). On the basis of known molecular weights and the relative content of these components, the complete enzyme appears to have the structure $\alpha_2\beta\beta'\sigma\omega_2$.

The enzyme without the σ subunit with the structure $\alpha_2\beta\beta'$ is called the core enzyme. Core enzyme has the ability to synthesize RNA, but it cannot initiate RNA synthesis from the DNA duplex (Vogt 1969; Ishihama et al., 1971). Sigma has no synthetic activity of its own. However, only in the presence of sigma can the enzyme transcribe phage DNA. The complete enzyme $\alpha_2\beta\beta'\sigma$ is called the holoenzyme.

d) Molecular weight and sedimentation properties.

The study of the physical properties of RNA polymerase, especially its size, has been difficult because it is a complex protein with several chains whose properties are functions of a number of variables. The variables which affect the sedimentation coefficient and the molecular weight are (i) ionic strength (Stevens et al., 1966), (ii) age (Richardson, 1966), (iii) the presence of polynucleotides (Smith et al., 1967) and (iv) substrates (Berg and Chamberlin, 1970). The holoenzyme aggregates at an ionic strength below 0.1 whereas aggregation of the core polymerase has been detected at an ionic strength as high as 0.2. Therefore, molecular weight determination should be done at high ionic strength in order to obtain reliable results. The degree of aggregation of the holoenzyme depends on the amount of the σ subunit which can vary from preparation to preparation. The sedimentation coefficient is affected by dissociation of the holoenzyme to a mixture of the core enzyme and the σ subunit

during the period of equilibrium sedimentation. The molecular weight of the E. coli core enzyme measured by equilibrium sedimentation method is found to be 383,000 which compares well with the value 380,000 - 400,000 determined on the basis of the molecular weights of the individual subunits of the structure $\alpha_2\beta\beta$. The molecular weights of the individual subunits have been determined by polyacrylamide gel electrophoresis after denaturation with SDS. They are in the range β' = 150,000 - 165,000; β = 145,000 - 155,000; σ = 86,000 - 95,000; and α = 39,000 - 41,000 and ω = 9,000 - 12,000. The variability of these values is due to lack of proper markers of precisely known molecular weight in the range over 90,000 (Burgess, 1968).

II. Catalytic Properties:

DNA-dependent RNA polymerase carries out three different types of reaction. They are: -

1) Template-directed synthesis of complimentary polyribonucleotides.

2) Template-directed synthesis of homopolymers of ribonucleotides.

3) Unprimed synthesis of polyribonucleotides.

a) The DNA-directed reaction.

The catalytic reaction of DNA-dependent synthesis of ribonucleic acid catalyses by RNA polymerase has been subdivided into four different steps. The commonly accepted steps are:

i) Template binding: the step in which the enzyme attaches itself to the template DNA and locates a site for initiation.

ii) Initiation: the step in which formation of first phosphodiester bond with subsequent release of inorganic pyrophosphate takes place.

iii) Elongation: in which addition of nucleoside monophosphate units to the 3' - OH terminus of the nascent RNA chain from the corresponding nucleoside triphosphates occurs.

iv) Termination: the step in which the RNA chain formed is released from the template and the enzyme.

These steps will each be considered in greater detail.

i) Template binding.

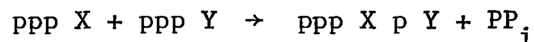
Interactions between RNA polymerase and helical or single stranded polynucleotides take place on mixing of the two in the absence of any divalent cation. Reaction between RNA polymerase and three different kinds of DNA, (Pappilloma DNA, Polyoma DNA and T7 DNA) have been studied by using the techniques: (i) zonal gradient sedimentation analysis (Richardson, 1966; Pettijohn and Kamiya, 1969), (ii) electron microscopy (Crawford, 1965; Harford and Beer, 1972; Murti, *et al*., 1972), (iii) the membrane filter technique (Jones and Berg, 1966; Hinkle and Chamberlin, 1972).

There are two kinds of binding between *E. coli* RNA polymerase and T7 DNA (Pettijohn and Kamiya, 1969). The majority of the polymerase

molecules dissociate from the template very easily. These weak binding sites on the template are called class B sites. There is another class of sites where binding between the enzyme and the template is very stable and is highly dependent on temperature. These sites are called class A sites. Only about eight holoenzyme molecules at saturation are bound in these complexes and their stability is very high; at 37°C the half time for dissociation has been estimated to be 30-60 hours (Hinkle and Chamberlin, 1972).

ii) Initiation of RNA chains.

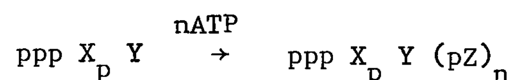
Bacterial RNA polymerase can initiate *de novo* RNA synthesis where two ribonucleoside triphosphate molecules couple to produce a dinucleoside tetraphosphate (Maitra, et al., 1967).



$$\text{ppp X} + \text{ppp Y} \rightarrow \text{ppp X p Y} + PP_i$$

RNA synthesis is exclusively initiated with purine nucleotides and the relative proportion of the chains initiated with ATP or GTP are characteristic of the DNA template used. Initiation can be quantitatively measured by using nucleoside triphosphates labelled in the γ-phosphate group, since the 5′-terminal triphosphate is conserved in the product RNA (Kleppe 1972; et al., 1972).

The dinucleotide formed as a result of initiation is then elongated by subsequent addition of nucleoside monophosphate to 3′-OH terminus of the growing chain at the direction of the template. The elongation reaction can be represented as follows:



$$\text{ppp } X_p \text{ Y} \xrightarrow{\text{nATP}} \text{ppp } X_p \text{ Y } (pZ)_n$$

Both initiation and elongation involves formation of phosphodiester bonds. But the difference is in the case of initiation the acceptor of the incoming nucleoside monophosphate moiety is the 3' -OH terminus of a nucleoside triphosphate whereas in elongation this the 3' -OH terminus of the growing polyribonucleotide or ribonucleic acid chain. The specificity of the initiation reaction can arise in two ways:

(1) there is template-directed specificity which specifies the substrate or ribonucleoside triphosphate for the reaction;

(2) there are different intrinsic sites in the enzyme for initiation and elongation and therefore substrates for initiation can be identified.

Initiation does not take place all over the DNA template: there are restricted regions on it where RNA polymerase can start a new chain. However, there can be multiple initiation sites on a DNA template. An initiator or promoter site is defined as a particular sequence in DNA recognised by RNA polymerase holoenzyme itself at which an RNA chain can be initiated. Watson-Crick rules of base pairing are applicable in determining the nucleoside sequence of RNA. The proper sequence of nucleotides in 5' - terminus of the RNA chain is determined by the sequence of nucleotides in the DNA template. This has been shown with synthetic templates of well characterised sequence (Terao, et al., 1972).

The postulate of site specificity of RNA polymerase for

initiation has been based on studies of RNA chain initiation with synthetic DNA templates. Kleppe and Khorana (1972) have shown using synthetic templates that initiation takes place exclusively with ATP or GTP. However when a synthetic template containing only purine bases is used, transcription takes place very slowly (Straat and Ts'o, 1969). These results and the fact that initiation reaction is bimolecular reaction supports the idea that bacterial RNA polymerase contains at least two kinds of binding sites for ribonucleoside triphosphates. One site should be specific for binding that nucleoside triphosphate which is bound to become the 5' - terminal triphosphate. The second site would bind the nucleoside triphosphate to be added to the first nucleoside triphosphate molecule. But it is quite conceivable that the second site involved in initiation may also take part in elongation.

The idea of RNA polymerase having initiation sites is further supported by pyrophosphate exchange studies involving the β and γ positions of the nucleoside triphosphates. The reaction of RNA polymerase was allowed to take place with poly d(A-T) and poly c(I-C) as templates. RNA polymerase directed by poly d(A-T) carries out an active exchange of PPi into UTP in the presence of catalytic amount of ATP. In other words the predominant reaction in these conditions is

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

but not

$$\text{pppA} + \text{pppU} \rightarrow \text{pppUpA} + \text{PPi}$$

Moreover, the requirement for ATP can be satisfied, with variable

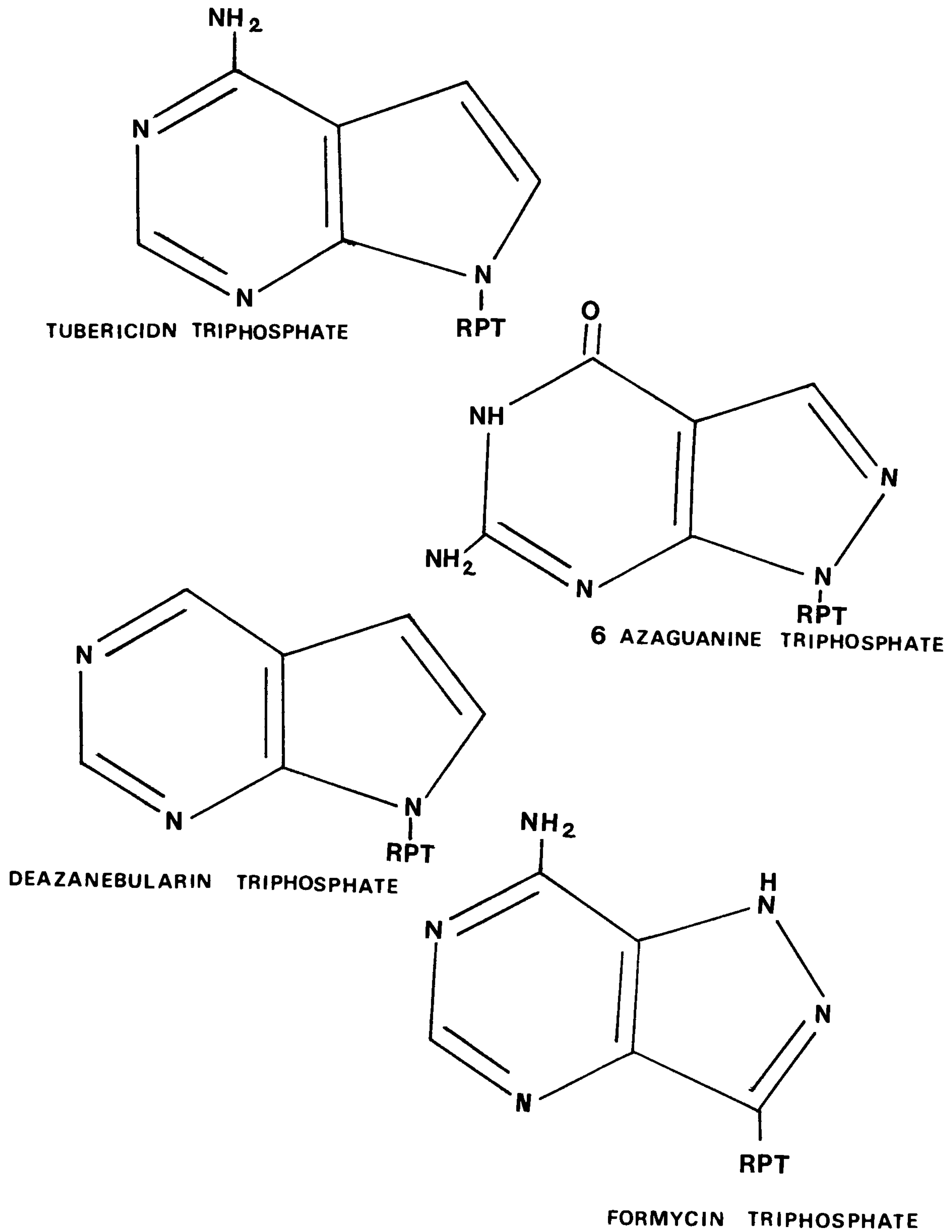
efficiency, by adenosine tetraphosphate, ADP and 5'-AMP. In the reaction directed by poly d(I-C) as template, an active exchange of pyrophosphate into CTP is absolutely dependent upon GTP. Thus the nucleoside initiation binding site appears to be specific for binding ATP or GTP as compared with UTP or CTP (Krakow and Fronk, 1969).

Further evidence on RNA polymerase having specific initiation sites comes from studies made with substrates which are different from usual substrates, having modified base and sugar moieties. A number of compounds have been synthesized which have structures analogous to purine nucleoside triphosphates. Examples are (i) tubercidin triphosphate (Nishimura et al., 1966), (ii) 6-azaguanine triphosphate (Darlix et al., 1971) and (iii) deazanebularin triphosphate (Ward and Reich 1972). Some of these analogs of purine nucleoside triphosphates used as substrates for the polymerisation reaction are shown in the Figure I. When these analogs were used to replace ATP and GTP, RNA synthesis is reduced remarkably (Ward and Reich, 1972). This is likely to happen as a result of reduction in chain initiation. So this is a good evidence for RNA polymerase having specific sites for the substrates of initiation that show a definite preference for the normal substrates ATP and GTP.

iii) Elongation of the RNA chain.

Elongation of the compound formed during initiation is the least complex of all the steps in the synthesis of RNA catalysed by DNA-dependent RNA polymerase. In the first step of RNA synthesis, called template binding, a binary complex is formed between RNA

FIGURE I



polymerase and the template DNA. In the next step, which is the initiation step the binary complex gets converted to a ternary complex by addition of the dinucleotide tetraphosphate. Conversion of the binary complex to a ternary one greatly increases its stability. This complex is stable at elevated ionic strength and low temperature. Ternary complex formation causes changes in the physical and chemical properties of the different constituents. The enzyme becomes more resistant to attack by trypsin (Khesin et al., 1967) and to heat denaturation (Stead and Jones, 1967). The region of the DNA template which is part of the complex becomes resistant to attack by DNase (Kameyama et al., 1969). Circular dichroism spectra of the DNA of the ternary complex show that the portion of the DNA bound to RNA polymerase undergoes transformation from one form to another (Beabealashvilly, 1972).

The chain elongation does not require the σ subunit. σ factor is released from the enzyme system as soon as the ternary initiation complex is formed. This is shown by sedimentation and electrophoresis studies. In zone sedimentation and electrophoresis of the ternary complex, the σ subunit is observed as an independent entity rather than part of the ternary complex (Gerard et al., 1972). This is also shown by *in vivo* studies of the ternary complex (Pettijohn et al., 1971).

The nucleotide sequence of ribonucleic acid chain formed during elongation is directed by the base sequence of the template DNA. In this case also, the Watson-Crick model of formation of hydrogen bonding is the guiding factor in determining the

sequence of nucleotide in the RNA chain. Is this rule followed one hundred percent by RNA polymerase? Can there be an incorrect base in the sequence of nucleotide formed when a template is copied? With alternating copolymers dAT or dAC-dTG as templates, frequency of formation of an incorrect sequence has been studied by analysing the product formed with the technique of nearest neighbour analysis. Instances of error frequencies found in these studies is of the order of one in three thousand (Bujard and Heidelberger, 1966).

Theoretically base pairs can be formed between C and U (or T) and G and U (or T). But in practice these pairing combinations do not normally dictate the synthetic reaction. Therefore the ability to form a base pair per se is not the only factor driving the elongation step of the RNA synthetic reaction.

RNA polymerase has specific sites for the substrates of initiation. There are evidences that similar specific sites are present in the enzyme for the substrates of elongation. This is shown by studies made using compounds with structures analogous to the normal substrates and DNA templates having modified DNA sequence. Substrate analogs are of the types in which the base has been altered by deamination, halogenation, alkylation and also purine and pyrimidines with modified ring structure. Modification in the nucleotide sequence of DNA has been achieved by deamination, halogenation, methylation and by mutagenic agents like hydroxylamine and ultraviolet light. When GTP was replaced by deazanebularin triphosphate (Fig. I page30) elongation proceeded as usual even though it cannot form any hydrogen bond with cytosine. Deanzanebularin triphosphate serves as an analog for ATP

and so is ambiguously incorporated into RNA (Ward, D., and Reich, E., 1972). Another observation which supports the argument that base pair alone is not a sufficient condition for elongation to proceed and is also more convincing is as follows: when methylated poly (rC) containing 3-methyl cytosine was used as a template extensive incorporation of UMP took place although methylation must block base pairing altogether (Ludhlen and Wilhelm, 1968).

Chain growth of the RNA molecules take place in the 5' to 3' direction. When the product formed by enzymatic synthesis in the *in vitro* condition is subjected to alkaline hydrolysis it yields a nucleoside tetraphosphate (pppNp) from the 5' end and a nucleoside (N) from the 3' end and the remaining nucleotides as 2' and 3' monophosphates (Bremer *et al.*, 1965). Further evidence for 5' -3' growth of RNA chains is derived from the demonstration that the ^{32}P-incorporated from the γ-^{32}P nucleoside triphosphates cannot be removed by chain elongation. Accordingly chain growth must proceed from the 3' -end (Maitra and Hurwitz, 1965).

iv) RNA chain termination.

Termination is the least understood all the different steps of *in vitro* RNA synthesis because there is no assay procedure specific for termination. However, in principle it can be visualized that termination must involve three steps: (1) termination of synthesis of the RNA chain; (2) release of the chain along with the enzyme from the template; and, finally, (3) separation of the RNA product and the enzyme.

The existence of three different kinds of RNA, tRNA, mRNA and rRNA gives rise to the speculation that there are termination regions in the DNA template. The sequence of nucleotides in the DNA template, recognised by RNA polymerase itself, at which synthesis RNA chain ends is called the terminator sequence. The terminator sequence for bacterial RNA polymerase has been identified with DNA templates from the following bacteriophages using the technique of hybridisation followed by mapping: T7DNA (Minkley and Pribnow, 1973), fd RFI (Takanami et al., 1971) and λDNA (Blattner and Dahlberg, 1972).

The role of ρ factor in termination. A protein termed the ρ factor, has been shown to influence RNA chain termination mediated by E. coli RNA polymerase. Roberts (1969) has isolated and purified the ρ factor in E. coli which is a tetrameric protein of molecular weight 200,000. It does not exert its influence at elevated ionic strength (Richardson, 1970). It can bind reversibly to DNA and it is released at high ionic strength or by denatured DNA. The length of the RNA formed depends upon the presence or the absence of the ρ factor. In the presence of the ρ factor, with T4DNA as template, E. coli RNA polymerase synthesizes RNA molecules consisting of about 2,000 nucleotides. But in the absence of the ρ factor the length of the product increased to 4,000 - 7,000 nucleotides (Witmer, 1971). The fact that such short transcripts are formed in the presence of ρ in vitro suggests that ρ - induced termination may not be relevant to the in vivo situation. The relationship between site specific termination and ρ induced termination as it occurs in vivo will require further study.

Effect of ionic strength on termination. Ionic strength is another parameter which is found to influence RNA chain growth and termination catalysed by DNA -dependent RNA polymerase. RNA synthesis at high ionic strength is more extensive than what it is at low ionic strength. Most of the RNA made at low ionic strength remaind attached to the enzyme DNA complex; higher ionic strength releases the product and reinitiation of RNA synthesis takes place (Richardson, 1968). The products made under these conditions are of discrete size indicating termination of RNA chains at specific sites (Maitra and Barash, 1969). Salt induced termination is likely to take place as a result of decrease in binding affinity of RNA polymerase for DNA even though it is part of the ternary complex due to increase in ionic strength.

D. H. cutirubrum DNA-dependent RNA polymerase:

General Introduction

RNA polymerase from H. cutirubrum has been studied to a considerable extent by Louis and Fitt (1971; 1971a, b; 1972a, c;). The most interesting observation about H. cutirubrum DNA-dependent RNA polymerase is that it has an unusually small molecular weight in comparison to RNA polymerases from other bacterial sources. It is an enzyme with two subunits of identical molecular weight of around 18,000. The two subunits are designated as α and β. The complete enzyme has one α and one β subunit and it is designated as $\alpha\beta$. The molecular weight of the complete enzyme ($\alpha\beta$) is about 36,000 which is about one tenth of the molecular weight of the enzyme from E. coli. The standard enzymatic reaction takes place in the presence of the complete enzyme.

Isolation and Purification

A very high concentration of salt (e.g., 2.5M-KCl-1M-NaCl, total 3.5M) is required throughout the different steps of isolation and purification. Bacteria grown in the complex medium of Sehgal and Gibbons as modified by Gochnauer and Kushner were harvested in the middle or late log phase. Then a smooth suspension of bacteria was made in a buffer of pH 8.6 having salt concentration mentioned earlier. The enzyme was released by sonicating the suspension for a given length of time at a particular intensity. The sonicated suspension was subjected to two centrifugations; the first one at low speed and the supernatant from this spin was centrifuged at a high speed in

in a preparative ultracentrifuge. Enzyme activity was found in the supernatant from the high speed centrifugation and was called the crude extract of the enzyme. The crude extract was taken through a series of steps to obtain a mixture of α and β subunits. The first step in the process of purification of the crude extract was to lower its pH to 4-5 when the DNA-dependent activity remained in the supernatant. The latter was concentrated to a smaller volume by dialysis against Ficoll solution and was then loaded onto a P-60 gel-filtration column prepared and run at high ionic strength (3.5 M). The active fractions from the P-60 gel-filtration was concentrated and subjected to hydroxylapatite column chromatography to obtain the subunits. A relative purification of 5,000 was obtained by this procedure, and the subunits were electrophoretically homogeneous.

Salt Requirement

A high salt concentration is essential for stability and activity of the complete enzyme. The salt concentration used for storage of the enzyme was 3.5 M and for maximum activity the salt requirement was between 2 and 2.5 M (Louis and Fitt, 1970). When salt was dialysed out the enzyme lost its activity completely and irreversibly within twenty four hours, so that it was a typical halophilic enzyme. In the presence of high salt the complete enzyme ($\alpha\beta$) maintained its activity at least for sixty days. However, the salt requirement for stability of the individual subunits was different. When salt was removed from the individual subunits by dialysis inactivation took place in markedly different rates. Protein α was fully active even after dialysis against salt free buffer for

four to twenty four hours and in the salt free condition.

Template requirement

The enzyme has a selective template requirement, so that the salt requirement for activity depended on the template used. With DNAs from eukaryotic sources (calf thymus and salmon sperm) as templates, activity was seen only in the absence of salt. In contrast, with DNAs from prokaryotes and bacteriophage T7, a high ionic strength was absolutely necessary for enzyme activity (Louis and Fitt, 1972a).

Role of Metal ions

H. cutirubrum RNA polymerase appeared to have an absolute requirement for both Mn^{++} and Mg^{++} (Louis et al., 1971; Louis and Fitt, 1971b). Mn^{++} was required to form a complex of the two subunits in the ratio 1:1 and Mg^{++} was apparently responsible for binding of the complex to DNA. When the complete enzyme ($\alpha\beta$) was passed through a column of Bio-gel P-60 in the presence of Mg^{++} and H. cutirubrum DNA the K_{av} was the same as that of the individual subunits. However in the presence of Mn^{++} the subunits associated with K_{av} corresponding to a molecular weight of about 36,000, as expected from the 1:1 complex of α and β. K_{av}, the average partition was calculated from the equation $K_{av} = (Ve - Vo)/(Vt - Vo)$ (Fischer, 1969) where Vo is the void volume of the column, Ve is the elution volume of the particular protein and Vt is the total column volume.

Effect of Antibiotics

The antibiotic rifampicin inhibits DNA-dependent RNA polymerase from H. cutirubrum (Louis and Fitt 1972). The concentration of rifampicin used in these studies was 1.2×10^{-6} M. Rifampicin inhibits the initiation step of the reaction, because incorporation of γ -labelled radioactive substrate was reduced considerably in the presence of the antibiotic, but the latter had little effect on chain elongation when added after the reaction had commenced. Moreover the inhibition by rifampicin could be relieved completely by adding excess of the β subunit in the reaction mixture. In the standard inhibition reaction, equal amounts of both of the subunits were present and an excess of the α subunit failed to prevent inhibition by the antibiotic.

Neither of the subunits alone could catalyse formation of the first phosphodiester bond. This was shown by inhibition studies. When the assay mixture containing only one subunit was incubated for the standard reaction and the other subunit was then added together with rifampicin no incorporation of radioactive substrates into an acid-in-soluble form took place.

It was therefore concluded that the β subunit contained the rifampicin binding site and was required for initiation (Louis and Fitt, 1972).

The Primed Reaction

RNA polymerases from other bacterial sources contain the subunit called the σ subunit which has a unique role in the catalytic reaction

(the role of the σ subunit has already been discussed while describing bacterial RNA polymerase in general).

RNA polymerase from H. cutirubrum apparently does not have a sigma-like factor and full activity requires α and β subunits together. However, the α subunit has some special properties. When one of the dinucleoside phosphates A_pA, A_pU or G_pU was added in the standarded assay mixture containing the α subunit only, the polymerisation reaction occurred at a rate which was comparable to that observed when both α and β subunits were present. The dinucleoside phosphate apparently acts as a primer by supplying the first phosphodiester bond, since no initiation of new chains could be detected under these conditions. The primed reaction required a DNA template, but it was insensitive to the inhibitor rifampicin. The length of the chain produced in the primed reaction was independent of the length of the oligonucleotide primer (Louis and Fitt, 1972b) but was inversely proportional to the concentration of the latter provided a certain minimum amount was present (Louis and Fitt, unpublished work).

The Roles of the Subunits in the Mechanism of the Reaction

The following conclusions can be drawn about the subunits on the basis of the inhibition studies and the nature of the primed reaction:

I) the α subunit contains the catalytic site, but is unable to initiate new chains;

II) the β subunit contains the inhibitor binding site;

III) α and β are required together for normal initiation.

E. Other work on H. cutirubrum DNA-dependent RNA polymerase

Chazan and Bayley (1973) have published results of preliminary experiments on H. cutirubrum DNA-dependent RNA polymerase. The enzyme obtained by these authors has many properties which are different from the properties of the same enzyme described by Louis and Fitt, discussed in the previous pages.

These authors isolated the enzyme in a complex form with DNA, protein and membrane. It was released from the complex by treatment with pancreatic deoxyribonuclease I. The isolated enzyme was partially purified by sephadex G-100 column chromatography. The authors claimed that direct comparison of specific activities between the complex and the partially-purified enzyme was not possible because initiation must take place with the isolated enzyme whereas elongation of nascent RNA chains alone could very well account for incorporation by the complex.

The complex had salt tolerance over a wide range of concentrations, whereas the partially purified enzyme was inactive at salt concentrations above 0.4 to 0.5 M when salmon sperm DNA was used as template. Streptovaricin and rifampicin did not inhibit the isolated enzyme, whereas Actinomycin D and Acriflavin, which are known to interact with the DNA template, did. Satisfactory incorporation was observed with a number of DNAs as templates: including salmon sperm DNA, heat denatured salmon sperm DNA, E.coli DNA, calf thymus DNA and H. cutirubrum DNA. Poly C and whole yeast RNA were not effective as templates.

The size of the isolated enzyme was roughly estimated by

the method of sucrose density gradient centrifugation. The molecular weight is reported to be in between 300,000 and 400,000 daltons. With respect to metal ion requirement the enzyme is twice as active in the presence of Mg^{++} as in the presence of Mn^{++}. Although activity was observed in a concentration range 10-80mM Mg^{++} 40mM Mg^{++} gave the maximum activity.

F. Inhibitors of RNA polymerase:

The reaction catalysed by DNA-dependent RNA polymerase is inhibited by two classes of compounds. One class causes inhibition by binding to the template DNA. These compounds form non-covalent complexes with DNA resulting in weakening of its template function. There are a number of these compounds reported in the literature. They include, Actinomycins, Proflavine, Chromomycin A , Methramycins, Echinomycin, Olivomycin, Anthracyclines, Rubiflavin, Hedamycin, Pluramycin, Distamycin A, Ethidium Bromide, Phleomycin, Anthramycin, Luteoskyrin, Kanchanomycin, Miracil D, Aflatoxin, 2-Acetylaminofluorene and Polyamines.

The second class of inhibitors are known as protein-specific inhibitors. Inhibition by these compounds takes place as a result of their interaction with the enzyme protein and there are very few of them. They include: rifamycin and its derivatives, streptovaricin, streptolydigin and α -aminitin. α -aminitin is a specific inhibitor certain types of eukaryotic RNA-polymerase. Similarly, streptolydigin and streptovaricin act on bacterial RNA polymerases only.

Rifampicin, a synthetic derivative of rifamycin B, has been extensively used to study the reaction mechanism of bacterial RNA polymerase.

Rifamycin and its derivatives.

The rifamycin antibiotics are fermentation products of Streptomyces mediterranei and were discovered in 1957. A large

number of rifamycin derivatives have been synthesized at the Lepetit Research Laboratories in Milan, Italy and by Ciba-Geigy in Basel, Switzerland. The biological activities of many of these compounds are not known and, without doubt, it will involve many years of work to determine them.

The major compound of fermentation, rifamycin B does not have any antibacterial activity. But on oxidation followed by hydrolysis it forms a compound called rifamycin S. It can inhibit the growth of gram positive bacteria at concentrations as low as 0.0025μg of antibiotic per ml (Sensi *et al*, 1967).

Structurally the rifamycins consists of a napthoquinone chromophore which is spanned by an aliphatic ansa chain. The relationship between chemical structure and antibacterial activity has been studied in order to synthesize derivatives with biological properties more potent than those obtained by natural fermentation. It has been found that modification of the side chain of the aromatic ring (as in Rifamycin S, Rifamycin SV, Rifamycin B and Rifampin) does not have any effect on the inhibition of the isolated enzyme where as *in vivo* bacterial growth was dependent upon the structure of the compound (Bickel *et al*., 1967; Kradolfer *et al*., 1967). Rifamycin B although very active with the isolated enzyme does not affect growth of bacteria. This is likely to happen as a result of lack of cell permeability (Maggi *et al*., 1968). Whereas modification in the aliphatic chain significantly changes both *in vitro* and *in vivo* effects. Hydrogenation of double bonds in the ansa chain reduces the inhibitory property of the molecule presumably by affecting the

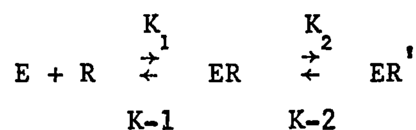
rigidity of the ansa chain (Wehrli and Staehelin, 1969). The detailed understanding of the effect of the chemical structure of rifamycin derivatives on their antibacterial activity will require further studies.

The reaction between RNA polymerase and rifampicin

Rifampicin inhibits the initiation step of the catalytic reaction. When it is added to the in vitro reaction mixture after prior incubation in the absence of the antibiotic, no inhibition was observed (Sippel and Hartmann, 1968). Complex formation between the enzyme and the drug takes place just on simple mixing of the two. This complex has been isolated by passage through a Sephadex column (Wehrli et al., 1968). Holoenzyme and the core enzyme bind the drug equally well with the same 1:1 stoichiometry. This indicates that it is not the sigma (σ) subunit that binds rifampicin. Experiments done with isolated subunits have proved that it is the β subunit that binds rifampicin (Wehrli et al., 1968). The reaction between rifampicin and the RNA polymerase β subunit was studied further by Heil and Zillig (1970) who isolated RNA polymerase and then the individual subunits from a rifampicin-resistant E. coli mutant. Studies of physical properties of the individual subunits showed that the β subunit from the resistant strain had a different electrophoretic mobility on cellulose acetate sheets from that of the β subunit from sensitive strains. Experiments involving mixed reconstitution also demonstrated the role of the β subunit in the inhibition by rifampicin. When all the subunits except β from the resistant mutant were used for reconstitution the enzyme was rifampicin

sensitive, while the enzyme obtained by using α and β' subunits from the sensitive mutant and β from the resistant strain was insensitive to rifampicin (Heil and Zillig, 1970).

On the basis of their kinetic studies, Yarborough et al., (1976) proposed a two step mechanism for interaction between RNA polymerase and rifampicin. According to this model, subsequent to bimolecular binding between the enzyme and rifampicin, the binary complex undergoes a slow unimolecular isomerisation:



$$E + R \underset{K_{-1}}{\overset{K_1}{\rightleftarrows}} ER \underset{K_{-2}}{\overset{K_2}{\rightleftarrows}} ER'$$

where E is the enzyme, R is rifampicin, ER and ER' are two isomers of rifampicin-enzyme complex and K_n is the rate constant, for the appropriate reaction.

This model of interaction between RNA polymerase and rifampicin is based on the following observations:

1. Rifampicin binds tightly to both holoenzyme and the core enzyme which indicates that the sigma (σ) subunit has no role in binding of rifampicin to RNA polymerase.

2. The Stoichiometry of binding between rifampicin and core enzyme or the holoenzyme is 1:1.

3. At high salt concentrations ($\geq$0.2M KCl), two molecules of rifampicin bind with the enzyme, since the enzyme exists as a dimer in these conditions.

4. Rifampicin is a non-competitive inhibitor with respect to nucleoside triphosphates.

5. The pseudo - first order rate constant is not dependent on rifampicin concentration, indicating the reaction is not a simple bimolecular one.

6. The rate of the forward isomerisation step is much greater than the backward step, i.e. $k_2 >> k_{-2}$

7. The rate of the forward step in isomerisation, k_2, decreases in the order of core enzyme > holoenzyme > the holoenzyme-T7DNA complex.

8. The elongation complex obtained by limiting the concentrations of one of the nucleotides bound rifampicin as well as the free holoenzyme or the holoenzyme-T7DNA complex. Therefore, the enzyme molecule actively engaged in elongation can still bind rifampicin.

9. The two step mechanism of action of rifampicin on RNA polymerase favours the observation of Johnston and McClure (1976). These authors claim rifampicin only partly inhibits the synthesis of dinucleotide tetraphosphates by RNA polymerase. This is explained by the following fact: there are two RNA polymerase-rifampicin complexes, only one of which is able to bind to the initiating triphosphates and catalyze the phosphodiester bond formation.

G. Aim of the work

These experiments had two principal aims. The first was to develop a modified method for the purification of the α and β subunits of H. cutirubrum DNA dependent RNA polymerase on a scale significantly greater than possible by the procedure described by Louis and Fitt (1972a). The second was to confirm the findings of Louis and Fitt (1972a) by an independent approach and to resolve the conflict between their results and those of Chazan and Bayley (1973) concerning the molecular weight and subunit composition of the enzyme and the effect of rifampicin on its activity.

II. EXPERIMENTAL

A. Materials

Chemicals and enzymes were purchased from the following suppliers.

Amersham/Searle Corp., Don Mills, Ont., Canada: ^{14}C-labelled nucleoside triphosphates and 2,5 - diphenyloxazole (PPO).

BDH (Canada) Ltd., Toronto, Ont., Canada: sodium dodecyl sulfate, specially pure.

Bio-Rad Laboratories, Richmond, Calif., U.S.A.: Bio-Gel P-60 Polyacrylamide gel beads, Bio-Gel HTP hydroxylapatite powder.

Calbiochem, Los Angeles, Calif., U.S.A.: cyclohexylaminopropane sulfonic acid (CAPS), tris (hydroxymethyl) methyl-aminopropane sulfonic acid (TAPS), tris (hydroxymethyl) methyl - 2 - aminoethane sulfonic acid (TES).

Canadian Laboratory Supplies Ltd., Montreal, P.Q., Canada: Triton X-100 non-ionic detergent.

Fisher Scientific Company, Ottawa, Ont., Canada: common laboratory chemicals.

Gallard-Schlesinger Chemical Mfg., Cork Place, L.I., N.Y., U.S.A.: Polyethyleneimine - (PEI) - Cellulose for thin layer chromatography.

General Biochemicals, Chargin Falls, Ohio, U.S.A.: E. coli DNA.

ICN Chemical and Radioisotope Division, Irvine, Calif., U.S.A.: ^{32}P-labelled nucleoside triphosphates.

Mann Research Laboratories, New York, N.Y., U.S.A.: rifampicin and sperm whale myoglobin.

Pharmacia (Canada) Ltd., Montreal, P.Q., Canada: Sephadex G-75, Blue-Dextran and Ficoll.

P-L Biochemicals, Inc., Milwaukee, Wis., U.S.A.: unlabelled nucleoside triphosphates.

Sigma Chemical Co., St. Louis, Mo., U.S.A.: glycine, Trizma base (Tris), adenosine, cytochrome type VI (horse heart), α -chymotrypsinogen A type II (ox pancreas), ovalbumin grade V, crystalline bovine serum albumin, pancreatic deoxyribonuclease (type I) and ribonuclease A (type IA).

Worthington Biochemical Corp., Freehold, N.J., U.S.A.: crystalline pancreatic deoxyribonuclease and ribonuclease.

B. Methods

1. Organism

Halobacterium cutirubrum strain NRC 34001 was used throughout this work.

2. Bacterial growth conditions

The growth medium was that of Sehgal and Gibbons (1960) which contained per 100 ml: 0.75 g Difco casamino acids; 1.0 g Difco yeast extract; 0.3 g sodium citrate; 0.2 g potassium chloride; 2.0 g magnesium sulfate (hydrated); 25 g sodium chloride; 2.3 mg ferrous chloride. The pH of the medium was adjusted to 6.5 - 6.8 using 10 N NaOH. After sterilization at 121°C for 30 minutes a precipitate formed that did not affect bacterial growth and slowly redissolved during storage of the medium. A 2% solution of agar in the medium was used for making slants and Petri dishes.

Stock cultures grown for 2-3 weeks at 37°C on agar slants were stored at 4°C.

Mass cultures were grown in Erlenmeyer flasks (125 ml and 1000 ml) with Morton closures. To obtain liquid cultures of the bacteria from slants, loop inocula were transferred to 60 ml of culture medium contained in 125 ml culture flasks. Incubation was performed at 37°C in a New Brunswick gyratory incubator shaker at 225 rpm until sufficient growth was observed spectrophotometrically (about 7 days). These cultures were used in making mass cultures; fresh stock liquid culture was always used when preparing a mass culture and the previous stock was thrown out. 5 ml of liquid culture were inoculated into 60 ml of medium. After 48 h of incubation, 30 ml of this starter culture were inoculated into 600 ml of medium and incubated for a further 48 h. The mass culture was harvested by centrifugation at 15,000 g for 6 minutes. The supernatant was discarded and the unwashed cells were used for the preparation of the crude extract.

A routine check on the purity of the cultures was made for each preparation by transferring suitable serial dilutions of the cultures in 25% NaCl to Petri dishes containing solid medium. The dishes were kept in plastic bags to prevent condensation and incubated at 37°C for about 7 days until which no more colonies appeared. The colonies were counted to obtain the viable cell count of the cultures and were inspected visually for contamination by other halophiles.

3. Buffers -

The complex buffers used had the following composition:

Buffer I: 3M-KCl-0.1M-magnesium acetate-8mM-2-mercaptoethanol-10 mM-Tris-HCl, pH 7.6;

Buffer II: Buffer I without 2-mercaptoethanol;

Buffer III: 3.1M-KCl-10mM-Tris-HCl, pH 7.6;

Buffer IV: 2.5M-KCl-1-M NaCl-10mM-Tris-HCl, pH 8.6.

4. Isolation and Purification of α and β subunits of H. cutirubrum DNA-dependent RNA Polymerase:

4.1 DNA-enzyme complex

The bacteria (0-11 g wet wt) were harvested after 48 hours of growth by centrifugation at 27000 g_{max} and were washed by resuspension in 25% (w/v) NaCl - 2% (w/v) $MgSO_4$, 7 H_2O - 0.2% (w/v) KCl (approximately 20 ml/g wet wt of cells). The washed cells were then suspended in buffer I (3 ml/g wet wt of cells) by slow addition of buffer while the bacteria were stirred with a bent glass rod. The

suspension was stirred magnetically until homogeneous (1-2 h) and then left in the refrigerator overnight. By the following morning the cells had lysed, and the suspension was centrifuged for 30 min. at 45000 g_{max} to give a clear supernatant, a soft, viscous, red layer and a small dark-red, dense pellet of cell debris and unbroken cells. The supernatant was decanted gently and discarded and the intermediate viscous layer was carefully transferred to a beaker. Buffer I (1.5 ml/g wet wt of cells) was added slowly with vigorous stirring to disperse the viscous layer, and a homogeneous suspension was obtained by magnetic stirring for up to 1 h , plus one or two strokes of a Potter-Elvehjem tissue homogeniser, if necessary. This suspension is referred to as the DNA-bound enzyme complex.

4.2 Crude extract

In order to isolate the soluble enzyme from DNA-enzyme complex deoxyribonuclease (1 mg/10 g wet wt of cells) was added to the suspension of the complex followed by incubation at 25°C for 1 h. The deoxyribonuclease-treated material was diluted with buffer I (1.5 ml/g wet wt of cells) and sonicated with a Bronwill Biosonik II sonic disintegrator (large probe; dial setting 70) for two 1 min. periods with an interval of 1 min. between them. The homogenate was then centrifuged at 45,000 g for 20 minutes to give an orange supernatant referred to as the crude extract.

4.3 Amicon Ultrafiltration

The crude extract was subjected to ultrafiltration using an Amicon model 52 cell (Amicon Corporation, Lexington, Mass., U.S.A.) at a pressure of 380 KPa (55 lb/in^2). It was first passed through an Amicon XM-50 membrane (approximate mol. wt. exclusion limit 50,000), followed by additional buffer I (10% of the volume of the crude extract) to give the XM-50 filtrate and supernatant. The XM-50 filtrate was then passed through an Amicon UM-10 membrane (approximate mol. wt. exclusion limit 10,000) to give the UM-10 supernatant and the filtrate. The DNA-dependent RNA polymerase activity passed through the XM-50 filter and was concentrated above the UM-10 filter.

4.4 Gel-filtration

The active UM-10 supernatant was purified by gel filtration through a column (2.5 cm x 85 cm) of either Bio-Gel P-60 polyacrylamide gel or Sephadex G-75. Gel filtration was carried out as described by Louis _et al._, (1971) with minor modifications. Buffer II was used in running these columns. Initially Buffer I was used, but this caused discoloration of the column.

4.5 Hydroxylapatite chromatography

A column of (0.9 cm x 10 cm) was prepared by using a slurry of Bio-Rad HTP hydroxylapatite powder (5 g) in buffer III. The column was run by downward flow at 5 ml/h. and was washed with

40 ml of buffer III. The P-60 concentrated pool was applied and elution performed successively with (i) buffer III (25 ml) and (ii) 3.1M-KCl-0.1-M-Na_2HPO_4-10mM-Tris-HCl, pH 7.6 (30 ml); 2 ml fractions were collected. Subunit β was eluted at stage (i) and α at stage (ii). The active fractions containing phosphate were dialysed against buffer II and the combined active fractions from each stage were then concentrated separately by ultrafiltration using an Amicon UM-10 membrane to give the purified α and β subunits.

5. Enzyme Assay:

H. cutirubrum DNA-dependent RNA polymerase activity was determined by three procedures referred to below as assays A, B & C respectively. All three assays depend on the incorporation of ribonucleoside triphosphates into an acid insoluble form. All concentrations are the final values taking into account the concentrations of buffer, salts etc. in the enzyme solution.

Assay A. The assay medium (0.125 ml) contained: 34-mM-tris-HCl buffer, ph 8.6; 2.1M-KCl; 40mM-$Mg(OAc)_2$; 0.2M-NH_4Cl; 3mM-2mercaptoethanol; 0.32mM-CTP, -GTP and -UTP; 0.12mM-α-[^{32}P]ATP (about 4000 dpm/nmole) or -[^{14}C]ATP (about 10,000 dpm/nmole); *H. cutirubrum* or *E. coli* DNA, 300 μg/ml; enzyme.

Assay B (Louis and Fitt, 1972a). The assay medium (0.1 ml) contained: 100mM-Tris (hydroxymethyl) methyl-2-aminopropane sulphonic acid-NaOH buffer, pH 8.6; 1.5M-KCl; 0.6M-NaCl; 0.1M-$MgCl_2$;

$10mM-MnCl_2$; $0.2M-NH_4Cl$;).15mM-CTP, -GTP and -UTP; 0.5mM- -[^{32}P]ATP (about 10,000 dpm/nmole); H. cutirubrum or E. coli DNA, 300 μg/ml; enzyme, 60μl, diluted at least ten told in buffer IV (so that the assay medium also contained 6mM-Tris-HCl buffer, ph 8.6).

Assay C (Chazan & Bayley, 1973). The assay medium (0.125 ml) contained: 34mM-Tris-HCl buffer pH 8.05; 1.2M-KCl; 40mM-$Mg(OAc)_2$; 0.2M-NH_4Cl; 3mM-2-mercaptoethanol; 0.32mM-CTP, -GTP and -UTP; 2μM-α-[^{32}P]ATP (about 300,000 dpm/nmole); salmon sperm DNA, 240 μg/ml; enzyme. Assays were also performed using 0.32mM-ATP and 2 M- ^{14}C -UTP (about 900,000 dpm/nmole, instead of radioactive ATP and non-radioactive UTP, in order to duplicate the conditions described by Chazan & Bayley (1973). Identical results, based on nmol of radioactive substrate incorporated, were obtained by the two variations of assay C, so α-[^{32}P]ATP was used in order to avoid the need for quench corrections.

For all assays incubation was done at 37°C for 60 minutes unless indicated otherwise. The assays were processed either by a modification of Bollum's technique (Method I), (Bollum, 1959, 1966, 1968), or by a modification (Method II) of the Furano washing procedure (Furano 1971).

Method I. After incubation a sample (100 μl in the case of A and C and 85 μl in the case of B) of the assay mixture was streaked on a numbered 2 cm x 3 cm strip of Whatman No. 3 filter paper. The strips were collected in cold 7% (w/v) $HClO_4$ (10 ml/strip) and the suspension was swirled (New Brunswick Scientific Co.,

rotary shaker) for at least 7 minutes. The strips were allowed to settle and the fluid was removed with an aspirator. The washing procedure was repeated successively with similar volumes of cold 7% (w/v) $HClO_4$, 1% (w/v) $HClO_4$ and ethanol. The papers were dried using an infrared lamp and individually placed into vials for counting.

Method II. This method was developed in the later part of the study. It gave us lower and very reproducible blank values and was used routinely thereafter. A fraction of the assay mixture (same amount as in method I) was streaked onto a Whatman No. 3 filter paper of diameter 18.5 cm which was divided into numbered 3 x 3 cm squares. Immediately after all the fractions had been applied, the filter paper was placed in a Buchner funnel and about 250 ml. of cold 7% (w/v) $HClO_4$ were added. The fluid was allowed to drop through the paper under gravity for about 1 min.; suction was then used to remove the remainder. Washing was repeated successively using similar volumes of cold 7% (w/v) $HClO_4$, 1% (w/v) $HClO_4$ and ethanol. Finally, the paper was dried with the help of an infrared lamp. The numbered squares were then cut out and transferred into vials for counting.

The radioactivity was counted to 2% accuracy in a Beckman LS-230 liquid scintillation counter after the addition of 10 ml of a 0.5% solution of 2,5-diphenyloxazole in toluene to each vial. Control assays without enzyme were performed and their radioactivity values were subtracted from the assay values. To determine the

specific radioactivity of radioactive nucleoside triphosphate, samples (10 µl) of the stock nucleoside triphosphate solution (0.5 mole/ml) were streaked on filter paper strips; strips were dried under infrared lamp and their radioactivities were counted as already described. A unit was defined as the amount of enzyme catalysing incorporation of 1nmole-[^{14}C]ATP or α[-^{32}P]ATP into an acid insoluble form per hour.

6. Protein Assay

Enzyme protein was assayed spectrophotometrically (Warburg and Christian, 1942) from the XM-50 filtrate fraction onwards. The protein content of the DNA-bound enzyme and the crude extract were determined by the Lane and Mavrides (1969) modification of the method of Gornall, Bardwill and David (1949) using α-chymotrypsinogen as the standard.

III. RESULTS

1. Studies on the isolation of H. cutirubrum RNA polymerase:

These experiments had two principal aims. The first was to develop a modified method for the purification of the α and β subunits of H. cutirubrum DNA dependent RNA polymerase on a scale significantly greater than possible by the procedure described by Louis and Fitt (1972a). The second was to confirm the findings of Louis and Fitt (1972a) by an independent approach and to resolve the conflict between their results and those of Chazan and Bayley (1973) concerning the molecular weight and subunit composition of the enzyme and the effect of rifampicin on its activity.

1.1 Preparation of the crude extract.

To obtain the crude H. cutirubrum DNA-dependent RNA polymerase in a form stable enough to take it through the different steps of purification in order to isolate the α and β subunits of the enzyme four different methods were tried for preparation of the crude extract.

1.1.1 Method I.

This was the method of Louis and Fitt (1971 , 1972) where sonication was used to release the enzyme from the cell suspension. The crude extract prepared this way had an activity

comparable to that observed before. However the pH 4 precipitation which was the next step in this method gave less satisfactory results, since a clear cut separation of the two activities was not achieved. Only about sixty per cent of the DNA-dependent RNA polymerase activity was recovered in the supernatant and it proved to be unstable during concentration by ultrafiltration or dialysis against a concentrated solution of Ficoll in buffer IV.

1.1.2 Method II.

Lieberman and Lanyi (1972) prepared H. cutirubrum threonine deaminase by freezing and thawing of bacterial suspension followed by DNase treatment. Attempts were therefore made to isolate H. cutirubrum RNA polymerase using freeze thawing of cell suspension. The freshly-harvested cells were suspended in Buffer I, using ml/g wet wt of bacteria. Dry ice in alcohol was used for freezing the suspension. It took about 10-15 minutes for the suspension to freeze completely and the melting process required 1-1½ h at 25°C. Freezing and thawing was done twice and yielded a red viscous fraction of broken bacterial cells. The viscous material was digested with DNase and then was diluted with buffer I. The solution thus obtained was centrifuged at 45,000 g_{max} for 20 minutes to give a supernatant that had as much as 80% of the activity obtained by method IV (see below). The crude extract was passed through a XM-50 membrane resulting in about 20-fold purification and about 25% loss in activity. In the next step the XM-50 filtrate was concentrated using a UM-10 membrane. Concentration resulted in complete loss of activity and it is probable

that the crude extract obtained by this method still contained degradative enzymes which destroyed the RNA polymerase.

1.1.3 Method III.

In another study, alumina grinding followed by DNase treatment were used to release the enzyme. Freshly-harvested bacteria were ground with alumina (0.5 g of alumina/g wet wt of bacteria) till a smooth homogeneous paste was obtained; this was digested with DNase to hydrolyse the DNA released during grinding. After DNase treatment, Buffer I (4 ml/g wet wt of bacteria) was added to the suspension, which was then centrifuged to give the crude enzyme in the supernatant. The crude extract was first passed through a XM-50 filter, and the filtrate was then concentrated by a UM-10 ultrafiltration. Once again, activity disappeared during concentration although expected level was observed in the crude extract and the XM-50 filtrate.

1.1.4 Method IV.

This method gave a highly active preparation and the activity was maintained during subsequent purification. The procedure is described in the materials and methods section and depends on the isolation of *H. cutirubrum* DNA-dependent RNA polymerase as a complex with DNA (plus unidentified colored substances, probably membrane fragments) after slow spontaneous lysis of *H. cutirubrum* cells in 3M-KCl (Kushner 1964).

Homogenisation of a suspension of freshly harvested *H. cutirubrum* cells as described by Chazan and Bayley (1973 did not give rise to breakage of cells. Even after 12-14 vigorous passes of the pestle the suspension did not become viscous and phase contrast

microscopy showed that essentially no cell breakage occurred. However, if the suspension was left in cold for 1-2 h , the cell walls weakened sufficiently for them to break during homogenisation, and almost complete lysis occurred without homogenisation if it was left overnight in the refrigerator.

Centrifugation of the lysed bacterial suspension at 45,000 g_{max} yielded a small dark pellet covered by a red viscous layer. The viscous layer contained 90-100% of RNA polymerase activity. On one occasion, when 10% of the enzyme activity remained in the 45,000 g_{max} supernatant, only one third of this residual activity could be recovered in the pellet by a further centrifugation at 229,000 g_{max} for 3 hours showing that it was largely in the soluble form (see below). Thus, the DNA enzyme complex we obtained must be significantly larger than that described by Chazan and Bayley (1973), which did not sediment at 40,000g.

The activity of the DNA enzyme complex was determined on several occasions using bacteria from either 24 h or 48 h cultures. The average yield of enzyme units per gram wet weight of bacteria from the 48 h cultures was found to be approximately double that from the 24 h cultures. Since the total yield of bacteria was also higher after 48 h, and no other difference was observed during subsequent purification of the enzyme, all further experiments were performed with 48 hr cultures. The variation of total activity as a function of growth of the culture has not been studied further.

1.2 Preliminary purification of the enzyme.

During initial studies of crude sonic extracts using as a

first purification step the pH 4 precipitation described by Louis and Fitt (1972), the separation of the DNA-dependent and RNA-dependent RNA polymerases of H. cutirubrum appeared less clear cut than that had previously been reported. Although no RNA-dependent activity remained in the supernatant a significant fraction of the DNA-dependent activity coprecipitated with it. This method was therefore abandoned in favour of ultrafiltration (section 1.3), which proved to be an extremely rapid and efficient step for purification of the DNA-dependent enzyme. However, more recent experiments (Fitt and Peterkin, private communication) have shown that a combination of both ultrafiltration and pH precipitation is even more effective and also appears to yield a stable enzyme.

1.3 Ultrafiltration.

Ultrafiltration was found to be very useful in partial purification of H. cutirubrum DNA-dependent RNA polymerase. A volume of 30 ml of crude extract took around 5-6 h to go through a XM-50 membrane in an Amicon Model 52 ultrafiltration cell which resulted in a purification of 30-60 fold with excellent recovery of activity.

Ultrafiltration was also used to concentrate the partially purified enzyme. The XM-50 filtrate was concentrated using a UM-10 membrane. PM-10 membranes could not be used to concentrate the enzyme, because they led to loss of activity.

1.3.1 Purification studies with Amicon PM-30 and UM-20 ultrafilters.

The procedure described above was used in the preparation of the highly purified subunits (section 1.6). Later in my work, I

studied ultrafiltration as a means of partial purification of the enzyme using filters PM-30 and UM-20 in addition to XM-50 filters.

Filters PM-30 and UM-20 have molecular weight exclusion limits of around 30,000 and 20,000 respectively. Tables (IV , V and VI)show the results of these experiments. By using XM-50 and PM-30 a purification of the order of 85-fold was obtained (Table V). When XM-50 and UM-20 filters were used, the purification was 95 fold (Table IV). However the best results were obtained when all the three filters XM-50, PM-30 and UM-20 were used. The purification achieved in this experiment was as high as 201-fold with an essentially quantitative recovery of the activity, so that this method holds much promise for future work.

1.4 Gel filtration.

Further purification of the partially purified enzyme was achieved by gel filtration using either Bio-Gel P-60 or Sephadex G-75. Both the gels gave the same order of purification. Our gel filtration experiments were performed as described previously by Louis and Fitt (1971), except that Buffer II was used. The active fractions were pooled and concentrated by ultrafiltration using a UM-10 membrane.

1.5 Hydroxyapatite chromatography.

The two subunits were separated by hydroxylapatite column chromatography after gel filtration. The active fraction of the enzyme prior to hydroxylapatite chromatography was in a buffer containing

TABLE IV

PURIFICATION OF SOLUBLE DNA-DEPENDENT - RNA POLYMERASE BY AMICON ULTRAFILTRATION

USING XM-50 AND UM-20 MEMBRANES

Fraction	Volume (ml.)	Activity (Units)	Protein (mg.)	Specific Activity (Units/mg.)
DNA-Bound Enzyme	13	912	665	1.37
Crude Extract	32	1056	377	2.80
XM-50, Filtrate	30	840	27	31.10
UM-20, Filtrate	30	786	6	131.00

TABLE V

PURIFICATION OF SOLUBLE DNA-DEPENDENT - RNA POLYMERASE BY AMICON ULTRAFILTRATION

USING XM-50 AND PM-30 MEMBRANES

Fraction	Volume (ml.)	Activity (Units)	Protein (mg.)	Specific Activity (Units/mg.)
DNA-Bound Enzyme	15	1053	900.0	1.17
Crude Extract	332	928	399.2	2.74
XM-50, Filtrate	30	960	24.0	40
PM-30, Filtrate	28	570	5.7	100

TABLE VI

PURIFICATION OF DNA-DEPENDENT - RNA POLYMERASE BY AMICON ULTRAFILTRATION

USING XM-50, PM-30 AND PM-20 MEMBRANES

Fraction	Volume (ml.)	Activity (Units)	Protein (mg.)	Specific Activity (Units/mg.)
DNA-Bound Enzyme	16	1046	1120	0.93
Crude Extract	34	952	428.4	2.24
XM-50, Filtrate	32	864	38.4	22.5
PM-30, Filtrate	32	992	12.8	77.5
PM-20, Filtrate	30	990	5.0	198

magnesium; and one of the elution buffers used was phosphate buffer. Therefore, before hydroxylapatite chromatography, the pooled and concentrated fraction was dialysed against buffer III which did not contain magnesium. The β subunit came out as a small peak during the wash with buffer III. The subunit was eluted from the column on washing with buffer III containing 0.2M phosphate, whereas Louis & Fitt (1972) eluted the α -subunit by applying buffer IV containing 0.1M phosphate. When I used 0.1M phosphate for elution, the α -subunit was not removed from the column.

1.6 Summary of the modified purification procedure

Table VII gives a summary of the isolation and purification of α and β subunits. The combined subunits in these experiments gave a specific activity of 8208. Louis and Fitt (1972) obtained a specific activity of 14,500 for the combined subunits but their β subunit was much more pure having been subjected to a second hydroxylapatite chromatography.

2. Studies of the effect of sonication on the molecular weight of H. cutirubrum RNA polymerase:

It had been claimed by Chazan and Bayley (1973) that the low molecular weight of the purified enzyme previously described from our laboratory (Louis and Fitt, 1971, 1972) could be due to cleavage of some larger molecules or multimeric complex during sonication of the cells to obtain the crude enzyme.

TABLE VII

PURIFICATION OF H. CUTIRUBRUM DNA-DEPENDENT - RNA POLYMERASE

Fraction	Volume (ml.)	Activity (Units)	Protein (mg.)	Specific Activity (Units/mg.)
Crude Extract	34	1274	1137	1.1
XM-50, Filtrate	30	1088	39	28
UM-10, Supernatant	8	1100	30	36
P-60, Concentrated Pool	6.	1104	1.870	590
Hydroxyeapatite Column:				
α-Subunit	2.5		0.025	
β-Subunit	3.5		0.081	
$\alpha + \beta$	6.	870	0.106	8208

TABLE VIII

RELEASE OF THE SOLUBLE ENZYME FROM THE DNA-ENZYME COMPLEX BY DNase DIGESTION

Fraction	Activity m(Units)	Recovery (%)	Specific Activity (Units/mg.)
1. DNA-Bound Enzyme	841	100	9.3
2. XM-50 Filtrate, Unsonicated	435	51	18.1
3. XM-50 Filtrate, Sonicated	497	59	12.5
2 + 3	932	1110	-

Results in the Table VIII show that sonication was not responsible for the low molecular weight of the soluble enzyme. In this experiment a DNA-enzyme complex was prepared as usual and then it was divided into two equal portions; one portion was treated with DNase and then sonicated, but the other portion was only treated with DNase. Both the fractions after dilution with the buffer I were centrifuged at 45,000 g_{max} for 20 minutes. The supernatants from the centrifugation were passed through an XM-50 membrane almost to dryness. It is very clear that the enzyme is released quantitatively even without sonication and this treatment was omitted during subsequent work in this laboratory.

2.1 Molecular weight of *H. cutirubrum* RNA polymerase

The molecular weight of the unseparated subunits using sonication and DNase treatment to obtain the crude extract was determined after the gel filtration step by analytical Bio-Gel P-60 as described by Louis *et al.*, (1971). It was found to be 19,100 (Fig.II) in buffer I which is in good agreement with the values of 17,800 - 20,000 previously reported for the mixture of α and β and of about 18,000 obtained for each subunit.

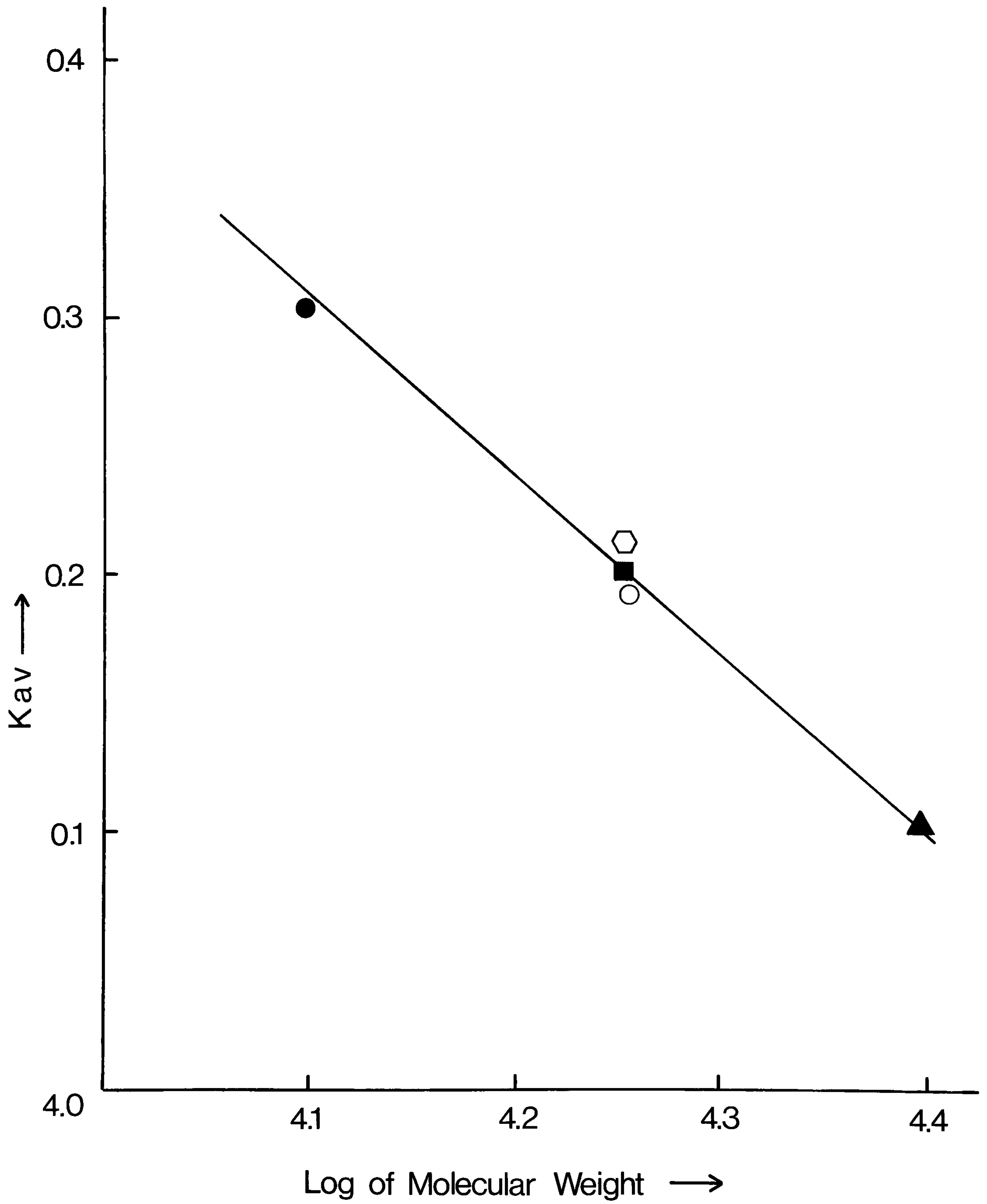
The molecular weight of the unseparated subunits, where the crude enzyme was obtained by DNase treatment alone, was similarly determined and it was found to be 18,400 (Fig.II), which conclusively shows that the sonication step was not responsible for the low molecular weight of the enzyme.

Fig. VI. Molecular weight of the sonicated and the unsonicated *H. cutirubrum* DNA-dependent RNA polymerase.

●, Cytochrome C; ■, Myoglobin; ▲, α- chymotrypsinogen;

○, sonicated DNA-dependent RNA polymerase;

⬡, unsonicated DNA-dependent RNA polymerase.


0.4
0.3
0.2
0.1
4.0
Kav ⟶
4.1
4.2
4.3
4.4
Log of Molecular Weight ⟶

3. Studies of conditions of assay of *H. cutirubrum* RNA polymerase:

The activity of the bound and the crude soluble enzyme was assayed using three different methods described in the materials and methods section. Table (IX) shows the reagents and their quantities in the different assays. Assay A has been developed in the course of present studies by progressive modification of assay C; we have found it to be the most reliable so far developed for use with both the bound and the soluble enzyme. Assay B is that used by Louis and Fitt (1971a, 1972b). Assay C is that described by Chazan and Bayley (1973).

Assay A was much more effective than either assays B or C for determination of the activity of the DNA-enzyme complex (Fig. III). Additional DNA was always added while assaying the bound enzyme, since it stimulated the activity by about 30%. In the case of the soluble enzyme after ultrafiltration assays A and B were both effective, but no reaction occurred at all in the conditions of assay C (Fig. IV), thus explaining the failure of Chazan and Bayley (1973) to detect the soluble enzyme described by Louis and Fitt.

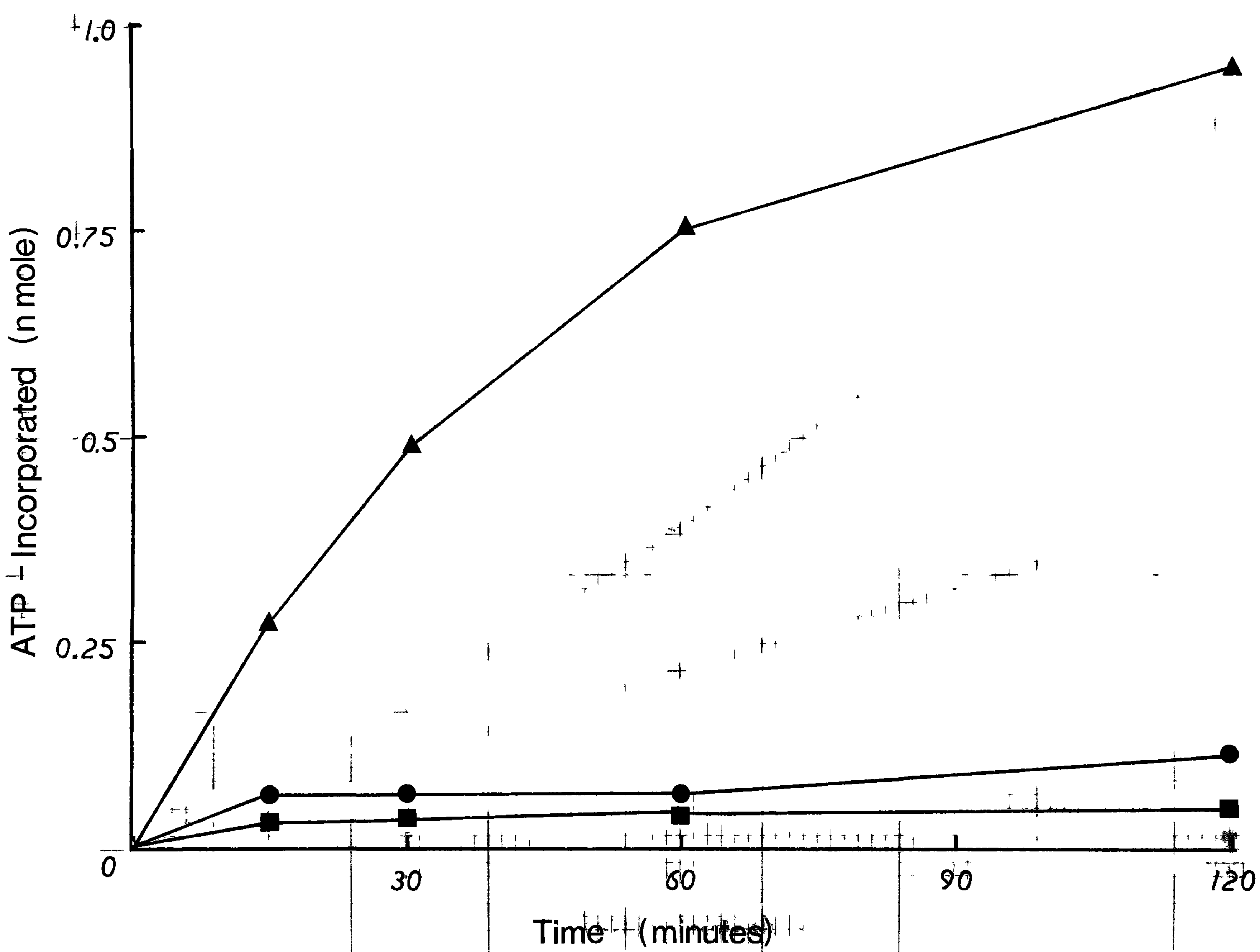
It should be emphasized that assay C is virtually a "nucleotide omission" assay with the non-radioactive nucleotides present at 160 times the concentration of the radioactive substrate. Its conditions are so far from optimum that enzymic properties deduced by its use are of doubtful significance. When the concentration of the radioactive nucleotide in assay C was increased to 0.32 mM, making it same as that of the non-radioactive ones, incorporation was increased 10-fold (after 60 minutes of incubation). Further changes in the total salt concentration

TABLE IX

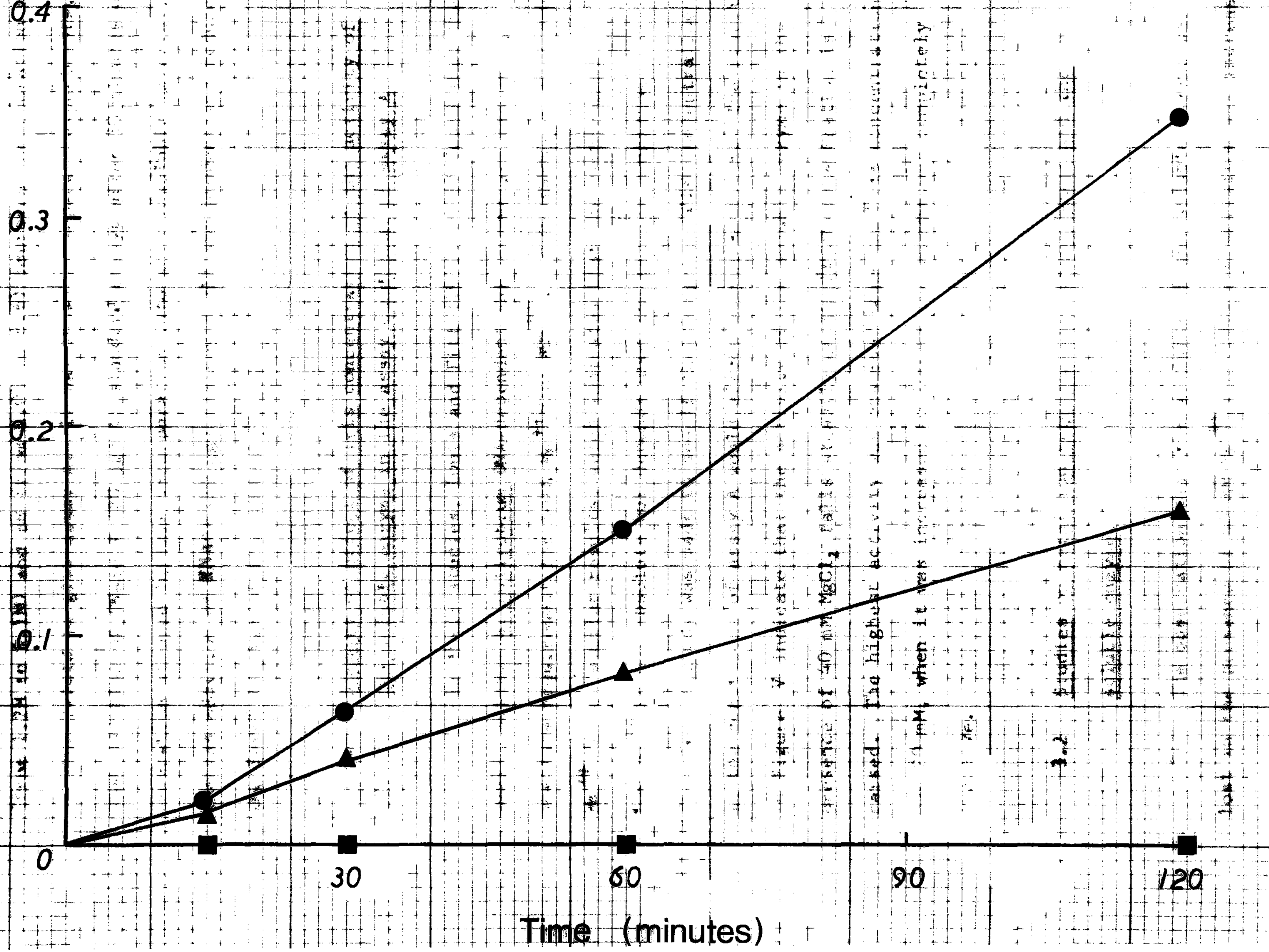
ASSAY A	ASSAY B	ASSAY C
Assay volume: 0.125 ml	Assay volume: 0.1 ml	Assay volume: 0.125 ml
1) 34mM-tris-HCl buffer pH 8.6	1) 100mM-tris buffer at pH 8.6	1) 34mM-tris-HCl buffer at pH 8.05
2) 0.2M-NH_4Cl	2) 1.5M-KCl	2) 1.2M-KCl
3) 2.1M-KCl	3) 0.6M-NaCl	3) 40mM-$Mg(OAc)_2$
4) 40mM-$Mg(OAc)_2$	4) 0.1M-$MgCl_2$	4) 0.2M-NH_4Cl
5) 3mM-2-mercaptoethanol	5) 10mM-$MnCl_2$	5) 0.32mM-CTP, -GTP, -UTP
6) 0.32mM-CTP, -GTP, -UTP	6) 0.2M-NH_4Cl	6) 2μM-α[^{32}P]ATP (about 900,000 dpm/nmole) or 2μM-^{3}H-UTP with 0.32M-ATP
7) 0.12mM-α-[^{32}P]ATP (about 4,000 dpm/nmole) or ^{14}C-ATP (about 10,000 dpm/nmole)	7) 0.15mM-CTP, -GTP, -UTP	7) Salmon sperm DNA 240/μg/ml
8) *H. cutirubrum* or *E. coli* DNA 300 μg/ml	8) 0.05mM-α-[^{32}P]ATP (about 10,000 dpm/nmole)	8) Enzyme
9) Enzyme	9) *E. coli* or *H. cutirubrum* DNA 300 μg/ml	
	10) Enzyme; 60 μl	

Figs. III and IV Activity of the (III) DNA-bound and (IV) soluble H. cutirubrum DNA-dependent RNA polymerase at different assay condition.

The time course of the reaction was followed by assay A, ▲ , assay B, ● , or assay C, ■ . Duplicate assays and a reagent blank were performed for each point on the curves. The assays contained 0.7 units of the DNA-bound enzyme or 0.08 units of the soluble enzyme (XM50 filtrate, Table VII) based on their activity in the standard assay A.



ATP - Incorporated (n mole)
1.0
0.75
0.5
0.25
0
30
60
90
120
Time (minutes)


ATP Incorporated (n moles)
0
0.1
0.2
0.3
0.4
Time (minutes)
30
60
90
120

(from 1.2M to 2.1M) and pH (from 8.05 to 8.6) improved incorporation a further 2-fold to give assay A. It was shown in separate experiments, that addition of DNase I (20 μg) to standard assays after 30 minutes of incubation stopped further incorporation into acid insoluble material, whereas addition of RNase (20 μg) destroyed all the product and reduced the level to that of the control.

3.1 Studies of effect of Tris concentration of the activity of the soluble enzyme in the assay condition of assay A

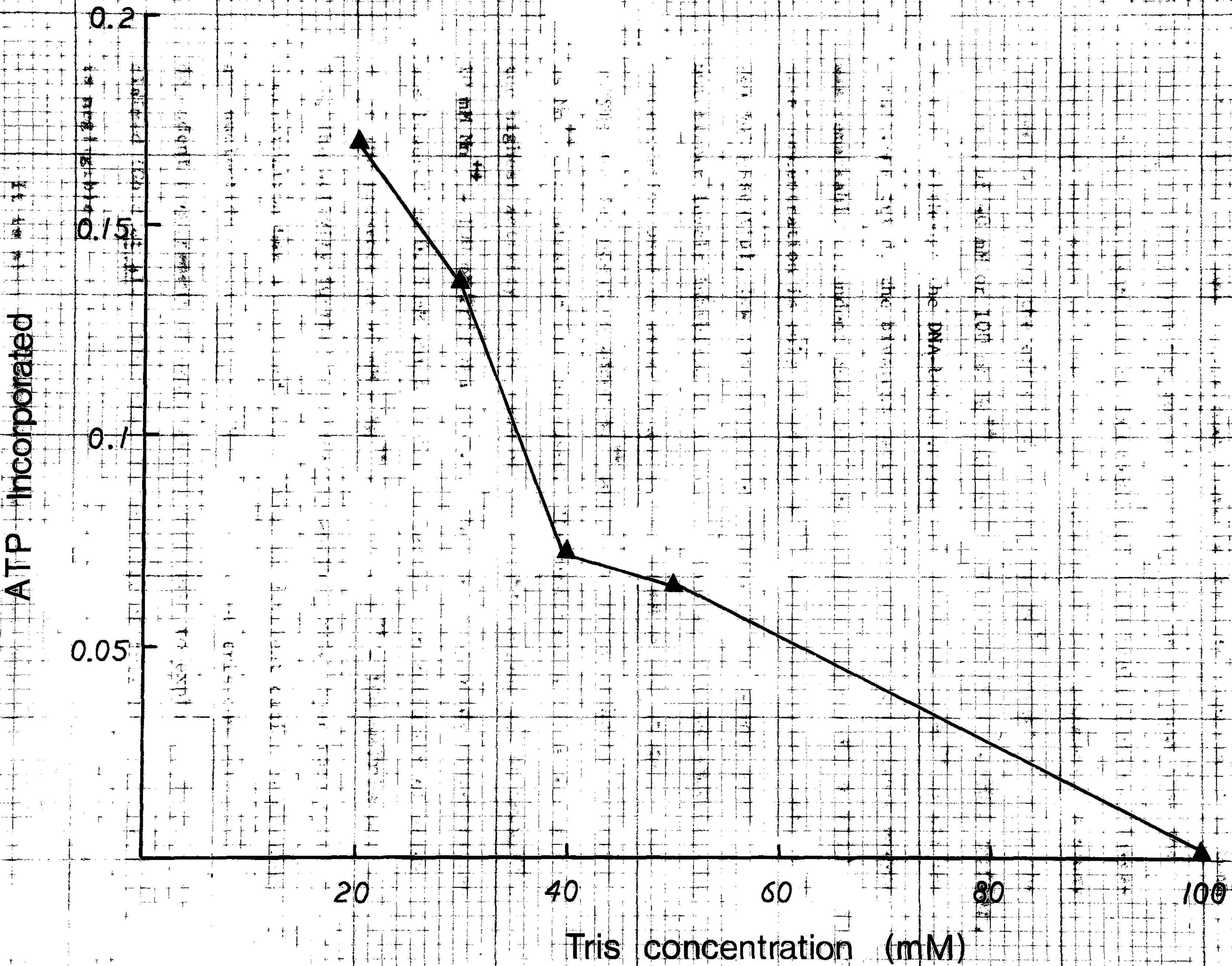
In their studies, Louis and Fitt (1971, 1972) found that the small, soluble H. cutirubrum DNA-dependent RNA polymerase has an absolute requirement for both Mg^{++} and Mn^{++}. But assay A without having Mn^{++} gives similar results, which indicates that the requirement for Mn^{++} is not absolute but depends upon the precise condition. Therefore a study was made to see if the change in Tris concentration in the conditions of assay A would affect enzyme activity. The results in Figure V indicate that the activity of the soluble enzyme in the presence of 40 mM $MgCl_2$ falls steadily as the Tris concentration is raised. The highest activity was observed when the Tris concentration was 20 mM, when it was increased to 100 mM the enzyme was completely inactive.

3.2 Studies on metal ion requirements of the bound and the soluble enzyme.

The observation that the activity of the soluble enzyme was lost in the presence of 40 mM Mg^{++} when tris concentration was increased

Fig. V Effect of tris concentration on the activity of the soluble enzyme in the conditions of assay A.

The activity of the soluble enzyme (XM50 filtrate, Table VII) was determined in the conditions of assay A (Experimental section) at the indicated tris concentrations. Each assay contained 0.14 units of enzyme activity (based on the standard assay A), plus an additional 4 μmol/ml of tris present in the enzyme dilution.

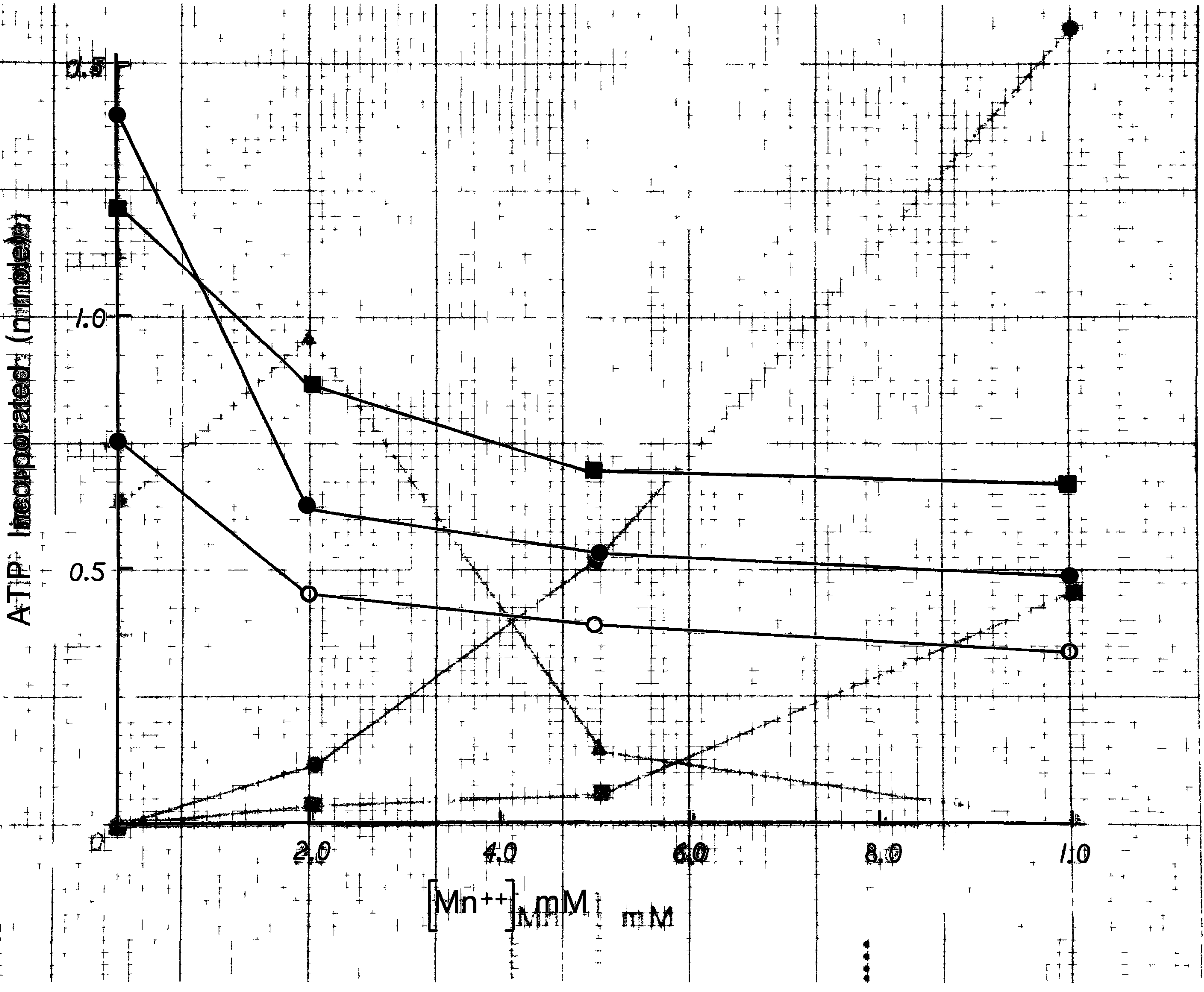

ATP Incorporated
0.2
0.15
0.1
0.05
20
40
60
80
100
Tris concentration (mM)

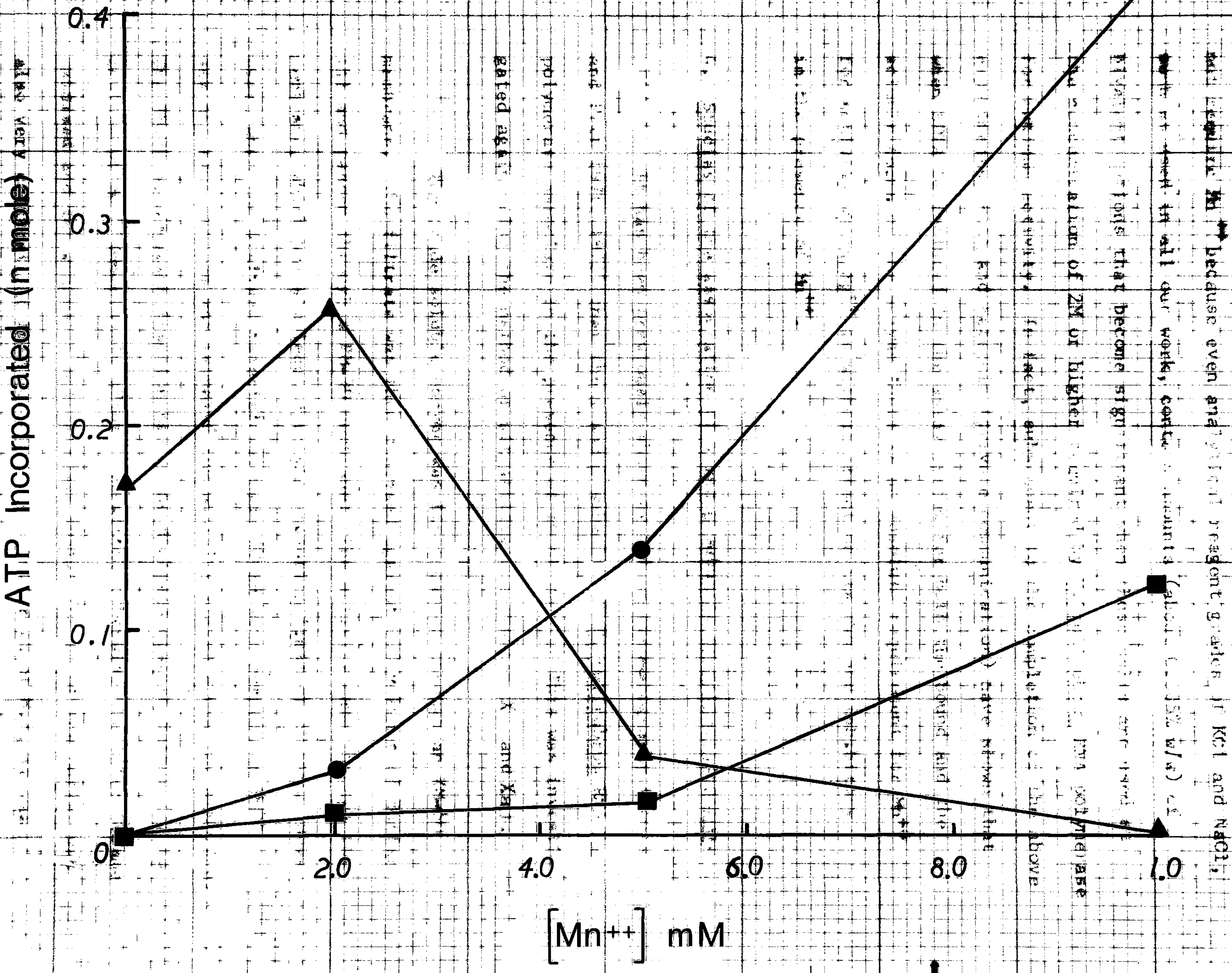
to 100 mM and the fact that Louis and Fitt (1972) have shown in their studies with the same enzyme that there is absolute requirement of both magnesium and manganese for enzyme activity led us to think that the requirement for manganese is dependent upon tris concentration. Investigations were made to find out the validity of this argument. A study was made to observe the effect of varying the Mn^{++} concentration in the presence of 40 mM or 100 mM Mg^{++} using 30 mM or 100 mM Tris-HCI buffer, pH 8.6. Although the DNA-bound enzyme was inhibited by Mn^{++} in all the conditions tried, the bivalent cation requirement of the soluble enzyme was remarkably dependent upon tris concentration (Figures VI & VII). When tris concentration is only 30 mM and Mg^{++} concentration was 40mM, Mn^{++} was not essential, low concentrations stimulated the enzyme about 30%, but higher levels inhibited it strongly. However with 100 mM-Tris, the concentration used in assay B, and either 40 mM or 100 mM $MgCl_2$, the enzyme was dependent on added Mn^{++} and the activity steadily increased as Mn^{++} concentration was increased. The combination tried that gave the highest activity in the case of the soluble enzyme was 40 mM-Mg^{++} - 10 mM Mn^{++} - 100 mM Tris HCl pH 8.6. When the nucleotide concentration was lowered to 0.15 mM which is the nucleotide concentration of assay B, in the presence of 40 mM - Mg^{++} - 10 mM - Mn^{++} - 100 mM Tris HCl pH 8.6, the activity further increased by another 50%. Therefore tris concentration has a combined effect both on divalent cation requirement and nucleotide concentration. The phenomenon of tris concentration dependent requirement of manganese is difficult to explain. It is claimed (Good _et al_., 1966) that the affinity of tris for Mg^{++} and Mn^{++} is negligible.

It is also difficult to establish that DNA-bound enzyme does

Figs. VI and VII Metal ion requirements of the DNA-bound (VI), and soluble enzyme VII).

The effect of changes in Mn^{++} concentration on the activity of (a) the DNA-bound enzyme and (b) the soluble P-60 fraction (Table I) was determined in the conditions of assay A (experimental section) using three different combinations of tris and Mg^{++} concentrations: 30mM-tris-40mM-Mg^{++}, ▲ ; 100mM-tris-40mM-Mg^{++}, ● ; 100mM-tris-100mM-Mg , ■ . The assays contained 0.9 units of the DNA-enzyme complex or 0.09 units of the P-60 fraction (based on the standard assay A), plus an additional 4 μmol/ml of tris present in the enzyme dilutions.


ATP Incorporated (n mole)
1.5
1.0
0.5
0
2.0
4.0
6.0
8.0
10
[Mn++] mM


ATP Incorporated (n mole)
0.4
0.3
0.2
0.1
0
2.0
4.0
6.0
8.0
1.0
[Mn++] mM

not require Mn^{++} because even analytical reagent grades of KCl and NaCl, such as used in all our work, contain amounts (about 0.005% w/w) of bivalent cations that become significant when these salts are used at the concentration of 2M or higher required by H. cutirubrum RNA polymerase for maximum activity. In fact, subsequent to the completion of the above experiments, Fitt and Peterkin (private communication) have shown that when EDTA is included in the assay system for both the bound and the soluble forms of the enzyme there is an absolute requirement for Mn^{++} for activity while Mg^{++} is without effect on its own and inhibitory in the presence of Mn^{++}.

4. Studies of the association of α and β subunits by ultrafiltration.

It has been previously shown by column chromatography (Louis and Fitt 1971, 1972) that the α and β subunits of H. cutirubrum RNA polymerase associate in the presence of 10 mM $MnCl_2$. This was investigated again using the method of ultrafiltration (Tables X and Xa).

The crude soluble enzyme was first passed through an XM-50 membrane. The filtrate was then dialysed against 100 volumes of Buffer III and passed through a PM-30 membrane: the activity came through the membrane, which has a molecular weight exclusion limit of 30,000. However, when the dialysed material was made 10 mM with respect to Mn^{++} and the same process was repeated the activity stayed above the membrane. The PM-30 supernatant fraction had to be dialysed against Buffer III to get rid of Mn^{++} immediately after filtration, otherwise the later became oxidised and the enzymatic activity was lost. The pH of the buffer was also very critical; if Mn^{++} was added to a buffer pH of 8.6, it was

TABLE X

ASSOCIATION OF α AND β SUBUNITS IN THE PRESENCE OF Mn^{++}

Fraction	Volume (ml.)	Activity (Units)	Protein (mg.)	Specific Activity (Units/mg.)
DNA - Bound enzyme	16	990	544	1.8
XM-50, Filtrate	35	1,015	13	78.3
PM-30, Filtrate (filtration without Mn^{++})	33 33	842	3.43	245.4
PM-30, Supernatant (filtration with Mn^{++})..	5.5	676	1.21	558

TABLE Xa

ASSOCIATION OF α AND β SUBUNITS IN THE PRESENCE OF Mg^{++}

Fraction	Volume (ml.)	Activity (Units)	Protein (mg.)	Specific Activity (Units/mg.)
DNA - Bound enzyme	12	744	408	1.82
XM-50, Filtrate	33	990	13.2	75
PM-30, Filtrate (filtration without Mg^{++})	33	924	3.6	256.6
PM-30, Filtrate (filtration with Mg^{++})	31	930	3.3	282

oxidised immediately, where as at a pH of 7.6 oxidation of Mn^{++} was not rapid.

When the experiments were repeated using Mg^{++} at concentrations of 10 mM and 100 mM instead of Mn^{++}, the activity came through the PM-30 membrane.

It can be seen that during this process of association a considerable degree of purification of the enzyme has been achieved. Therefore this particular step was a very good potential to be used in the isolation of the two subunits. The results also demonstrate that Mn^{++} causes association of the subunits while Mg^{++} does not.

5. Studies on the stability of *H. cutirubrum* RNA polymerase in different salts.

A preliminary study was made in order to observe the effect of different grades of salts on the stability of RNA polymerase. Buffers were made using reagent and biological grades of sodium chloride and potassium chloride. The biological grade contains much less impurities than the reagent grade. The crude enzyme after passage through the XM-50 membrane was dialysed against these buffers. Six different buffers were tried and they are listed in the Table XI. Activity was determined daily for seven days. Though these observations do not lead to any conclusive relationship between stability and quality of salt, however it is obvious that when biological grades of salt was used the activity was lost more slowly.

TABLE XI

STABILITY OF THE CRUDE SOLUBLE ENZYME IN DIFFERENT GRADES OF SALTS

Buffer	1st day units/ml	2nd day units/ml	3rd day units/ml	4th day units/ml	6th day units/ml	7th day units/ml
3.1 M KCl (Reagent Grade) 0.01 M Tris pH 7.6	36.6	25	21	7	12.4	-
3.1 M KCl (Biological Grade) 0.01 M Tris pH 7.6	32.4	41	40	12	9	-
3.0 M KCl (Biological Grade) 0.1 M Magnesium Acetate 0.01 M Tris, pH 7.6	46.2	53.6	24	11	11.25	-
3.0 M KCl (Reagent Grade) 0.1 M Magnesium Acetate 0.01 M Tris, pH 7.6	32	20	14	12	-	-
3.0 M NaCl (Biological Grade) 0.1 M Magnesium Acetate 0.01 M Tris, pH 7.6	53.4	39	41.	24.3	18	-
2.5 M DCl (Reagent Grade) 1.0 M NaCl (Reagent Grade) 0.01 M Tris, pH 8.6	20.2	16.5	13.5	15.7	11.2	-

6. Studies of the effects of rifampicin and streptolydigin on the growth of H. cutirubrum and on the activity of its RNA polymerase.

Rifampicin and streptolydigin are two inhibitors of bacterial DNA-dependent RNA polymerase. H. cutirubrum RNA polymerase has been shown to be inhibited by rifampicin (Louis and Fitt 1971, 1972). Chazan and Bayley (1973) in their study with the same enzyme from the same organism did not observe any inhibition of the enzyme by rifampicin. It was one of the purposes of this study to resolve these conflicting observations. Effects of rifampicin and streptolydigin on the growth of H. cutirubrum were studied in order to arrive at a correlation with the in vitro situation. But the in vitro effect was studied with rifampicin alone.

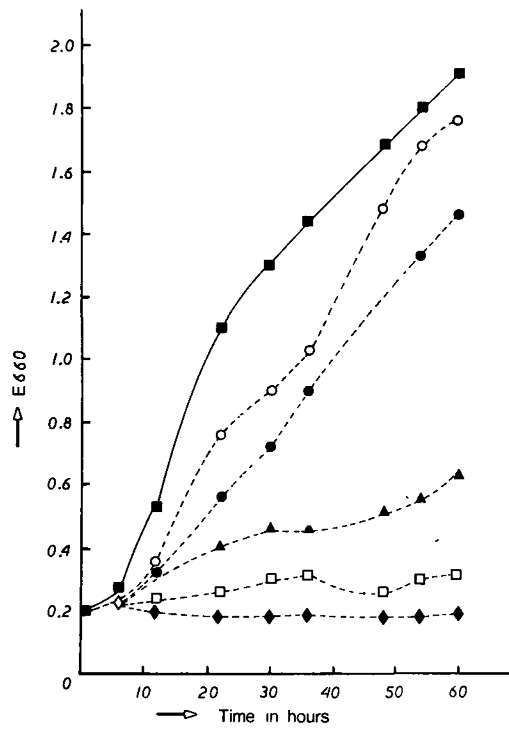
Both rifampicin and streptolydigin inhibited growth of H. cutirubrum. Results of these experiments are shown in the Figures VIII and IX and the Tables X and XI. The concentration required for complete inhibition of growth was altogether different in the two cases. Complete inhibition of growth required a rifampicin concentration of 12 μg/ml of the medium; but with streptolydigin the corresponding concentration was much higher (150 μg/ml). Some intermediate concentrations were also tried with both of the inhibitors. They are shown in the figures and the tables.

Since the original method for isolation and purification and also the assay of Louis and Fitt (1971, 1972) were modified in these studies, the effect of rifampicin on the bound and the soluble enzyme were examined in greater detail. Rifampicin had relatively little effect on the bound enzyme (Table XIV) whereas it was once again observed that

Fig. VIII Effect of rifampicin on the growth of <u>H. cutirubrum</u> cell.

■ , control; ○ , 5 μg/ml; ● , 6 μg/ml; ▲ , 8 μg/ml
□ , 9.5 μg/ml; ◆ , 12 μg/ml.

Experiments were run using some more concentrations of rifampicin apart from those shown in the diagram. They are shown in the Table XII in order to avoid overcrowding the diagram.


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
E 660
10
20
30
40
50
60
Time in hours

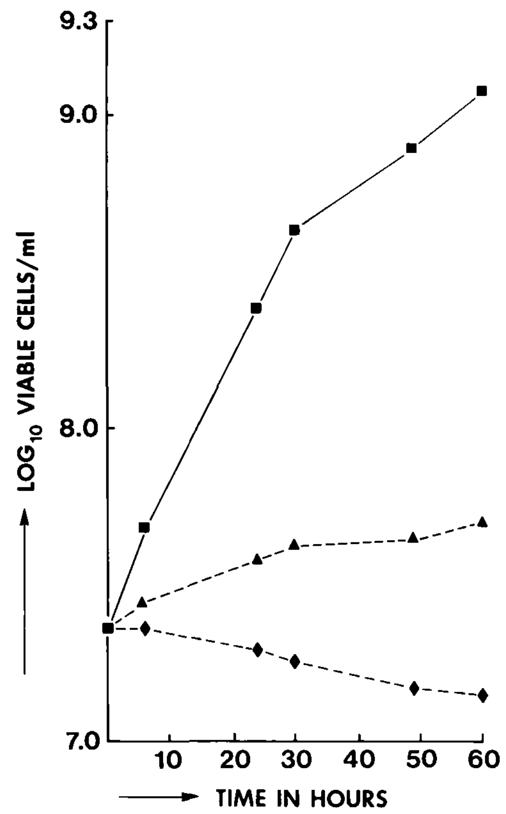

9.3
9.0
8.0
7.0
LOG10 VIABLE CELLS/ml
10
20
30
40
50
60
TIME IN HOURS

TABLE XII

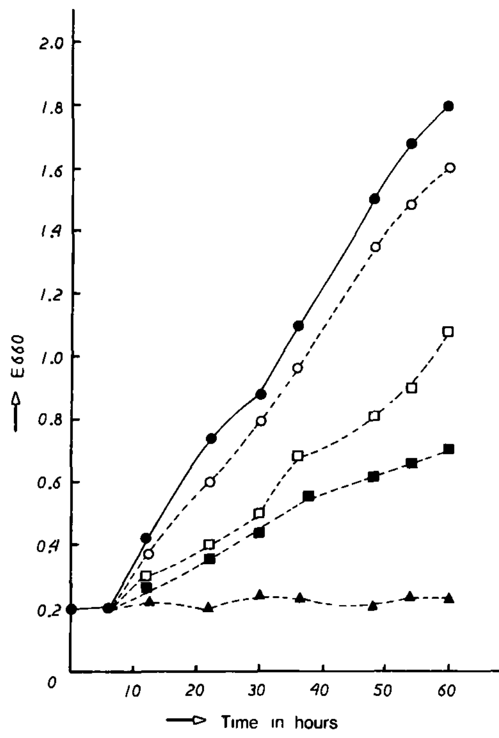
EFFECT OF RIFAMPICIN ON THE GROWTH OF H. CUTIRUBRUM CELLS

Rifampicin concentration = 2.5 μg/ml			Rifampicin concentration = 7 μg/ml		
Time (Hours)	E_{660}	% Inhibition	Time (Hours)	E_{660}	% Inhibition
0	0.23	0	0	0.23	0
6	0.24	7.6	6	0.23	13.0
12	0.55	5.4	12	0.32	39.6
24	1.00	10.0	24	0.49	55.4
30	1.18	9.2	30	0.61	53.0
36	1.30	10.3	36	0.65	55.0
48	1.60	4.7	48	0.71	58.0
54	1.72	4.6	54	0.81	55.0
60	1.80	5.7	60	0.90	53.0

Fig. IX Effect of streptolydigin on the growth of *H. cutirubrum* cells.

●, control; ○, 10 μg/ml; □, 75 μg/ml; ■, 100 μg/ml
▲, 150 μg/ml.

Note: Experiments were run using some more concentrations of streptolydigin apart from those shown in the diagram. They are shown in the Table XIII in order to avoid over-crowding the diagram.


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
E 660
10
20
30
40
50
60
Time in hours

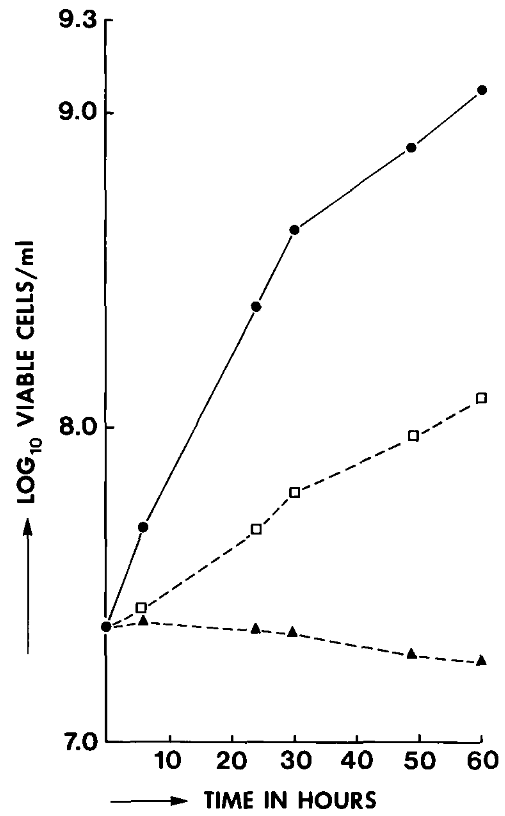

9.3
9.0
8.0
7.0
LOG10 VIABLE CELLS/ml
10
20
30
40
50
60
TIME IN HOURS

TABLE XIII

EFFECT OF STREPTOLYDIGIN ON THE GROWTH OF *H. CUTIRUBRUM* CELLS

Streptolydigin concentration ≑ 5 μg/ml			Streptolydigin concentration ≑ 125 μg/ml		
Time (Hours)	E_{660}	% Inhibition	Time (Hours)	E_{660}	% Inhibition
0	0.19	0	0	0.19	0
6	0.19	0	6	0.19	0
12	0.40	4.7	12	0.25	40.5
24	0.71	4.2	24	0.33	55.4
30	0.84	4.5	30	0.41	53.4
36	1.04	5.4	36	0.50	54.5
48	1.41	6.0	48	0.53	64.6
54	1.58	6.0	54	0.56	66.6
60	1.70	5.5	60	0.60	66.6

TABLE XIV

EFFECT OF RIFAMPICIN ON THE DNA-BOUND ENZYME

Rifampicin conc. (M)	% Inhibition
12×10^{-7}	5.6
24×10^{-7}	10.0
36×10^{-7}	10.0
48×10^{-7}	24.0

TABLE XV

EFFECT OF RIFAMPICIN ON THE CRUDE SOLUBLE ENZYME

Rifampicin conc. (M)	% Inhibition
6×10^{-7}	69
12×10^{-7}	79
18×10^{-7}	92
24×10^{-7}	100

the soluble crude enzyme was highly sensitive to it (Table XV). 1.2 x 10^{-7} M Rifampicin inhibited the soluble enzyme 80% and the bound enzyme only 6%. E. coli RNA polymerase requires rifampicin concentration of 2 x 10^{-7} M for complete inhibition (Nakamura and Yura, 1976), similar to that required in the case of the H. cutirubrum enzyme.

Chazan and Bayley (1973) claimed that their enzyme preparations were not inhibited by rifampicin at a concentration as high as 50 μg/ml which is equivalent to 2.4 x 10^{-7} M. It appears possible that their observations were affected by the assay conditions they used, which were not suitable for valid measurement of RNA polymerase activity (see Section 3).

7. Studies of the effects of methanol, ethanol and DMSO on the growth of H. cutirubrum.

Rifampicin and streptolydigin are sparingly soluble in water. The growth medium for H. cutirubrum has a salt concentration of 3.5 M which reduces the solubility of the inhibitors in aqueous solution. Therefore they had to be dissolved in organic solvents and then dispersed in the medium. The solubility of rifampicin in ethanol and methanol are 2.6% (w/w) and 0.44% (w/w) respectively. A volume of 1.65 ml of either methanol or ethanol was required in a volume of 60 ml of the medium to obtain a rifampicin concentration 10 μg/ml of the medium. If the volume of ethanol or methanol were reduced, in other words when a more concentrated solution of the inhibitor was used, it was no longer in solution as soon as it came into contact with the medium. At this solvent concentration, there was no concentration dependent effect of the

inhibitor rifampicin on the growth of H. cutirubrum. A study was made to observe the effects of ethanol and methanol on the growth of this bacterium. Figures X and XI are the results of these experiments. Methanol is more toxic than ethanol for the cells. Ethanol inhibited the growth completely at a concentration of 0.04 ml/ml of the medium and the same value for methanol was 0.02 ml/ml of the medium.

Finally, DMSO was found to be a very suitable solvent for the inhibitors. Not only it has great solubility but it did not effect the growth at all even at the highest concentration used in the medium in these studies, viz., 0.013 ml/ml (Figure XII).

Fig. X Effect of methanol on the growth of H. cutirubrum cells.

■, control; △, 0.005 ml/ml; ○, 0.01 ml/ml; ▲, 0.02 ml/ml.

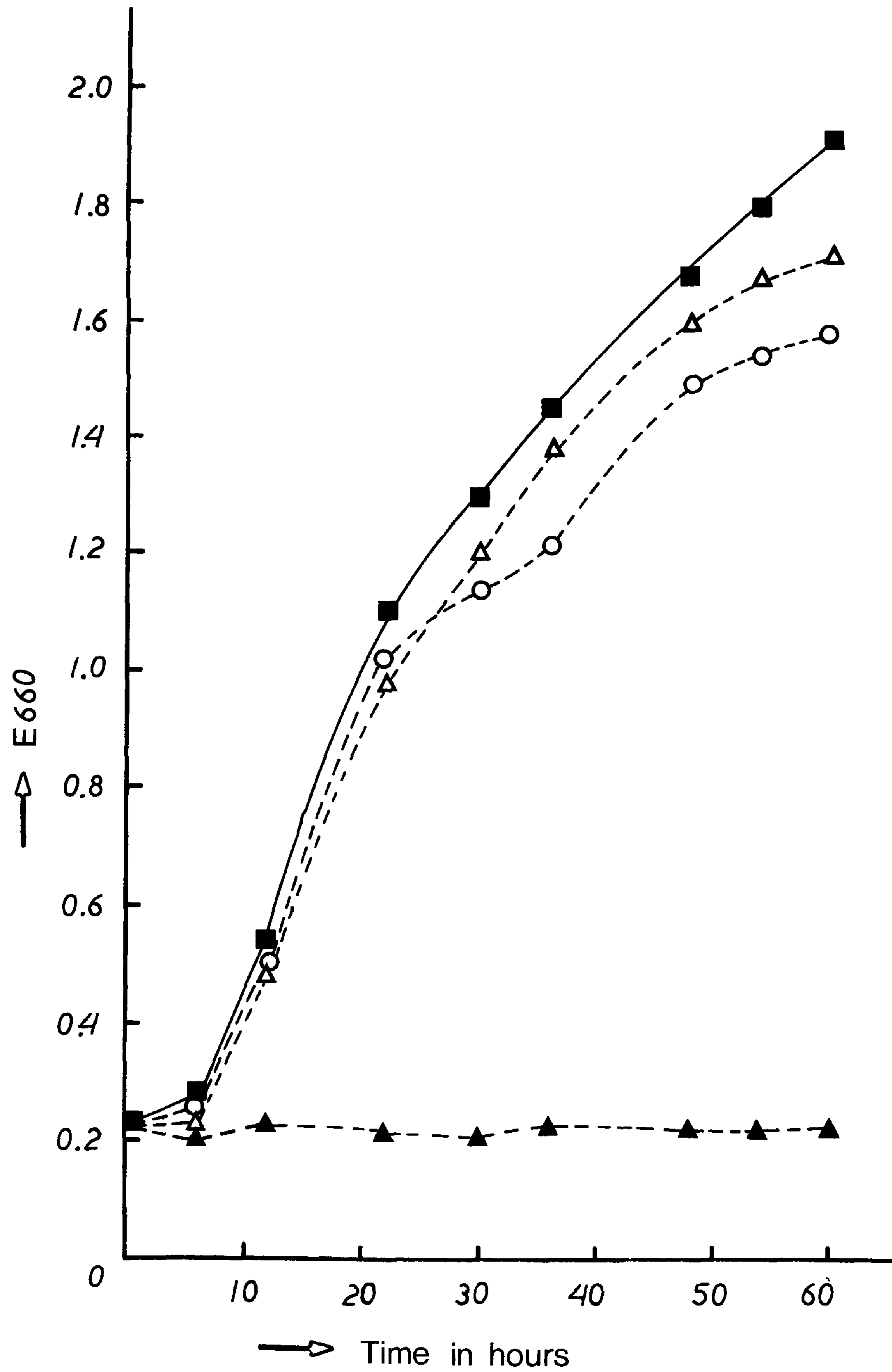

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
E660
10
20
30
40
50
60
Time in hours

Fig. XI. Effect of ethanol on the growth of *H. cutirubrum* cells.

■, control; ○, 0.005 ml/ml; ●, 0.01 ml/ml;

△, 0.02 ml/ml; ▲, 0.04 ml/ml.

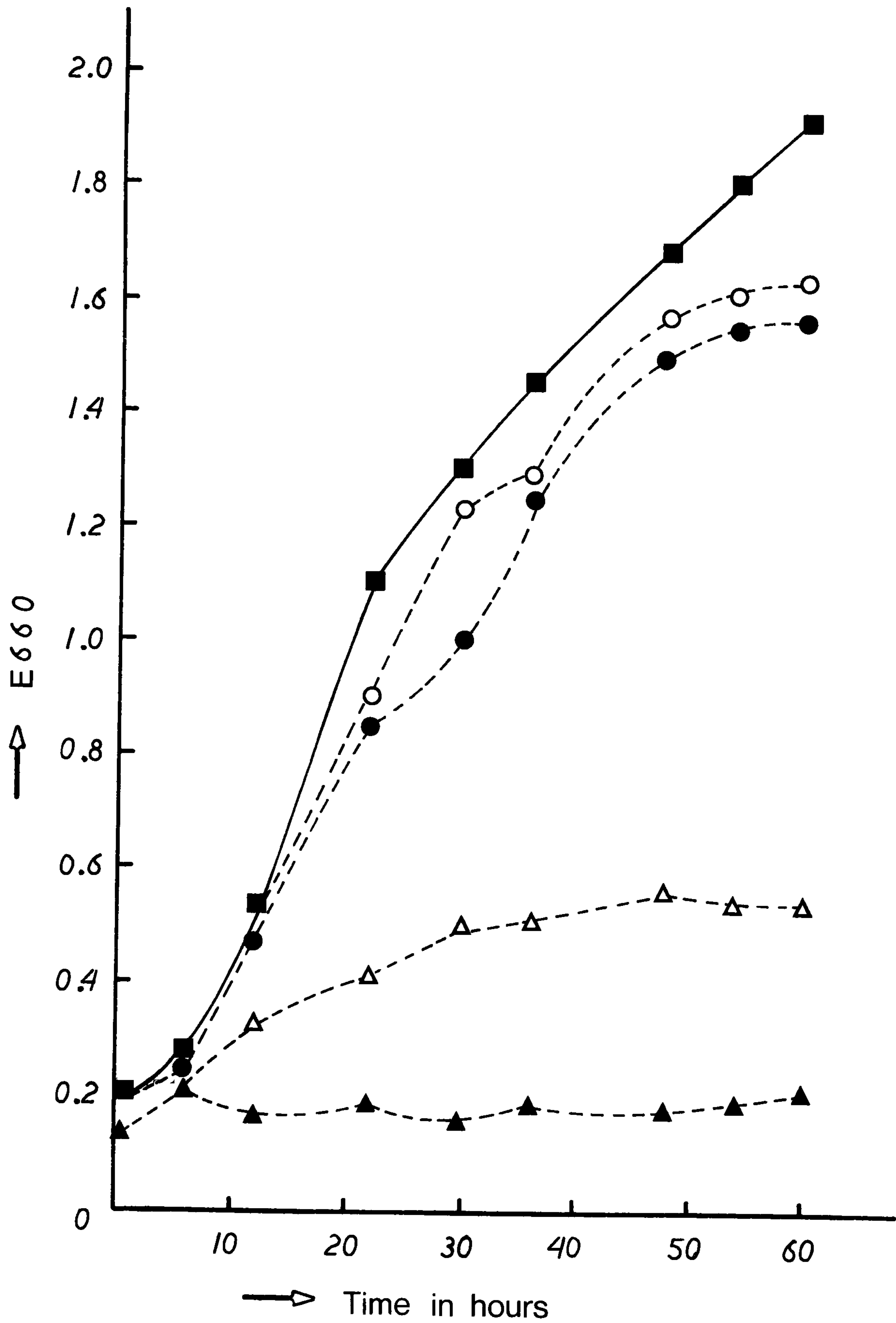
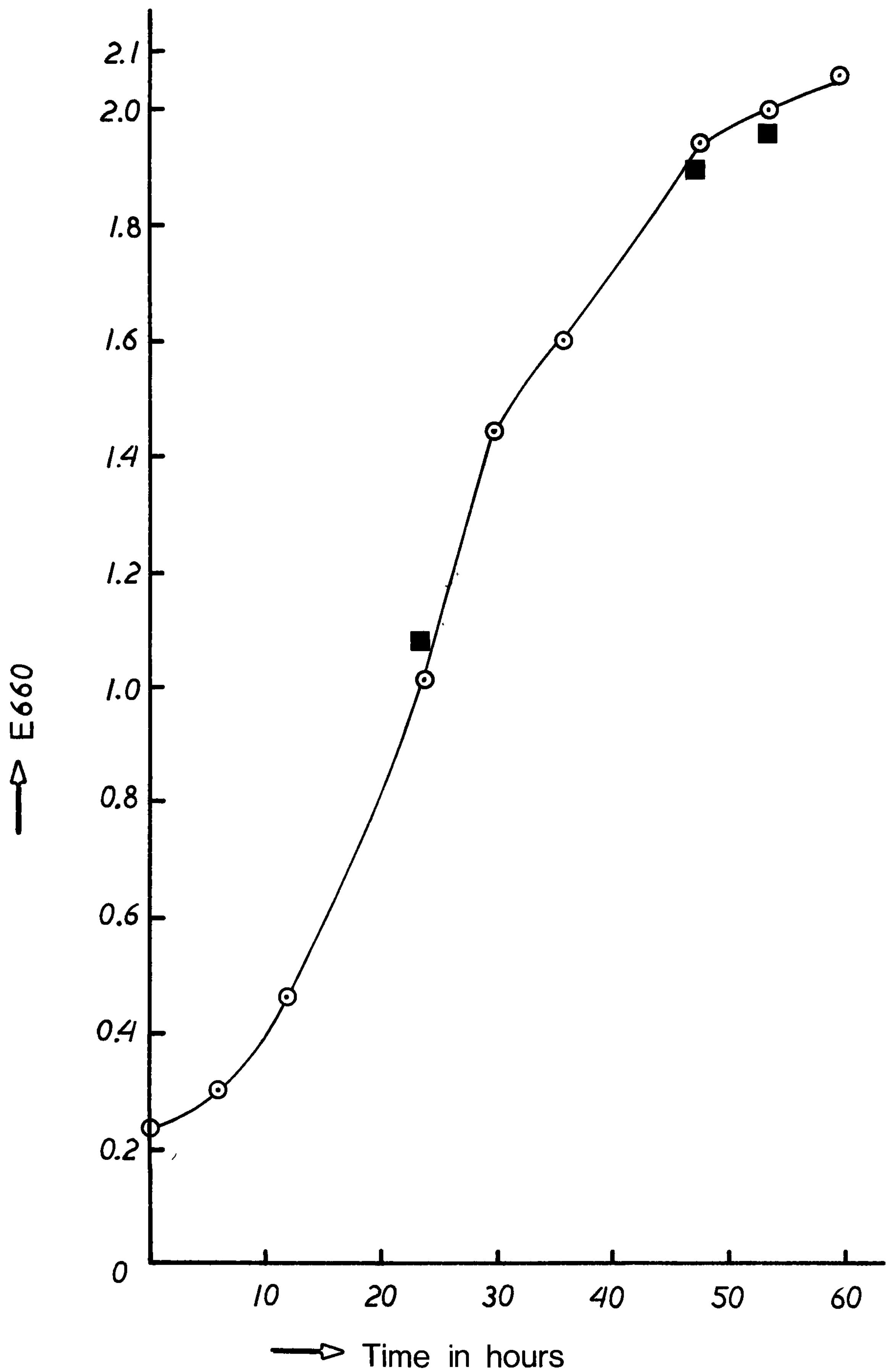

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
E660
10
20
30
40
50
60
Time in hours

Fig. XII Effect of DMSO(0.013 ml/ml) on the growth of *H. cutirubrum* cells.

○, control; ■, DMSO.


2.1
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
E660
Time in hours

IV. DISCUSSION

H. cutirubrum DNA-dependent RNA polymerase described by Louis and Fitt (1971, 1972) has many interesting properties, different from those of the corresponding enzyme from other prokaryotic sources described in the literature. It was intended in the beginning of this work to extend the studies on this topic in a number of areas: (i) the different steps of the transcription reaction catalysed by this enzyme (ii) detailed study of the primed reaction and (iii) structual studies.

Our first aim was to find a suitable method which would give higher yields of the enzyme in a stable form in order to carry out the planned experiments. While I was still investigating methods of isolation and purification, Chazan and Bayley (1973) published the results of their experiments which have been discussed in the introduction and results sections. At this point, I directed my efforts to an attempt to resolve the conflicts between the reports from the two different groups on H. cutirubrum DNA-dependent RNA polymerase, especially with regard to its molecular weight and its reaction with the inhibitor rifampicin. Many of the planned experiments were not completed because of instability of the enzyme, which remains the major obstacle to further experiments with it. However, the conflicts between two different reports on the same enzyme were resolved definitively.

It appeared from the very beginning that the method of isolation and purification of H. cutirubrum RNA polymerase was extremely critical

for its stability and activity and much time and effort were devoted to a study of this process. The work of Chazan and Bayley (1973) was helpful in arriving at a modified method of isolation primarily because of their demonstration that the enzyme could be isolated as a complex with DNA.

The enzyme in the form of complex with DNA and other materials, described below as the DNA-enzyme complex, is much more stable than the free enzyme. Preliminary attempts were made to purify the DNA-enzyme complex by density gradient centrifugation. Gradients consisting of layers of 10%, 20%, 30% and 40% sucrose in Buffer II were made using a pipette. The crude DNA-enzyme complex was layered on top of the gradient and was allowed to settle overnight under gravity. (Tulp and Bont (1975) used a special kind of apparatus to separate cells of heterogeneous size by unit gravity sedimentation). This process was repeated two further times with the active fractions (previously dialysed to 5% Sucrose). A purification of the order of 50 fold was obtained by this method and it is probable that even better and more reproducible results could be obtained by the use of linear gradients and more carefully controlled fractionation techniques. In these conditions centrifugation at low speed may be helpful. The soluble form of the enzyme could then be released by DNase treatment and depending upon the protein content and specific activity it could directly be subjected to HTP chromatography without prior gel filtration in order to separate the subunits. Thus reducing the time required for overall purification of the enzyme to about 48 hours which may be very useful in stabilizing the enzyme.

In *E.coli* *in vitro* transcription in isolated nucleoids has

been studied by a number of authors (Lubin 1969; Pettijohn et al., 1970; Murooka and Lazzarini 1973; Giorno et al., 1975). Similar studies can be undertaken in H. cutirubrum with the purified DNA enzyme complex. In previous studies with the solublised RNA polymerase of H. cutirubrum it was found to catalyse the formation of short RNA chains containing no more than 70 - 80 nucleotides on average and it was suggested that this might be due to loss of some essential factors during purification (Louis and Fitt, 1972). In physiological conditions cells must be making RNA of different size and it could be of interest to study the size of the product made by the enzyme when it is still attached to DNA. Analysis of the detailed nature of the complex and a comparative study of different enzymatic properties of the purified complex and the soluble enzyme will be valuable from the standpoint of understanding the mechanism of transcription in this bacterium. Moreover such a study will highlight the physiological differences between an organism which grows in extreme ionic condition and E. coli. We already know that RNA polymerase of H. cutirubrum is much smaller than the corresponding E. coli enzyme. Does the small size of the enzyme anyway affect transcription in this organism? Are the RNA products (rRNA, mRNA, tRNA) made by the nucleoids of the two different organisms different in nature? These are some of the aspects which can be examined using the purified complex.

In these studies, the crude soluble DNA-dependent RNA polymerase was not subjected to pH precipitation followed by concentration by dialysis against Ficoll solution as described by Louis and Fitt (1971, 1972), because during my initial studies the activity always disappeared during the concentration step. Instead, ultrafiltration through Amicon filters of appropriate pore size, was successfully used to achieve partial

purification and concentration.. pH - Precipitation as a method of purification of the crude enzyme was abandoned early in the studies described in this thesis, because some difficulty was experienced in obtaining as sharp a separation of the RNA-dependent and DNA-dependent RNA polymerase activities as that described by Louis and Fitt (1971, 1972a). In retrospect, it appears that this was a mistake, since Fitt and Peterkin (private communication) have found that a combination of pH precipitation and ultrafiltration can improve the stability of the enzyme and gives a rapid method for its partial purification.

Partial purification of the enzyme by the method of ultrafiltration developed in these studies is a considerable improvement from previous work and will be useful in further work on this enzyme. The amount of enzyme sample that can be loaded to polyacrylamide gel columns is limited by the volume of the solution and the protein concentration unless industrial scale equipment is available. Thus it is essential to improve the earlier pre-gel filtration steps if the scale of preparation is to be increased. The purification achieved by Louis and Fitt (1971) prior to P-60 gel filtration step was 4.2-fold as against 32-fold in these studies (Table VII, page 68). Subsequent studies using a combination of three successive filters afforded a 200-fold purification (TableVI, page 66) and finally, filtration through Amicon XM-50, and PM-30 membranes, followed by retention of $\alpha\beta$ complex above a PM-30 membrane in the presence of Mn^{++}, gave a 310 fold purification (Table X, page 86). So in principle use of the later procedure should permit a 75-fold increase in the amount of the enzyme that can be applied to a P-60 gel filtration column. A better purification is obtained when a column of bigger size is used. Louis and Fitt (1971) obtained a purification of 980-fold after P-60 column chroma-

tography using a 5 cm column as against 527 fold (Table I, page 68) in my studies, or in their earlier work, when a 2.5 cm column was used. It has been shown that an increase in scale of preparation increases the stability of E. coli RNA polymerase subunits (Dr. P. Palm, private communication to Dr. P.S. Fitt). If this were also true for H. cutirubrum RNA polymerase then, the method of partial purification by serial ultra-filtration may be of great help in the rapid preparation of the subunits on a greatly increased scale.

The HTP column chromatography step, in which the two subunits are separated, could be modified. In my experiments, the α-subunit was not eluted from HTP column by buffer containing 0.1 M phosphate as previously found; instead the phosphate concentration had to be raised to 0.2 M. It seems probable that an improved purification could be achieved if a linear concentration gradient of phosphate in 3.1 M KCl-10mM-Tris-HCl were used instead of a simple two step washing and elution technique. Ion exchange resins eg. DEAE-cellulose, QAE-sephadex or phosphocellulose, especially the latter which has been valuable with other RNA polymerases could also be tried, since hydroxylapatite columns are not very satisfactory on account of their poor flow rate.

The precise metal ion requirements of this enzyme are not yet clear. It was found that the requirement for Mn^{++} was dependent upon the concentration of Tris in the assay and the behaviour of the bound and the soluble enzyme was not the same in this regard. (See result section 3.2, page 78). On the other hand the assay medium developed in these studies does not have any added Mn^{++} in it, although the two subunits associate only in the presence of Mn^{++} not Mg^{++} (See the result section 4, page 85).

Louis and Fitt (1971, 1972) found that H. cutirubrum RNA polymerase had an absolute requirement for both Mg^{++} and Mn^{++} in the conditions they used for assay. However, unpublished studies by Fitt and Peterkin (private communication) have now demonstrated that DNA-dependent RNA polymerase of H. cutirubrum, H. salinarium and H. halobium are all inactive in the presence of EDTA when no exogenous divalent cation is supplied and that, in these conditions activity is restored by Mn^{++} alone while Mg^{++} has no effect even at Tris concentrations used in the assay A. The presence of trace impurities in analytical grades of KCl and NaCl (which must be used at very high concentrations) and possibly also in the other reagents, makes it difficult to demonstrate specific divalent cation requirements of a halophilic enzyme. Four other H. cutirubrum enzymes, alkaline phosphatase (Fitt and Peterkin 1976), polynucleotide phosphorylase (Peterkin and Fitt 1971), a phosphodiesterase (private communication from Dr. P.S. Fitt) and RNA dependent RNA polymerase (Louis and Fitt 1971a,b) are now known to require Mn^{++} for optimum activity. In contrast, E. coli alkaline phosphatase does not require a divalent cation and most bacterial polynucleotide phosphorylase and most phosphodiesterases are stimulated by or require Mg^{++}. So it is conceivable that many H. cutirubrum enzymes require Mn^{++} and this cation may play a special role in conditions of extreme intracellular ionic strength.

Even though the template requirement of the enzyme was not investigated extensively during this work, the quality of the DNA template was found to be very critical in the assay. H. cutirubrum DNA prepared in the laboratory according to Moore and McCarthy (1969) modification of the method of Marmur (1961) gave the best results. At times, E. coli DNA from commercial sources was used, but results were not reliable even

with DNA from the same manufacturer and some batches gave no activity even though the same enzyme was fully active with a different template preparation. Further, the template activity of solutions of DNA decreased with time and they had to be replaced at regular intervals of 7-10 days.

The washing technique developed in these studies for processing the enzyme assay, a modification of the Furano washing procedure (see page 56) was of great help. In the previous method for this purpose, a modification of Bollums technique (see page 55) the blanks were always very high and they were not reproducible. By using this technique blanks were reduced 6-10 times from the previous ones and incorporation of the order of 6-10 times over the blank was observed in the assays. Results obtained using this technique were very reproducible.

The results of the studies of the effect of rifampicin on H. cutirubrum RNA polymerase confirmed that the enzyme is typical of its type in being inhibited by this compound, in agreement with the preliminary observations of Louis and Fitt (1972). However, my results suggest that Louis and Fitt had to use a very high concentration $(1.2 \times 10^{-6}$ M) as against $(1.2 \times 10^{-7}$ M) used in these studies of the inhibitor because they did not realise that rifampicin is insoluble in aqueous media containing very high concentrations of NaCl and KCl. It seems probable that this is the reason why Chazan and Bayley (1973) did not observe any inhibition of the soluble enzyme when using a rafampicin concentration of 2.2×10^{-7}M. In the present studies, both the bound and soluble forms of the enzyme were inhibited by the antibiotic as was the growth of the bacteria.

Finally the work presented in this thesis clearly demonstrates that H. cutirubrum DNA-dependent RNA polymerase exists in the low molecular

weight form consisting of two separable subunits previously described by Louis and Fitt (1971, 1972). The failure of Chazan and Bayley (1973) to detect the soluble enzyme and their claims to have found another RNA polymerase in H. cutirubrum of high molecular weight that was not inhibited by rifampicin were based on the use of a faulty assay procedure. These authors used an assay in which the concentration of the one of the four nucleotide triphosphate substrates was 160 times less than that of others. For practical purpose this is equivalent to a nucleotide ommision assay of the type used in RNA polymerase studies to demonstrate dependence of a catalytic activity in the presence of a complete mixture of the nucleotide units. As would be expected, I observed very little activity in their conditions with the bound enzyme and none at all with the soluble enzyme. On the other hand when assay conditions appropriate for RNA polymerases were used I experienced no difficulty in demonstrating the activity of the enzyme whether in the bound or soluble form. Chazan and Bayley's demonstration that H. cutirubrum RNA polymerase can be isolated as a complex with DNA (and other components) was very valuable. However the method they described was not effective in my hands, because complete lysis of the cells is essential before the enzyme can be isolated as the complex. In particular, vigorous homogenisation of a suspension of the cells in a Potter-Elvehjem hand homogenizer did not lead to their breakage contrary to the claims of Chazan and Bayley provided cells were freshly harvested, in agreement with general experience with bacteria. Lysis of the cells by this technique was only possible if they had already been allowed to stand for an hour or more in the lysis buffer containing 1M KCl-0.1M - magnesium acetate - 10mM-Tris-Hcl, pH 7.6. In these conditions the bacteria are unstable and lyse spontaneously within a few hours, so

that clearly, hand homogenisation becomes possible after some destruction of the cell walls has already occurred. In contrast microscopic examination of a suspension of freshly harvested bacteria that had been homogenised showed that little or no breakage occurred and that the bacteria were unusually vigorous and motile, possibly as a result of thorough aeriation of the suspension.

Thus my results agree with the findings of Louis and Fitt on all the three major points where the observations of Chazan and Bayley were altogether different: subunit structure and composition, molecular weight and reaction towards the inhibitor rifampicin.

In addition, these studies have led to the development of an improved assay procedure for H. cutirubrum RNA polymerase that has largely overcome the problems connected with the need to use Mn^{++} at a comparatively alkaline pH and to the demonstration that a rapid and effective preliminary purification of the enzyme is possible by sequential ultrafiltration in the absence and presence of Mn^{++}. This new method of isolation should make possible for the first time on a large scale purification of H. cutirubrum DNA-dependent RNA polymerase and thus open the way to a detailed study of the relationship between its structures and functions.

SUMMARY

DNA-dependent RNA polymerase from the extreme halophile *Halobacterium cutirubrum* has been isolated as a complex with DNA by slow spontaneous lysis of bacteria in 3M-KCl which sedimented completely at 45,000 x g. Controlled DNase digestion of the complex with or without subsequent sonication released the enzyme quantitatively in a soluble form; this preparation was active and was excluded by ultrafilters with molecular weight exclusion limit of 50,000. The ultrafiltrate upon gel filtration and subsequent hydroxyapatite chromatography gives a higher yield of the purified and subunits of the enzyme as described by Louis and Fitt (Biochem. J. 127, 69-80, 1972). The purified and subunits chromatograph together in an analytical gel filtration column (Bio Gel P-60) corresponding to molecular weight of around 18,000. Neither of the subunits alone was active. In the presence of 10 mM Mn^{++}, an equimolar mixture of the two subunits was fully active and was retained above ultrafilters with a molecular weight exclusion limit of 30,000. During the course of the present study, a new assay valid for both the bound and the soluble forms of the enzyme was developed and it was also found that the divalent cation requirement of the soluble enzyme depended upon the ionic strength of the buffer.

REFERENCES

Abram, D. and Gibbons, N.E. (1960). Canad. J. Microbiol. 6, 535.
Abram, D. and Gibbons, N.E. (1961). Canad. J. Microbiol. 7, 741.
Aitken, D.M. and Brown, A.D. (1969). Biochim. Biophys. Acta. 177, 351.
Aitken, D.M., Wicken, A.J. and Brown, A.D. (1970). Biochem J: 116, 125.

Bauer, R.J. and Carlberg, D.M. (1973). Canad. J. Biochem. 51, 621.
Baxter, R.M. and Gibbons, N.E. (1956). Canad. J. Microbiol. 2, 599.
Baxter, R.M. (1959). Canad. J. Microbiol. 5, 47.
Bayley, S.T. (1966). J. Mol. Biol. 15, 420.
Beabealashvilly, R., Ivanov, V., Minchenkova, L. (1972). Biochim. Biophys. Acta. 259, 35.
Berg, D. and Chamberlin, M. (1970). Biochemistry. 9, 5055.
Berg, D., Barret, K. and Chamberlin, M. (1971). Methods in Enzymology. Vol. XXI, p. 506.
Bergey's Manual of Determinative Bacteriology, 7th ed. (1957). Ed. by Breed, R.S., Murray, E.G.D. and Smith, N.R.
Bergey's Manual of Determinative Bacteriology, 8th ed. (1974). Ed. by Breed, R.S., Murray, E.G.D. and Smith, N.R.
Bickel, H., Knusel, F., Kump, W. and Neipp, L. (1967). Anti-Microb. Ag. Chemother. 1966, 352.
Blattner, F. and Dahlberg, J. (1972). Nature (New Biol). 237, 232.
Bollum, F.J. (1959). J. Biol. Chem. 234, 2733.
Bollum, F.J. (1966). Procedures in Nubleic Acid Research. Ed. by Cantoni, G.L. and Davies, D.R. Harper and Row, New York, Vol. 1, p. 296.
Bollum, F.J. (1968). Methods in Enzymology. Vol. XII B, p. 169.
Bremer, H., Konrad, M., Gaines, K. and Stent, G.S. (1965). J. Mol. Biol. 13, 540.
Brown, A.D. (1963). Biochim. Biophys. Acta. 75, 425.
Bujard, H. and Heidelberger, C. (1966). Biochemistry. 5, 3339.
Burgess, R.R. (1969). J. Biol. Chem. 244, 6160.
Burgess, R.R. and Jendrisak, J.J. (1975). Biochemistry. 14, 4634.

Cazzulo, J.J. and Vidal, M.C. (1972). J. Bacteriol. 109, 437.
Chamberlin, M. and Berg, P. (1962). Proc. Nat. Acad. Sci. USA 48, 81.
Chazan, L.L. and Bayley, S.T. (1973). Canad. J. Biochem. 51, 1297.
Cheah, K.S. (1969). Biochim. Biophys. Acta 180, 320.
Cheah, K.S. (1970). FEBS Lett. 7, 301.
Christian, J.H.B. and Watho, J. (1962). Biochim. Biophys. Acta 65, 506.
Crawford, L. (1965). J. Mol. Biol. 14, 593.

Darlix, J., Fromageot, P. and Reich, E. (1971). Biochemistry. 10, 1525.
Dundas, I.E.D. (1972). Eur. J. Biochem. 27, 376.

Fischer, L. (1969). In Laboratory Techniques in Biochemistry and Molecular Biology Vol 1, p. 157. Ed. by Work, T.S. and Work, E. New York: John Wiley and Sons Inc.
Fitt, P.S. and Peterkin, P.I. (1976). Biochem. J. 157, 161.
Fitt, P.S., Peterkin, P.I. and Barua, N.N. (1975). Biochem. J. 149, 719.

Furano, A.V. and Harris, M.I. (1971). Biochim. Biophys. Acta. 247, 291.

Gerard, G., Johnson, J. and Boezi, J. (1972). Biochemistry. 11, 989.
Girorno, R., Stamato, T., Lydersen, B. and Pettijohn, D. (1975). J. Mol. Biol. 96, 217.
Gochnauer, M.B. and Kushner, D.J. (1969). Canad. J. Microbiol. 51, 1297.
Good, N.E., Winget, G.D., Winter, W., Connolly, T.N., Isawa, S. and Singh, R.M.M. (1966). Biochemistry 5, 467.
Good, W.A. and Hartman, P.A. (1970). J. Bacteriol. 104, 601.
Gornall, A.G., Bardawill, C.J. and David, M.M. (1949). J. Biol. Chem. 177, 751.
Griffiths, E. and Bayley, S.T. (1969). Biochemistry. 8, 541.

Harford, A. and Beer, M. (1972). J. Mol. Biol. 69, 179.
Harrison, F.C. and Kennedy, M.E. (1922). Trans. Royl Soc. Canada. V, 16, 101.
Heil, A. and Zillig, W. (1970). FEBS Lett. 11, 165.
Higa, A. and Cazzulo, J.J. (1975). Biochem. J. 147, 267.
Hinkle, D. and Chamberlin, M. (1972). J. Mol. Biol. 70, 157.
Hinkle, D. and Chamberlin, M. (1972). J. Mol. Biol. 70, 186.
Hochstein, L.I. and Dalton, B.P. (1968). J. Bacteriol. 95, 37.
Hochstein, L.I. and Dalton, B.P. (1968a). Biochem. Biophys. Acta. 167, 638.
Holmes, P.K. and Halvorson, H.O. (1963). Canad. J. Microbiol. 9, 904.
Holmes, P.K. and Halvorson, H.O. (1965). J. Bacteriol. 90, 316.
Holmes, P.K., Dundas, I.E.D. and Halvorson, H.O. (1965). J. Bacteriol. 90, 1159.
Hubbard, J.S. and Miller, A.B. (1969). J. Bacteriol. 99, 161.
Hurwitz, J., Bresler, A. and Diringer, R. (1960). Biochem. Biophys. Res. Commun. 3, 15.

Ingram, M. (1947). Proc. R. Soc. B, 134, 181.
Ishihama, A., Murakami, S., Fukuda, R., Matsukage, A. and Kameyama, T. (1971). Mol. Gen. Gent. 111, 66.

Johnston, D.E. and McClure, W.R. (1976). In RNA Polymerase (R. Losick and M. Chamberlin eds.). Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 413.
Jones, O. and Berg, P. (1966). J. Mol. Biol. 22, 199.

Kameyama, T., Matsukage, A., Tanaka, Y. (1969). J. Cell. Physiol. 74: Suppol. 219.
Khesin, R.B., Astaurovia, O.B., Shemakin, M.F., Kamzolova, S.G. and Manyakov, V.F. (1967). Mol. Biol. 1, 617.
Kim, E.K. and Fitt, P.S. (1977). Biochem. J. 161, 313.
Klebahn, H. (1919). Mitt. Inst. Allgem. Botan. Hamburg. 4, 11.
Kleppe, R. and Khorana, H.G. (1972). J. Biol. Chem. 247, 6149.
Kradolfer, F., Neipp, L. and Sackmann, W. (1967). Anti-microb. Ag. Chemother. 1966, 359.
Krakow, J. and Fronk, E. (1969). J. Biol. Chem. 244, 5988.
Kushner, D.J. and Bayley, S.T. (1963). Canad. J. Microbiol. 9, 53.
Kushner, D.J. (1964). J. Bacteriol. 87, 1147.
Kushner, D.J. and Onishi, H. (1966). J. Bacteriol. 91, 653.

Lane, E.A. and Mavrides, C. (1969). Anal. Biochem. 27, 363.

Lanyi, J.K. and Stevenson, J. (1969). J. Bacteriol. 98, 611.
Lanyi, J.K. (1972). J. Biol. Chem. 247, 3001.
Lanyi, J.K. (1974). Bacteriological Review. Vol. 38, p. 272.
Larsen, H. (1962). In The Bacteria, Vol. 4, p. 297. Ed. by Gunsalus, I.C. and Stainier, R.Y. New York: Academic Press Inc.
Larsen, H. (1967). In Advances in Microbial Physiology. Vol. I, p. 97. Ed. by Rose, A.H. and Willkinson, J.F. Academic Press Inc., New York.
Liebel, V., Kaplan, J.G. and Kushner, D.J. (1969). Can. J. Biochem. 47, 1095.
Lieberman, M.M. and Layni, J.K. (1971). Biochim. Biophys. Acta. 245, 21.
Lieberman, M.M. and Layni, J.K. (1972). Biochemistry. 11, 211.
Louis, B.G. and Fitt, P.S. (1971a). Biochem. J. 121, 621.
Louis, B.G. and Fitt, P.S. (1971b). Biochem. J. 121, 629.
Louis, B.G. and Fitt, P.S. (1971c). FEBS Lett. 14, 143.
Louis, B.G. and Fitt, P.S. (1972a). Biochem. J. 127, 69.
Louis, B.G. and Fitt, P.S. (1972b). Biochem. J. 127, 81.
Louis, B.G. and Fitt, P.S. (1972c). Biochem. J. 128, 755.
Louis, B.G., Peterkin, P.I. and Fitt, P.S. (1971). Biochem. J. 121, 635.
Lubin, M. (1969). 39, 219.
Ludhlum, D. and Wilhem, R.C. (1968). J. Biol. Chem. 243, 2750.

Maggi, N.
Maitra, U. and Hurwitz, J. (1965). Proc. Nat. Acad. Sci. USA. 61, 1494.
Maitra, U., Nakata, Y. and Hurwitz, J. (1967). J. Biol. Chem. 242, 4908.
Maitra, U. and Barash, F. (1969). Proc. Nat. Acad. Sci. USA. 64, 779.
Marmur, J. (1961). J. Mil. Biol. 3, 208.
Mescher, M.F., Strominger, J.L. and Watson, S.W. J. Bacteriol. 120, 945.
Minkley, E. and Pribnow, D. (1973). J. Mil. Biol. 77, 255.
Moore, R.L. and McCarty, B.J. (1969). J. Bacteriol. 99, 248.
Murti, J., Prescott, D. and Pene, J. (1972). J. Mol. Biol. 69, 179.
Murooka, Y and Lazzarini, R.A. (1973). J. Biol. Chem. 248, 6248

Nakamura, Y. and Yura, T. (1976). Mol. Gen. Gent. 145, 227.
Nishimura, S., Harada, F. and Ikehara, M. (1966). Biochim. Biophys. Acta. 129, 301.
Norberg, P. and Hofsten, B.V. (1969). J. Gen. Microbiol. 55, 251.
Norberg, P. and Hofsten, B.V. (1970). Biochim. Biophys. Acta. 220, 132.
Norberg, P., Kaplan, J.G. and Kushner, D.J. (1973). J. Bacteriol. 113, 680.

Ochoa, S., Burma, D.P., Kroger, H. and Weill, J.D. (1961). Proc. Nat. Acad. Sci. USA. 47, 670.

Peterkin, P.I. and Fitt, P.S. (1971). Biochem. J. 121, 613.
Pettijohn, D. and Kamiya, T. (1969). J. Mil. Biol. 29, 275.
Pettijohn, D.E., Clarkson, K., Kossman, C.R. and Stonington, O.G. (1970). J. Mil. Biol. 52, 281.
Pettijohn, D., Stonigton, O. and Kossman, C. (1971). Nature (London). 228, 235.
Pugh, E.L. WasseF, M.K. and Kates, M (1971). Canad. J. Biochem. 49, 953.
Richardson, J. (1966a). Proc. Nat. Acad. Sci. USA. 55, 1616.

Richardson, J. (1966b). J. Mol. Biol. 21, 83.
Richardson, J. (1970). Cold. Spring. Harbor Symp. Quant. Biol. 35, 127.
Roberts, J.W. (1969). Nature. 224, 1168.

Sehgal, S.N. and Gibbons, N.E. (1960). Canad. J. Microbiol. 6, 165.
Sensi, P., Maggi, N., Furesz, S. and Maffi, G. (1967). Anti-microb. Ag. Chemother. 1966, 699.
Sippel, A. and Hartman, G. (1968). Biochim. Biophys. Acta. 157, 218.
Smith, D.A., Martinez, A.M., Ratliff, R.L., Williams, D.L. and Hayes, F.N. (1967). Biochemistry. 6, 3057.
Soo-Hoo, T.S. and Brown, A.D. (1967). Biochim. Biophys. Acta. 135, 164.
Stead, N. and Jones, O. (1967). Biochim. Biophys. Acta. 145, 679.
Steensland, H. and Larsen, H. (1969). J. Gen. Microbiol. 55, 325.
Stevens, A. (1960). Biochem. Biophys. Res. Commun. 3, 92.
Stevens, A. (1961). J. Biol. Chem. 236, PC 43.
Stevens, A., Emery, A.J., Jr. and Sternberger, N. (1966). Biochem. Biophys. Res. Commun. 24, 929.
Stoeckenius, W. and Kunau, W.H. (1968). J. Cell. Biol. 38, 337.
Straat, P. and Ts'o, P.O.P. (1969). J. Biol. Chem. 244, 6263.

Takanami, M.T., Okamoto, T. and Sugiura, M. (1971). J. Mol. Biol. 62, 81.
Terao, T., Dahlberg, J. and Khorana, H.G. (1972). J. Biol. Chem. 247, 6157.
Tulp, A. and Bont, W.S. (1975). Analytical Biochemistry. 67, 11.

Vogt, V. (1969). Nature (London). 223, 854.

Warburg, O. and Christian, W. (1942). Biochem. L. 310, 384.
Ward, D. and Reich, E. (1972). J. Biol. Chem. 247, 705.
Wehrli, W., Knusel, F. and Staehelin, M. (1968) Biochim. Biophys. Acta. 157, 215.
Wehrli, W. and Staehelin, M. (1969). Biochim. Biophys. Acta. 182, 24.
Weiss, S.B. and Gladstone, L. (1959). J. Am. Chem. Soc. 81, 4118.
Weiss, S.B. (1960). Proc. Nat. Acad. Sci. USA. 46, 1020.
Witner, H.J. (1971). J. Biol. Chem. 246, 5220.

Yarborough, L.R., (1976). Biochemistry. 15, 2669.

Zillig, W., Zechel, K. and Halbwachs, H. (1970). Hoppe Seyler's Z. Physiol. Chem. 351, 221.