# RNA POLYMERASES FROM AN EXTREME HALOPHILE

By B. Gregory Louis

presented to the

Division of Sciences

School of Graduate Studies

in partial fulfilment of the requirements

for the degree of Doctor of Philosophy

Department of Biochemistry

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DEDICATION

This thesis is dedicated to

Dr. W. Horsley Gantt,

who first stimulated my interest in the biological sciences, and by whose continued interest and encouragement I have immeasurably profited.

## ABSTRACT

DNA-dependent and RNA-dependent RNA polymerases from the extreme halophile Halobacterium cutirubrum have been separated and purified to electrophoretic homogeneity. Their specificities for templates and substrates and the complementarity of products and templates have been demonstrated.

The DNA-dependent polymerase consists of two polypeptide chains, α and β, both of molecular weight 18,000. The functional entity has thus a mol. wt. of 36,000, less than a tenth that of Escherichia coli RNA polymerase. Proteins α and β are separate in solution, but associate in the presence of $Mn^{2+}$ and are required in equimolar amounts for optimum activity. Greatest activity and stability are displayed at monovalent salt concentrations above 2 M. Unlike the E. coli polymerase, the H. cutirubrum DNA-dependent enzyme requires $Mg^{2+}$ in order to bind to DNA. When these conditions are all satisfied the enzyme recognizes the same initiation signals as E. coli RNA polymerase and copies the template accurately. In the absence of salt the template specificity is altered and the two chains become unstable, the β chain extremely so. The α protein is capable of RNA synthesis in the absence of β provided an oligonucleotide

primer is supplied; the primer is then incorporated into the 5' end of the growing chain. Thus α can bind the template and substrates (except for the initiating nucleotide), form phosphodiester bonds, translocate, terminate and possibly release as well. Data are presented which imply that the β chain contains, or at least participates in the formation of, the binding site for the initiating nucleotide, and is responsible for the salt-dependent template specificity of the enzyme. It probably has also a role in chain termination. The purified α protein or αβ complex has no activity in the absence of double-stranded DNA.

The RNA-dependent polymerase is a single polypeptide chain of molecular weight 17,000-18,000. It produces RNA complementary to a wide variety of synthetic or natural RNA templates of high molecular weight, but has limited activity with tRNA as template, and none at all with double-stranded RNA or with native or denatured DNA. It does not possess 'CCA-adding' or reverse transcriptase activity. The enzyme bears greater functional resemblance to the DNA-dependent RNA polymerases of other prokaryotes (in their RNA-directed reaction) than to the bacteriophage-induced replicases. It resembles protein α of the DNA-dependent polymerase in its amino acid composition and in its response to ionic strength.

The RNA-dependent polymerase is functionally related to neither the α nor the β component of the DNA-dependent enzyme; none of the three polymerase proteins will substitute for any other.

The occurrence of an RNA-dependent RNA polymerase in an organism whose DNA-dependent polymerase, unlike most others, lacks the capacity for RNA-directed RNA synthesis, suggests that there may be a physiological role for the RNA-directed reaction in vivo.

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## ABBREVIATIONS

Abbreviations and spelling conform to the conventions of the Biochemical Journal (vol. 126, pp. 1-19, 1972) with the following exceptions:

a) RNase and DNase are substituted for RNAase and DNAase

b) Liquid-scintillation counter is abbreviated LSC in s. 6.

c) cpm, dpm and rpm are substituted for c.p.m., d.p.m. and r.p.m.

Temperature is expressed in degrees Celsius throughout, and is indicated only by the symbol $^{o}$.

# 1. INTRODUCTION

## 1.1. DNA-dependent RNA polymerase

### 1.1.1. Historical background

The belief that genetic information flows from DNA to RNA to protein - the central dogma, as it is called - was firmly established by the late 1950's. The Kornberg enzyme (Lehman, Bessman, Simms & Kornberg, 1958; Bessman, Lehman, Simms & Kornberg, 1958; Bessman, Lehman, Adler, Zimmerman, Simms & Kornberg, 1958; Adler, Lehman, Bessman, Simms & Kornberg, 1958) was characterized and highly purified, and all were certain that the genetic replicase had been found. As it was equally clear that polynucleotide phosphorylase, discovered by Grunberg-Manago & Ochoa (1955), had not the specificity to be the genetic transcriptase, an enthusiastic search was well under way for an enzyme capable of producing RNA complementary to the genetic material. A number of investigators (Heidelberger, Harbers, Leibman, Takagi & Potter, 1956; Paterson & LePage, 1957; Canellakis, 1957; Edmonds and Abrams, 1957; Herbert, 1958; Hecht, Zamecnik, Stephenson & Scott, 1958; Preiss, Dieckmann & Berg, 1961) observed terminal addition of one or more nucleotides to RNA molecules, but could not demonstrate extensive polymerization. Weiss first successfully identified an enzyme in mammalian liver (Weiss & Gladstone, 1959; Weiss, 1960) which required

all four ribonucleoside triphosphates and produced RNA labelled throughout the polynucleotide chain when radioactive substrates were supplied.

Reports claiming the existence of an Escherichia coli enzyme with similar properties followed within a few weeks of the full paper by Weiss (Stevens, 1960; Hurwitz, Bresler & Diringer, 1960) in which DNA dependence was demonstrated and physical separation from polynucleotide phosphorylase was achieved. Weiss and Nakamoto (1961a) demonstrated the presence of the enzyme in Micrococcus lysodeikticus (now known as M. luteus); Ochoa, Burma, Kröger & Weill (1961), in Lactobacillus arabinosus and Azotobacter vinelandii (now A. agilis). Ehrlich ascites tumour cells were shown to possess net RNA-synthesizing activity (Burdon & Smellie, 1960a,b; Burdon, 1960; Burdon & Smellie, 1961a,b), as were pea embryo cells (Huang, Maheshwari & Bonner, 1960). The complementarity of product and template was soon proved by base composition analysis (Stevens, 1961; Furth, Hurwitz & Goldmann, 1961a) and by nearest-neighbour analysis of the products formed on a poly d(A-T) template (Furth et al., 1961b), or on a natural template (Weiss & Nakamoto, 1961b). Geiduschek, Nakamoto & Weiss (1961) hybridized the product of $T_2$ coliphage DNA-directed synthesis with isolated $T_2$ DNA and showed that hybridization would not occur with other DNA in the same conditions, thus confirming that the M. luteus enzyme

accurately transcribed the $T_2$ DNA.

RNA polymerase is an extremely complex enzyme system and is subject to influence from an extremely wide range of external conditions and factors. Its characterization proceeded apace over the next eight years, but an abundance of conflicting results was obtained, and confusion abounded likewise among those who attempted to form a coherent picture of RNA synthesis and its regulation. The reason for this became apparent early in 1969 with the publication of the finding (Burgess, Travers, Dunn & Bautz in that year) that a protein factor, capable of modifying the template specificity of E. coli RNA polymerase, could be separated from the enzyme by phosphocellulose chromatography. The presence of the σ factor, as it was called, led to increased rates of transcription of a wide variety of templates, stimulation being greatest in the case of double-stranded DNA (Burgess, 1971) or when chain initiation became rate-limiting at low substrate concentrations (Niyogi, 1972). The rate of chain elongation was unaffected by σ, but more RNA chains were initiated (Travers & Burgess, 1969). Initiation was stimulated at specific sites on natural templates (Burgess, Travers, Dunn & Bautz, 1969; Berg, Barrett, Hinkle, McGrath & Chamberlin, 1969; Krakow & von der Helm, 1970), and σ was released for re-use once initiation had taken place. The transcription of fd replicative form, for example, was asymmetric in the presence of σ and synthesis was mainly restricted to three

discrete RNA molecules, whereas without σ the enzyme produced heterogeneous RNA from both strands of the template (Sugiura, Okamoto & Takanami, 1970; Takanami, Okamoto & Sugiura, 1970). Even before the σ factor had been fully characterized as to function, it was obvious that many of the discrepancies in previous results could be explained on the basis of the variable amount of σ present in the various preparations of RNA polymerase used.

In order not to share unduly in the confusion which prevailed throughout the sixties, we shall abandon the historical discussion at this point and turn to a detailed consideration of a part of what is now known or thought about RNA polymerases (whose name is beginning to be legion), with particular reference to bacterial systems free from bacteriophage infection. The discussion will necessarily be limited to those aspects of RNA polymerase properties and function which are relevant to the work to be presented.

### 1.1.2. Overall function of RNA polymerase

The 'ideal' RNA polymerase makes, when needed, RNA complementary to specific sequences of the template. Unfortunately for the investigator, that deceptively simple sentence implies a hideous complexity of regulatory apparatus. The enzyme must recognize the right template, the right initiation and termination loci and the right time to

function, as well as faithfully copy the template once initiation is triggered. Although all this must and does happen in vivo with a high degree of reliability, the isolation of the enzyme inevitably removes some part of the apparatus that makes it so. Negative regulation can no longer be observed - the enzyme sets to work as soon as the necessary materials are supplied - and the recognition of what should and what should not be copied is usually impaired as well (the removal of some or all of the σ factor in early purifications is an excellent example of the sort of difficulty encountered in in vitro studies). For practical purposes, therefore, the function of RNA polymerase is defined as making RNA complementary to the template - the restrictions as to specificity and temporal regulation being removed - and the investigator accepts as part of the task of characterization the necessity of determining why and how his polymerase differs from the ideal.

### 1.1.3. Occurrence of RNA polymerase

Table 1.1 lists a number of the sources from which RNA polymerases have been isolated and studied, along with some basic properties to which reference will later be made.

### 1.1.4. RNA polymerase from prokaryotes

As a class, the RNA polymerases of prokaryotes other than the extreme halophiles are quite similar. Although the

Table 1.1. Some properties of RNA polymerases from various sources

Template specificity is defined in the following terms: Broad, accepts native and denatured DNA from homologous and heterologous sources; Native DNA, accepts only double-stranded DNA but from homologous or heterologous sources; Denatured DNA, accepts only single-stranded DNA but from homologous or heterologous sources; Restricted, accepts DNA from homologous source only or with much greater activity than other DNA. Where the enzyme is active with more than one divalent cation, that most commonly used is specified. Nuclear RNA polymerases whose existence is controversial (i.e. may be due to mitochondrial contamination) are omitted.

| Source | Mol. Wt. | Subunit Comp. | Divalent Cations Needed | Template Specificity | Rifampicin Sensitivity | Reference |
|---|---|---|---|---|---|---|
| E. coli | 495,000 | $\alpha_2\beta\beta'\sigma$ | $Mg^{2+}$ | Broad | + | see text |
| A. vinelandii (agilis) | 495,000 | $\alpha_2\beta\beta'\sigma$ | $Mg^{2+}$ | Broad | + | Krakow & von der Helm (1970) |
| M. lysodeikticus (luteus) | similar to E. coli | | $Mg^{2+}$ | Broad | + | Gumport & Weiss (1969) |
| Pseudomonas putida | 506,000 | $\alpha_2\beta\beta'\sigma$ | $Mg^{2+}$/ $Mn^{2+}$ | Broad | + | Johnson, DeBacker & Boezi (1971); Gerard, Johnson & Boezi (1972) |
| Ps. aeruginosa | - | $\alpha_2\beta\beta'\sigma$ | - | Broad | + | Whiteley & Hemphill (1970) |
| Bacillus subtilis | 457,000 | $\alpha_2\beta\beta'\sigma$ | $Mg^{2+}$ | Broad | + | Losick et al. (1970), Avila et al (1971) & Hussey et al. (1972) |
| B. stearothermophilus | - | - | $Mg^{2+}$ | Broad | ? | Remold-O'Donnell & Zillig (1969) |
| Anacystis nidulans (a blue-green alga) | 436,000 | $\alpha_2\beta\beta'\sigma$ | $Mg^{2+}$ | Broad | + | Herzfeld & Zillig (1971), von der Helm & Zillig (1967) |

Table 1.1. continued....

| Source | Mol. Wt. | Subunit Comp. | Divalent Cations Needed | Template Specificity | Rifampicin Sensitivity | Reference |
|---|---|---|---|---|---|---|
| Coconut nuclei polymerase I | - | - | $Mn^{2+}$ | Native DNA | ? | Mondal, Mandal & Biswas (1972) |
| polymerase II | | | $Mg^{2+}$ | Native DNA | ? | |
| Calf thymus polymerase I | 13S | - | $Mn^{2+}$ | Restricted | - | Blatti et al. (1970) |
| polymerase II | 400,000? | ABCD | $Mn^{2+}$ | Restricted | - | Weaver, Blatti & Rutter (1971) |
| Rat liver nuclei polymerase I | - | - | $Mn^{2+}$ | Restricted | - | Seifart (1970) |
| polymerase II | 400,000? | ABCD | $Mn^{2+}$ | Restricted | - | Weaver, Blatti & Rutter (1971) |
| Human placenta | - | - | $Mn^{2+}$ | Restricted | - | Voigt, Kaufman & Matthaei (1970) |
| Bovine brain nuclei | - | - | $Mn^{2+}$/ $Mg^{2+}$ | Broad | - | Singh & Sung (1972) |
| Saccharomyces cerevisiae nuclei | 440,000 | - | $Mn^{2+}$ | Denatured DNA | | Frederick, Maitra & Hurwitz (1969); Dezelee, Sentenac & Fromageot (1972) |
| S. carlsbergensis nuclei | | | | | | |
| polymerase A | 18S | - | $Mn^{2+}$ | Broad | - | Brogt & Planta (1972) |
| polymerase B | 16S | - | $Mn^{2+}$ | Broad | - | |
| polymerase C | - | - | $Mn^{2+}$ | Broad | | |
| Zea mays nuclei polymerase I | - | - | $Mg^{2+}$ | Restricted | - | Strain, Mullinix & Bogorad (1971) |
| polymerase II | - | - | $Mg^{2+}$ | Restricted | - | |
| Tetrahymena pyriformis nuclei | - | - | $Mn^{2+}$ | Denatured DNA | - | Byfield, Lee & Bennett (1970); Kurtz & Pearlman (1972) |

Table 1.1. continued....

| Source | Mol. Wt. | Subunit Comp. | Divalent Cations Needed | Template Specificity | Rifampicin Sensitivity | Reference |
|---|---|---|---|---|---|---|
| *Zea mays* chloroplasts | >500,000 | - | $Mg^{2+}$ | Restricted | - | Bottomley, Smith & Bogorad (1971 |
| *Neurospora crassa* mitochondria | 64,000 | 1 chain | $Mg^{2+}$/ $Mn^{2+}$ | Restricted | + | Küntzel & Schäfer (1971) |
| Rat liver mitochondria | 64,000 to 68,000 | 1 chain | $Mg^{2+}$/ $Mn^{2+}$ | Restricted | + | Reid & Parsons (1971) |
| Yeast mitochondria (species not stated) | 450,000 to 500,000 | - | - | - | - | Eccleshall & Criddle (1972) |
| $T_7$-infected *E. coli* | 107,000 | 1 chain | $Mg^{2+}$ | Restricted | - | Chamberlin, McGrath & Waskell (1970) |
| $T_3$-infected *E. coli* | 110,000 | 1 chain | $Mg^{2+}$ | Restricted | - | Dunn, Bautz & Bautz (1971) |

E. coli enzyme is by far the best studied, therefore, data obtained with other polymerase preparations will be adduced as needed in the discussion which follows.

### 1.1.4.1. Purification

Various methods have been employed to obtain highly-purified preparations of RNA polymerase. Chamberlin and Berg (1962) achieved 150-fold purification in 45% yield by streptomycin/protamine precipitation, ammonium sulphate fractionation and then DEAE-cellulose ion exchange chromatography. The protamine step proved difficult to reproduce in the hands of others, and a modified procedure was developed by Zillig, Fuchs & Millette (1966), involving differential centrifugation, DEAE-cellulose chromatography, ammonium sulphate fractionation, sucrose-density-gradient centrifugation and sucrose-density-gradient electrophoresis. Purification was about the same as that of Chamberlin & Berg (1962), but the yield was only 13%. Phase partition systems were introduced with good results by Babinet (1967) and by Bibilashvili & Savochkina (1971), and various modifications of the purification of Chamberlin & Berg have been published, but the method of choice at the present time is probably that of Burgess (1969a; Burgess & Travers, 1971). The crude extract was treated with DNase, centrifuged, and fractionated with ammonium sulphate; chromatography on DEAE-cellulose was followed either by phosphocellulose

and agarose-gel chromatography or by glycerol gradient centrifugation, the former yielding 'core' enzyme free from σ factor and the latter holoenzyme ($\alpha_2\beta\beta'\sigma$). The phosphocellulose-purified enzyme was active (56% yield), at least 98% pure and free of nucleases; the removal of σ was responsible for the relatively low yield and specific activity. The method could be used to prepare from 100 µg to 900 mg of pure RNA polymerase.

1.1.4.2. Physical characterization

The E. coli RNA polymerase was initially difficult to characterize owing to its strong tendency to aggregate (Richardson, 1969). At low ionic strengths, the core polymerase forms aggregates up to hexamers, while the holoenzyme dimerizes (Berg & Chamberlin, 1970), and in conditions consistent with full activity both 13 S and 24 S forms exist (Seifert & Zillig, 1969). That the 13 S form is the active particle was concluded on the following grounds (Richardson, 1969): (a) some preparations do not readily aggregate, (b) the enzyme is assayed in dilute solutions, which favour dissociation, (c) binding of polynucleotides favours dissociation as well; under conditions in which free enzyme sediments at 21 S, complexes between RNA polymerase and small polynucleotides sediment at 13-15 S.

Molecular weights in the range 360,000 to 440,000 have been reported for the 13 S form of the polymerase from E. coli (Richardson, 1966a; Preiss & Zillig, 1967; Maitra & Hurwitz, 1967; Berg, Barrett, Hinkle, McGrath & Chamberlin, 1969; Burgess, 1969b). Core polymerase consists of four subunits of three types in the structure $\alpha_2\beta\beta'$, where the molecular weights of the three subunits are: α, 39,000; β, 155,000; β', 165,000, in addition to a small protein (ω, 9000 daltons) for which no function or necessity has been determined (Burgess, 1969b). The σ factor has a molecular weight of 95,000 (Burgess & Travers, 1970); hence the molecular weight of 495,000 specified in Table 1.1 for the holoenzyme. The data of Burgess were obtained using polymerase from E. coli K12; similar results were obtained by King and Nicholson (1971) with E. coli B RNA polymerase, but the molecular weights determined were generally higher: α, 42,000; β, 172,000 (actually a mixture of β and β'); core enzyme, 460,000; the discrepancy was due to incomplete removal of σ from the core enzyme preparation. The core enzyme dissociated with age, yielding an αβ and an αβ' complex; the dissociation was enhanced by storage in the presence of 0.1 M 2-mercaptoethanol (King & Nicholson, 1971), although disulphide linkages are not involved in subunit association (Burgess, 1969b).

Solvent perturbation and optical rotatory dispersion studies (Nicholson, 1971) indicate a helix content of 13% for the core polymerase. A large proportion of the aromatic amino acid residues (trp, 30%; tyr, 54%) was accessible to perturbants, indicating that many are at or near the surfaces of the protein; on the other hand, RNA polymerase has an unusually low content of tyrosine and tryptophan (Burgess, 1971).

The core enzyme contains two tightly-bound zinc atoms per molecule, one or both of which may participate in chain initiation (v.i.) or specifically in substrate binding at the initiation site (Scrutton, Wu & Goldthwait, 1971).

1.1.4.3. Conditions of assay

The basic requirements for in vitro polymerase activity are independent of source. Maximum activity requires DNA, all four ribonucleoside triphosphates, a divalent metal and (in some instances) a sulphydryl reagent. The last is not necessary for activity, but is usually included in the buffers used during purification (Hurwitz & August, 1963); both core enzyme and σ factor contain highly reactive sulphydryl groups essential to activity (Sümegi, Sanner & Pihl, 1971, 1972). The pH is usually 7.5-8.

Ionic strength and the exact divalent cation used have complex effects which will be discussed in separate sections. The incubation temperature for E. coli polymerase is either $30^{o}$ or $37^{o}$; temperature optima for the plant enzymes can range as high as $48^{o}$, while B. stearothermophilus RNA polymerase has a temperature optimum of $50^{o}$ in vitro (Remold-O'Donnell & Zillig, 1969).

### 1.1.4.4. Steps in the polymerase reaction

The following are considered to be the major events in RNA synthesis by template-dependent RNA polymerases:

Binding of the enzyme to the template.

Initiation. The term refers to the formation of the first phosphodiester bond.

Elongation. This refers to chain growth beyond the dinucleotide stage.

Termination. The cessation of elongation.

Release. Dissociation of the enzyme, template and newly-synthesized RNA chain.

All of these have been subdivided into two or more individual processes:

Burgess (1971) has described four stages in template binding. The enzyme first binds to the template in a non-specific, readily reversible manner which is subject to

inhibition by high ionic strength, RNA and heparin, and for which σ factor is not required. The polymerase then locates a specific initiation site which may or may not be a 'proper' site for chain initiation. (Initiation can occur at many points on the template, only a few of which are genetically appropriate or 'proper'.) Strand separation occurs with the formation of a more stable initiation complex, the decay of which is accelerated at high ionic strength or at low temperature. This complex is unaffected by rifampicin but forms more readily at 'proper' initiation sites if σ is present. Finally, a change occurs which renders the complex resistant to rifampicin. The change is stabilized by σ, in the absence of which it readily reverses if initiation does not immediately follow, and is blocked by rifampicin. It is probable that the change is normally coincident with, although (Bautz & Bautz, 1970) it is not caused by, the binding of the terminal nucleoside triphosphate, which also stabilizes the complex (Goldthwait, Anthony & Wu, 1970). The latter is the first step in initiation, and is also blocked by rifampicin (ibid.).

Initiation consists of three processes. The first NTP is bound in a separate site called the initiation site (ibid.) which accepts purine nucleotides only and has a higher $K_m$ than the elongation site. The latter, which accepts

all four NTP, probably binds the second NTP, but there may be a third group to which the 3'-hydroxyl group of the growing chain attaches, and there is slight evidence to suggest that the second NTP may attach directly to this 3'-binding site. In either case, the formation of the first phosphodiester bond, with concomitant release of pyrophosphate, terminates the initiation step.

Translocation of the enzyme with respect to the DNA, binding of the incoming NTP and formation of the next bond are the three substeps of chain elongation. Since the energy of translocation is supposed to come from the cleavage of the high-energy phosphate bond, it might be better to group bond formation and translocation together; the outline given is that of Goldthwait et al. (op.cit.), who were primarily concerned with studying the initiation process.

Recognition of a stop signal is not necessarily a prerequisite to termination in vitro. The causes of non-specific termination are poorly understood, but low ionic strength was until recently thought to lead to product inhibition by RNA and therefore to termination (Burgess, 1971). Release does not follow termination of this type (Fuchs, Millette, Zillig & Walter, 1967; Bremer & Konrad, 1964). The product inhibition hypothesis has been questioned by Jenkins, Crist & Jones (1971) who showed that very long RNA molecules were spontaneously released at limiting RNA polymerase

concentration. At $\mu>0.1$, on the other hand, RNA chains of specific size are synthesized and released (Maitra & Barash, 1969; Maitra, 1970; Qasba & Zillig, 1969); these terminate predominantly with uridine at the 3' end. (RNA synthesis runs from 5' to 3', an intact purine nucleoside triphosphate being incorporated at the 5' end: Niyogi & Stevens, 1964; Bremer, Konrad, Gaines & Stent, 1965; Maitra, Novogrodsky, Baltimore & Hurwitz, 1965; Maitra & Hurwitz, 1965; Krakow & Horsley, 1967.) Below $\mu = 0.12$, re-initiation does not take place (Millette, Trotter, Herrlich & Schweiger, 1970; Maitra, Lockwood, Dubnoff & Guha, 1970).

At $\mu<0.15$, termination can be stimulated by a protein factor called $\rho$, a tetramer of molecular weight 200,000 (Roberts, 1969). RNA chains so terminated are released but re-initiation does not take place (Maitra, Lockwood et al., op. cit.; Richardson, 1970). The termination mediated by $\rho$ occurs at specific sites on DNA, and one or more $\rho$ termination sites may precede a $\rho$-independent termination site. The $\rho$ factor binds both to DNA and to the enzyme (Darlix, Sentenac & Fromageot, 1971).

1.1.4.5. Template specificity

1.1.4.5.1. Properties of the DNA

The recognition of the DNA template by E. coli polymerase holoenzyme is not limited to specific initiation sequences. The effect of template length on activity has been studied

for both core enzyme (Ishihama, Murakami, Fukuda, Matsukage & Kameyama, 1971) and holoenzyme (Cato & Jones, 1972). Shearing was found to increase template activity for the core enzyme, whereas it decreased activity with the holoenzyme, causing rapid inactivation as template size fell from $4 \times 10^6$ to $1\text{-}2 \times 10^5$. Above four million, however, the effect of shearing on holoenzyme recognition was slight. Other forms of damage to the DNA have similar effects; the introduction of single-strand nicks increased core enzyme but not holoenzyme activity (Ishihama et al., op. cit.; Chessin & Summers, 1970), in the latter case causing increases in nonspecific initiation but also in premature termination and non-productive template binding. The introduction of thymine dimers by UV irradiation also resulted in diminished template activity for holoenzyme (Chessin & Summers, 1970).

The mechanism by which recognition takes place is not understood. One early idea (Szybalski, Kubinski & Sheldrick, 1966) was that pyrimidine clusters in the DNA acted as initiation and termination signals for transcription, an idea which has received some circumstantial corroboration in that asymmetric transcription has correlated well with the occurrence of such clusters on the active strand. Langridge (1967) found that synthetic double helical DNA with pyrimidines on one strand and purines on the other had slightly different

structure from natural DNA, and Summers & Szybalski (1968) suggested that the modified structure might be recognized in a pyrimidine-rich region by the RNA polymerase.

A means whereby specific sequence recognition might be possible is apparent from the work of Gabbay, DeStefano & Sanford (1972). There are theoretically ten different sequence-determined intercalation sites on double-helical DNA into which aromatic residues might specifically fit, and the use of reporter molecules has shown that the differences are real as well as theoretical in several sites at least. It should be noted that the resulting hypothesis, viz., that recognition involves the intercalation of specific aromatic amino acid residues on RNA polymerase between base pairs in specific sequence, would explain and is to some extent supported by the finding of Nicholson (see s. 1.1.4.2.) that a large proportion of the aromatic amino acids of E. coli polymerase are external despite their hydrophobic nature. Intercalation would be thermodynamically favoured because of the removal of water. Non-specific binding (the 'loose' binding of Stead & Jones, 1967) might result from the intercalation of only one or two residues on the binding site, while the more stable ('tight-binding') complex would form when the DNA sequence permitted full interaction with the protein.

### 1.1.4.5.2. Properties of the σ factor

The binding of RNA polymerase to DNA is severely restricted in the presence of σ factor, presumably because of an increased preference of the enzyme for specific initiation sites. For example, Jones & Berg (1966) found that core polymerase binds to some 35-70 sites on $T_7$ coliphage DNA, while Richardson (1966b) obtained a value of about 50. Similarly, Müller (1971) found that core enzyme will bind $T_4$ DNA to an extent approaching the physical limit. In the presence of σ, E. coli polymerase binds tightly at less than four sites on $T_7$ DNA unless the reaction system is overloaded with enzyme (Dausse, Sentenac & Fromageot, 1972); the number of specific initiation sites may be as low as one (Fukuda & Ishihama, 1971). With $T_4$ DNA, the σ factor was found to stimulate release of non-specifically bound polymerase and to restrict attachment to a small number of sites from which long RNA molecules are normally initiated; "early quitter" polymerase molecules (Müller & Bremer, 1969) do not bind specifically to these sites and form less stable complexes (Müller, 1971).

No report has appeared in which the σ factor has been stated to prevent transcription of heterologous templates. The factor seems rather to stimulate specific initiation than

to reduce non-specific activity, except as noted above for $T_4$ DNA.

1.1.4.5.3. Properties of the core enzyme

In the absence of σ factor, the core enzyme transcribes almost any nucleic acid. Although there are variations in the rate of transcription from one template to another, these variations appear to be more closely related to the conditions of assay than to any physiological function (cf. Niyogi, 1972). It may be concluded with moderate assurance that functional specificity is conferred on the E. coli polymerase by the properties of the σ factor and that transcription in its absence is non-specific. (The participation of other factors yet unknown in the determination of template specificity is not improbable.)

1.1.4.6. Substrate specificity

In the last few years, studies with nucleotide analogues have yielded useful information regarding the substrate interactions of the E. coli polymerase. Fromageot and co-workers investigated the triphosphates of 8-azaguanosine and formycin, analogues of guanosine and adenosine respectively (Fromageot, Darlix & Sentenac, 1970; Darlix, Fromageot & Reich, 1971). Chain initiation was found to be slower and release did not occur when the analogues were substituted

for the two purines, but elongation was unaffected. On calf thymus DNA, fewer initiation sites were available to the analogues than to the normal purines. As a result of studies with analogues unable to assume the anti conformation, which proved to be competitive inhibitors, Kapuler & Reich (1971) postulated that during elongation, substrates were in the syn conformation when first bound to the enzyme but underwent a change to anti upon formation of the phosphodiester bond, pointing out that the poor reversibility of the synthesis (low rate of pyrophosphorolysis) might be in part due to the coupling of such a change to bond formation. Studies with a wide variety of analogues suggested to Asano and co-workers that separate binding sites exist for each NTP (Asano, Kurashina, Anraku & Mizuno, 1971; Asano, Anraku & Mizuno, 1971), although the data seemed to be equally consistent with different substrate affinities at the elongation and initiation sites previously postulated.

1.1.4.7. Effect of divalent cations

A divalent cation is required for polymerase activity. From polymerase to polymerase, however, there is considerable variation in qualitative and quantitative optima. The E.coli enzyme, for example, is stimulated to nearly the same extent

by both $Mg^{2+}$ and $Mn^{2+}$ at their respective optimum concentrations, $Mn^{2+}$ being slightly more effective and effective at considerably lower concentration. The precise figures are dependent on the template used. It has become common to assay the E. coli enzyme in the presence of $Mg^{2+}$ alone, probably because problems of oxidation are avoided (see s. 6.2.). For other polymerases $Mn^{2+}$, or a mixture of the two, may be required (see Table 1.1.), either absolutely or with significant preference.

No divalent cation is required for DNA binding (Richardson, 1966b). In fact, binding is decreased by 20-25% at the normal concentrations of assay (Anthony, Zeszotek & Goldthwait, 1969). Substrate binding, both for initiation and for elongation, is dependent on the presence of $Mg^{2+}$, $Mn^{2+}$ or $Co^{2+}$ (Goldthwait, Anthony & Wu, 1970; Fuchs, Millette, Zillig & Walter, 1967). Adding excess $Mg^{2+}$ could cause release and re-initiation even after the cessation of synthesis at low ionic strength (Fuchs et al., op. cit.); addition of $Mn^{2+}$ or $Co^{2+}$ once the reaction had come to a halt had no effect, although their presence in an actively synthesizing reaction mixture sufficed to maintain activity if the ionic strength was above 0.12.

### 1.1.4.8. Effect of ionic strength

The effect of ionic strength on termination and

release has already been mentioned (s. 1.1.4.4.). To recapitulate, specific termination does not occur below an ionic strength of 0.1 and re-initiation does not occur below 0.12. Termination mediated by ρ factor, on the other hand, does not occur above μ = 0.15.

Ionic strength is also important in template binding. Increasing μ produced non-competitive inhibition of binding, 90% dissociation being caused at μ = 0.2 (Anthony, Zeszotek & Goldthwait, 1969). Free enzyme and enzyme which had bound to DNA but had not bound any substrate was inhibited above μ = 0.13 and no synthesis occurred at μ ≥ 0.2 (Fuchs et al., op. cit.), but the rate of elongation increased with salt concentration up to μ = 0.36, provided the salt was added after initiation.

Taking all factors into account, therefore, it would seem that unless the ionic strength can be locally varied at each polymerase molecule throughout the reaction, or the action of the enzyme molecules synchronized, the value μ = 0.12-0.13 should be rigidly maintained in E. coli polymerase assays.

1.1.4.9. Subunit function

The degree to which the three subunit types of core polymerase participate in the various stages of transcription

is only poorly understood at the present time. Subunits β and β' have been implicated in certain processes, and the role of σ as specificity determinant seems established, but no specific function has been ascribed to α.

The mixture of β and β' separated from α by sucrose gradient centrifugation binds to DNA, while α does not (Zillig, Fuchs, Palm, Rabussay & Zechel, 1970; Zillig, Zechel, Rabussay, Schachner, Sethi, Palm, Heil & Seifert, 1970). Low concentrations of urea dissociated the enzyme into αβ and αβ' complexes, both of which bound DNA (Ishihama, 1972), indicating that both β and β' possess template binding sites. It has been clearly shown by dissociation and mixed reconstitution that the property of resistance to rifampicin, which blocks binding of the first NTP, is transferred when α and β' from rif-sensitive polymerase are reconstituted together with β from rif-resistant polymerase, and lost when the reverse experiment is performed (Heil & Zillig, 1970); β is also known to be the only subunit to which rifampicin binds (Zillig, Zechel et al., op. cit.), and was concluded to be involved in initiation and probably to contain the initiation site of the enzyme (not to be confused with initiation sites on DNA; see s. 1.1.4.4.). The conclusion is confirmed by the finding (Ishihama, 1972) that the ββ' complex contains both substrate-binding sites. Similar dissociation and mixed reconstitution experiments with streptolydigin-resistant

mutants (Heil & Zillig, 1970) implicate β in translocation, the step believed to be prevented by that antibiotic. Both β and β' bind to α, but β alone binds to σ (Ishihama; Zillig et al., opera cit.).

The fact that β' is particularly sensitive to trypsin has allowed a verification of its role in template binding (Lecocq, 1971). Incubation of polymerase with trypsin resulted in a parallel decrease in its overall synthesizing activity and in the binding of DNA. Loss of activity was prevented by preincubation with DNA, and it was shown that the conditions of trypsin incubation used had negligible effects on α or β.

### 1.1.4.10. Further protein factors influencing synthesis

#### 1.1.4.10.1. M factor

M factor was isolated by Davison, Pilarski & Echols (1969). Its effect is to stimulate transcription, and it does so independently of σ function (Davison, Brookman, Pilarsky & Echols, 1970), yet early in the reaction. Its effect is lessened by increasing the enzyme-DNA ratio (Burgess, 1971), which has led Burgess to suggest that it may act to destabilize non-productive binding between RNA polymerase and DNA. Other postulated roles include the prevention of premature termination; perhaps the factor is that which is missing in 'early quitter' polymerase molecules.

A factor called H, smaller than M but exerting a similar influence, has recently been described (Jacquet, Cukier-Kahn, Pla & Gros, 1971).

1.1.4.10.2. CAP and ψ factor

Two proteins are known to affect the transcription of specific parts of the genome. ψ, which stimulates rRNA synthesis (Travers, Kamen & Schleif, 1970; Travers, Kamen & Cassel, 1970), is particularly fascinating in that it is one of the components of Qβ replicase; it is possible that the factor becomes an endogenous replicase for rRNA upon interaction with RNA polymerase, but the suggestion is the purest speculation. The other is catabolite-gene-activating protein (CAP) which stimulates transcription of the lac operon *in vitro* (see Burgess, 1971).

1.1.4.11. Non-transcriptive synthesis

DNA-dependent RNA polymerase has two non-transcriptive capabilities. In the absence of the other substrates both *E. coli* and *M. luteus* RNA polymerases catalyse a DNA- and $Mn^{2+}$-dependent incorporation of ATP into poly A (Chamberlin & Berg, 1964; Fox & Weiss, 1964). The activity was completely inhibited by very low concentrations of the other substrates, and denatured DNA only served as template. Poly U, poly C and

poly G could also be synthesized (Fox & Weiss, 1964).

A. agilis and E. coli RNA polymerases both catalyse both poly (A,U) and poly (I,C) synthesis in the absence of template when the appropriate substrates (only) and $Mn^{2+}$ are incubated with the enzymes (Smith, Ratliff, Williams & Martinez, 1967; Krakow & Karstadt, 1967; Sternbach & Eckstein, 1970). In neither case does homopolymer synthesis occur in the absence of the three nonessential substrates, as it does when DNA is present.

### 1.1.4.12. RNA-directed RNA synthesis

RNA polymerase will accept as template both single- and double-stranded RNA, synthesizing RNA complementary to the template as well as poly A and poly U (Gomatos, Krug & Tamm, 1964; Krakow & Ochoa, 1963; Karstadt & Krakow, 1970; Niyogi & Stevens, 1965b; Fox, Robinson, Haselkorn & Weiss, 1964; Straat, Pongs & Ts'o, 1971). Reaction rates are template-dependent, but a wide range of both natural and synthetic templates are 'transcribed'. The reaction directed by poly U and to a lesser extent by mixed ribopolymers is stimulated by σ factor (Niyogi, 1972), while that directed by poly C appears to be unaffected by σ. Levels of activity are generally much lower than those obtained in the transcription of DNA, for which reason the RNA-directed reaction has

been largely neglected over the years. It has probably been generally assumed that the activity represents an in vitro artefact. I shall have occasion to refer again to this point later.

1.1.4.13. Primed synthesis

In 1965, Niyogi & Stevens (1965a) demonstrated a priming effect of oligonucleotides complementary to homopolymer templates directing RNA synthesis. The oligonucleotide primers were incorporated into the 5' end of the new chain and their presence increased the rate of synthesis. Niyogi & Wilton (1969) presented evidence that reversible binding of the primer to the template was involved. Very recently, Niyogi (1972) showed that addition of oligonucleotides to both ribo- and deoxyribopolynucleotide-directed reaction mixtures obviated the stimulatory effect of σ and reduced the incorporation of γ-labelled NTP into chain termini, indicating that the primers were bypassing the initiation step. No mention was made of experiments with natural templates.

1.1.4.14. Preliminary report regarding a DNA-dependent RNA polymerase from Halobacterium cutirubrum

It has been claimed (Chazan & Bayley, 1971) that there is a DNA-dependent RNA polymerase in H. cutirubrum which requires $Mg^{2+}$ only and is rifampicin-insensitive, and which

sediments at a rate comparable to the E. coli polymerase. The enzyme was isolated as a DNA-membrane-enzyme complex and freed from the DNA by centrifugation after treatment with DNase, following which it was inactive in the presence of salt. It seems probable that the method of purification failed to free the enzyme from membrane material, which can bind to the polymerase and interfere with its further purification, as well as giving high apparent molecular weight values. No further data have been reported, and we have not detected any activity in the absence of $Mn^{2+}$ in our crude extracts.

### 1.1.5. Other DNA-dependent RNA polymerases

An attempt to be exhaustive on the subject of RNA polymerase would occupy far too much space in this thesis. A great deal has been left unsaid even about the E. coli and related enzymes, and it will not be necessary to refer to phage-induced or eukaryote enzymes in any detail. Mention should be made, however, of two phage-induced and two mitochondrial enzymes to which reference will later be necessary.

#### 1.1.5.1. $T_3$- and $T_7$-induced RNA polymerases

The first 'really different' RNA polymerase to be isolated was the $T_7$ coliphage-specific enzyme (Chamberlin,

McGrath & Waskell, 1970). The enzyme was a single polypeptide chain of molecular weight 107,000 and transcribed only the late genes of $T_7$ DNA. A similar enzyme was soon isolated from E. coli infected with the related coliphage $T_3$ (Dunn, Bautz & Bautz, 1971; Maitra, 1971) and it was found that even the slight heterologous nature of DNA from $T_7$ was enough to reduce to a low level its template activity for $T_3$ polymerase and vice versa.

1.1.5.2. Neurospora crassa and rat liver mitochondrial RNA polymerases

Similar to the $T_3$ and $T_7$ polymerases in their restricted template specificities, the mitochondrial enzymes from the fungus N. crassa (Küntzel & Schäfer, 1971) and rat liver (Reid & Parsons, 1971) are remarkable chiefly in being smaller still (64,000 and 64,000-68,000 daltons, respectively). Both are single chains and both require $Mg^{2+}$ and $Mn^{2+}$ together, in 10:1 ratio, for optimum activity. They differ from the phage-induced enzymes in being sensitive to rifampicin.

## 1.2. RNA-dependent RNA polymerase

The Qβ replicase is to RNA-dependent polymerases what the E. coli enzyme is to DNA-dependent polymerases. It can be highly purified and has been well characterized. The

enzyme contains three host proteins, none of which is associated with E. coli RNA polymerase, and one phage-coded protein (Kamen, 1970; Kondo, Gallerani & Weissman, 1970), and has a highly restricted template specificity (August, 1969; Spiegelman, Pace, Mills, Levisohn, Eikhom, Taylor, Peterson & Bishop, 1968). The initiating nucleotide is invariably GTP and the enzyme has no other catalytic activities (August, 1969), both of which properties are common to all phage replicases so far studied. $Mg^{2+}$ is not required for template binding but is necessary to subsequent processes. Nothing is known about subunit function.

Other replicases are, so far as is known, similar. The RNA viruses of mammalian cells, when they induce replicases, have enzymes of similar function and restricted specificity. They do not resemble the RNA-dependent enzyme of H. cutirubrum, as will be shown.

Two non-viral RNA-dependent RNA polymerases have so far been reported (in addition to that I have isolated). The sources are Chinese cabbage leaf chloroplasts (Astier-Manifacier & Cornuet, 1971) and macrophage cells (Neuhoff, Schill & Jacherts, 1970). The little that is known about these enzymes will be related later (s. 4.3.3.).

## 1.3. Halophilism and halophile enzymes

Halophilic organisms are those requiring salt

concentrations above 0.5 M for growth; extreme halophiles, those requiring above 3.0 M (Baxter & Gibbons, 1956). The limits of the various classifications appear in Table 1.2. The extremely halophilic bacteria include both gram-negative rods (genus Halobacterium, family Pseudomonadaceae) and gram-variable cocci (genera Micrococcus and Sarcina, family Micrococceaceae).

The first question generally asked about the extreme halophiles is whether they have very active pumping mechanisms for ions, so that the intracellular ionic strength is low. The answer is yes but no. There is indeed a highly active sodium pump, but its purpose is to maintain low (relative to the exterior) sodium concentrations and not low overall ionic strength. Christian & Waltho (1962) performed the only study in the literature of comparative intracellular salt concentrations (Table 1.3.), and found that the intracellular ionic strength of Halobacterium salinarium, for example, slightly exceeded that of the surrounding medium. Since it also considerably exceeded the solubility of the salts in question at physiological temperatures, and since starvation for prolonged periods, which inactivated the sodium pump, did not result in the release of potassium, it was concluded (Ginzburg, Sachs & Ginsburg, 1970; Ginzburg, Ginzburg & Tosteson, 1971) that the $K^+$ must be bound within

Table 1.2. Classification of bacteria according to salt requirement for growth

| Class | Examples | M NaCl |
|---|---|---|
| Nonhalophiles | Escherichia coli | |
| | Desulfovibrio desulfuricans | 0 to 0.4 |
| Halotolerant bacteria | Bacillus cereus | |
| | Staphylococcus aureus | 0 to 1.7 |
| Moderate halophiles | Micrococcus halodenitrificans | |
| | Vibrio costicolus | 0.5 to 3.5 |
| Extreme halophiles | Halobacterium cutirubrum | 3.0 to saturation |
| | Sarcina morrhuae | |

Table 1.3. Intracellular ionic contractions in various bacteria

Intracellular concentrations are expressed as moles per kg cell water.

| | Staphylococcus aureus | Vibrio costicolus | Halobacterium salinarium | Sarcina morrhuae |
|---|---|---|---|---|
| NaCl in medium (M) | 0.15 | 1.0 | 4.0 | 4.0 |
| KCl in medium (M) | 0.025 | 0.0004 | 0.032 | 0.032 |
| $Na^+$ in cells | 0.098 | 0.684 | 1.37 | 3.17 |
| $K^+$ in cells | 0.680 | 0.221 | 4.57 | 2.03 |
| $Cl^-$ in cells | 0.008 | 0.139 | 3.61 | 3.66 |

the cell. Unfortunately, the dry weight of the bacteria is insufficiently high to accomodate any special binding compounds in adequate quantity, and there is not enough protein to account for the $K^+$ bound, so the nature of the binding loci is difficult to imagine. In fact, the salt content of Halobacteria resembles the flight of the bumblebee in being theoretically impossible, unless perhaps the existence of submicroscopic salt crystals within the bacterium be postulated.

### 1.3.1. Nucleic acids of halophiles

The high-molecular-weight nucleic acids of halophiles do not differ significantly from those of other organisms (Larsen, 1967). The extreme halophiles, however, possess a separable satellite DNA (about 11-36% of the total) with a generally lower percentage of G-C base pairs (57-60% as contrasted with 63-68% for the major component: Joshi, Guild & Handler, 1963; Moore & McCarthy, 1969a). An exception was the one photosynthetic extreme halophile tested, with a single component of 70% G-C (Moore & McCarthy, 1969a). It was shown by hybridization experiments (Moore & McCarthy, 1969b) that the Halobacterium strains tested had considerable sequence homology, but were quite different from the halophilic cocci and the moderate halophiles. The satellite

was non-repetitive and, if episomal in the usual sense, was abnormally large.

The genetic code in the extreme halophiles appears to be the same as that of other organisms (Bayley & Griffiths, 1968a,b).

## 1.3.2. Enzymes of extreme halophiles

### 1.3.2.1. Purification and stability

Enzymological studies of halophile enzymes have been hampered by the difficulty of obtaining pure, active preparations of such enzymes in reasonable yield. The reason for the difficulty is that the absence of salt generally leads to inactivation which is often totally irreversible and rarely more than fractionally reversible at best. For example, Holmes & Halvorson (1965) reported that their highly-purified (in 0.5% yield after purification at low ionic strength and reactivation by dialysis) malic dehydrogenase required 4.3 M NaCl for stabilization *in vitro*. A similar result was obtained with electrophoretically (95%) homogeneous isocitrate dehydrogenase (3% yield after reactivation: Hubbard & Miller, 1969). The dehydrogenases both displayed the same requirements in the crude state as when purified. Crude lactic dehydrogenase of *H. salinarium* required 2 moles of salt per mole of enzyme for stability

(Baxter, 1959), while crude H. cutirubrum malic enzyme required 3 M NaCl or KCl, the latter being less effective (Cazzulo & Vidal, 1972).

The two purified dehydrogenases described above are the only halophile enzymes to have been purified beyond the initial stages. Attention is drawn to the low yields obtained by the technique of inactivation, low-salt purification and reactivation, as well as to the possibility of alteration in the enzymic properties during such a procedure.

Concurrently with the early phases of the present work, the polynucleotide phosphorylase of H. cutirubrum was purified 200-fold in 25% yield, using steps which did not involve the absence of salt (Peterkin, 1970; Peterkin & Fitt, 1971). The enzyme, which catalysed all three characteristic reactions of polynucleotide phosphorylase from other sources, had a molecular weight of less than 12,000 and was membrane-bound in the intact cell, though it could be released by gentle methods without loss of activity. Full stability required the presence of 4 M NaCl, and the activity required 2 M or higher salt concentration.

1.3.2.2. Activity

All enzymes so far isolated from extreme halophiles, with the exception of the fatty acid synthetase of H. cutirubrum,

an enzyme known to have no function *in vivo* (Pugh, Wassef & Kates, 1971), require 2-4 M salt for optimum activity. There is, however, a considerable difference in the response of different enzymes to specific monovalent cations. *H. cutirubrum* catalase activity, for example, is stimulated by KCl to the greatest extent and then by NaCl, LiCl and $NH_4Cl$ in that order (Lanyi & Stevenson, 1969), while malic enzyme is stimulated strongly by $NH_4Cl$, weakly by KCl and not at all by NaCl or LiCl (Cazzulo & Vidal, 1972). KCl specifically (3.8 M) is required by *H. cutirubrum* tRNA synthetases for acylation, while KCl, NaCl and $NH_4Cl$ are all needed for transfer (Griffiths & Bayley, 1969).

## 1.4. Purposes of the present work

The work here presented forms a part of the basic studies preliminary to a detailed examination of the interaction of template, enzyme and substrate in nucleic acid synthesizing systems operating in limiting conditions of ionic strength. The original intent was to isolate, purify and characterize the DNA-dependent RNA polymerase of *H. cutirubrum*. The discovery of a separate RNA-dependent RNA polymerase from the same organism resulted in the expansion of the work to include its purification and characterization as well.

Specific objectives in characterization included:

1. The establishment of conditions for optimum activity
2. The proof of polymerase specificity
3. The determination of physical structure and subunit function

Most of the work to be presented has appeared in print (Louis & Fitt, 1971a,b,c; 1972a,b,c; Louis, Peterkin & Fitt, 1971).

# 2. EXPERIMENTAL

## 2.1. Materials

Chemicals and enzymes were purchased from the following suppliers:

Amersham/Searle Corp., Don Mills, Ont., Canada: $^{14}C$- and $^{3}H$-labelled nucleoside triphosphates and 2,5-diphenyloxazole (PPO); BDH (Canada) Ltd., Toronto, Ont., Canada: sodium dodecyl sulphate, specially pure; Bio-Rad Laboratories, Richmond, Calif., U.S.A.: Bio-Gel P-2 and P-60 polyacrylamide gel beads (100-200 mesh), Bio-Gel HTP hydroxylapatite powder and Dowex 50W-X4 cation exchange resin; Calbiochem, Los Angeles, Calif., U.S.A.: cyclohexylaminopropane sulphonic acid (CAPS), tris(hydroxymethyl)methylaminopropane sulphonic acid (TAPS), tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid (TES) and high-molecular-weight wheat germ RNA; Canadian Laboratory Supplies Ltd., Montreal, P.Q., Canada: Triton X-100 non-ionic detergent; E-C Apparatus Corp., Philadelphia, Pa., U.S.A.: Cyanogum 41 and N,N,N',N'-tetramethylethylenediamine; Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, L.I., N.Y., U.S.A.: dinucleoside monophosphates and Serva diethylaminoethyl-(DEAE)- and polyethyleneimine-(PEI)-cellulose for thin-layer chromatography; General Biochemicals, Chagrin Falls, Ohio, U.S.A.:

Escherichia coli DNA, Bacillus subtilis DNA and poly d(A-T); ICN Chemical and Radioisotope Division, Irvine, Calif., U.S.A.: $^{32}$P-labelled nucleoside triphosphate; Mann Research Laboratories, New York, N.Y., U.S.A.: rifampicin, bacitracin and sperm whale myoglobin; Miles Laboratories, Inc., Kankakee, Ill., U.S.A.: oligonucleotides, poly A, poly U, poly (A,U), poly A·poly U and E. coli mixed 16 S and 23 S ribosomal RNA; New England Nuclear, Boston, Mass. U.S.A.: $^{3}$H-ApA, tritiated by the Wilzbach procedure; Packard Instrument Co., Inc., Lagrange, Ill., U.S.A.: 1,4-bis-2-(4-methyl-5-phenyloxazoyl)benzene (dimethyl POPOP); Pharmacia (Canada) Ltd., Montreal, P.Q., Canada: Sephadex G-50 dextran gel, Sepharose 4B agarose gel, 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 c type VI (horse heart), α-chymotrypsinogen A type II (ox pancreas), ovalbumin grade V, crystalline bovine serum albumin, yeast transfer RNA and yeast high-molecular-weight RNA; Worthington Biochemical Corp., Freehold, N.J., U.S.A.: venom phosphodiesterase and crystalline pancreatic deoxyribonuclease and ribonuclease.

Halobacterium halobium Verhoeven NRC 34020 DNA, prepared as described below (s. 2.2.5.), was a gift from Mrs. P.I. Peterkin. Coliphage $T_7$ DNA, separated chains of $T_7$ DNA and

unlabelled and $^{14}C$-labelled $T_7$-specific RNA species were provided by Dr. W.C. Summers. Coomassie Brilliant Blue R250 was donated by Canadian Industries Ltd., Montreal, P.Q., Canada, and streptolydigin by Upjohn Co., Kalamazoo, Mich., U.S.A.

## 2.2. Methods

### 2.2.1. Organisms

Halobacterium cutirubrum strain NRC 34001, originally isolated from salted buffalo hides (Lochhead, 1934), was used throughout this work.

### 2.2.2. Growth conditions for H. cutirubrum

The culture medium (Sehgal & Gibbons, 1960) contained, per 100 ml of aqueous solution: Difco casamino acids, 0.75 g; Difco yeast extract, 1.0 g; sodium citrate, 0.3 g; KCl, 0.2 g; $MgSO_4 \cdot 10H_2O$, 2.0 g; NaCl, 25.0 g; $FeCl_2$, 2.3 mg; NaOH, to pH 6.5. 2% (w/v) agar was added for agar slants and plates. The liquid medium contained a small amount of precipitate after sterilization (20-30 min at 121°) which apparently did not affect growth.

Stock cultures were grown on agar slants at 37° for 2-3 weeks. Aeration was maintained by momentarily loosening the tube caps every three or four days. The cultures were

stored at 4° and replaced by transfer at 6-month intervals.

Culture flasks with Morton closures (Bellco Glass Inc., Vineland, N.J., U.S.A.) were used for growth of mass cultures. 125 ml flasks containing 60 ml of liquid medium were inoculated from a stock culture and incubated at 37° in an incubator shaker (New Brunswick Scientific Co., Inc., New Brunswick, N.J., U.S.A.). Rotation was such that each point on the shaker platform described a circle of 1 in diameter 225 times per minute. After 6 days' incubation, the flasks were stored at 4°. For mass culture, 5 ml of the liquid stock thus obtained were added to similar flasks which were incubated as above for 48 h. One-litre flasks containing 600 ml of liquid medium were inoculated with 30 ml aliquots of these starter cultures and incubated in the same conditions. Extra starter culture flasks were stored at 4° for subsequent use as liquid stock. The mass culture was harvested after 72 h, in late log or early stationary phase (Gochnauer & Kushner, 1969), by centrifuging at 15,000 g for 15 min at 4°. The supernatant was poured off and the unwashed cells were used for the preparation of the crude extract (s. 2.2.4.1.).

As a routine check on the purity of the stocks, two agar slants were inoculated with about 1 ml of liquid culture at each transfer. These were incubated for about one week

at 37° and examined visually for contamination by H. halobium.

2.2.3. Buffers

The pH of buffers refers to the value at 25°. High-salt buffer had the following composition: 2.5 M KCl, 1 M NaCl, 10 mM tris-HCl, pH 8.6.

2.2.4. Purification of H. cutirubrum DNA-dependent and RNA-dependent RNA polymerases

2.2.4.1. The crude extract

The conditions of suspension and lysis of the cells appear to be critical, not only for obtaining the best yield of both activities, but also for achieving separation of the two enzymes at the pH 4 precipitation step (s. 2.2.4.2.2.). It was therefore essential to follow the described procedure as closely as possible. All operations were carried out at 0-5°.

2.2.4.1.1. Suspension of the bacteria

A bent glass rod was used to mix the harvested bacteria (s. 2.2.2.) with 2 ml/g (wet weight) of high-salt buffer. The buffer was added slowly, with careful stirring, as one might prepare a thick soup from a dry mix in the kitchen. Each 15 g of bacteria was suspended in 25 ml of

buffer, and the suspension (ca. 35 ml) was transferred to a 50 ml beaker of standard dimensions. The centrifuge bottles were rinsed with a further 5 ml of buffer which was then added, with stirring, to the suspension in the beaker. In each beaker was placed a teflon-covered magnetic stirrer bar (1 in x 0.375 in, with rounded ends), and the suspension was stirred magnetically for 45-60 min on a Thomas model 15 stirrer (Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.) set at about 4.5 on its dial.

2.2.4.1.2. Sonication

Ultrasonic disruption was carried out with a Biosonik II sonicator (Bronwill Scientific Co., Rochester, N.Y., U.S.A.). The probe was positioned about 2 cm above the bottom of the beaker and sound was applied (dial setting: 70) for 30-45 s. Normally, the suspension changed by 30 s from a milky red which verged on pink to a darker, clear-looking red colour; the longer sonication times were occasionally required to complete this colour change. The viscosity of the suspension increased only slightly, and there was no difficulty in handling it after sonication.

2.2.4.1.3. Centrifugation

Gross cell debris were removed by centrifuging the lysed cell suspension for 30 min at 27,000 g. The supernatant

was further centrifuged at 78,000 to 81,000 g (Beckman L2-65B ultracentrifuge: Type 65 or 40 rotor at 35,000 rpm or type 30 at 30,000 rpm) for 135 min. Both pellets were discarded and the ultracentrifugation supernatant (crude extract) was stored at $4^{o}$.

2.2.4.2. Separation of DNA-dependent and RNA-dependent activities

The characterization of the two polymerase activities required their separation. The purification procedure was therefore developed with the aim of separating them first and purifying them separately afterwards, despite the duplication of labour involved. Two methods have been employed with success, although the conditions of reproduction are so critical that neither method is entirely satisfactory. Acid precipitation (s. 2.2.4.2.2.), which is easier to repeat successfully than ammonium sulphate precipitation (s. 2.2.4.2.1.), has replaced the latter in the standard purification procedure.

2.2.4.2.1. Ammonium sulphate precipitation

The crude extract was dialysed against 200 vol of 10 mM tris-HCl buffer, pH 8.6, for 2 h. The buffer was replaced and the dialysis repeated. Sufficient solid KCl and NaCl was added to bring the concentration of KCl to

0.087 M and that of NaCl to 0.035 M. These starting concentrations were critical to the success of the separation, but the enzymes, particularly the DNA-dependent enzyme, lost activity at low ionic strength. In the absence of a conductivity meter, therefore, it was necessary to assume that the dialysis had removed essentially all of the original KCl and NaCl, and the assumption was usually justified: aliquots of the dialysed extracts were saved and subsequently analysed for $Cl^-$ by titration with standard mercuric nitrate solution after tungstate-sulphuric acid deproteinization, using diphenylcarbazone as internal indicator (Schales & Schales, 1941).

Solid $(NH_4)_2SO_4$ was added to bring the solution to 25% of saturation (calculated for $25^o$ according to Green & Hughes, 1955). The suspension was stirred 30 min and the precipitate was removed by centrifugation (30 min at 27,000 g) and immediately resuspended in the minimum of high-salt buffer. The supernatant was brought to 35% of saturation, stirred and centrifuged as before, and the process was repeated once more, this time at 75% of saturation. The resuspended pellets and the final supernatant were dialysed against high-salt buffer to remove ammonium sulphate. The entire separation was carried out at $0\text{-}5^o$.

2.2.4.2.2. Acid precipitation

The crude extract was adjusted by slow, cautious addition of 1 M HCl to a pH between 4.4 and 4.8 and stirred for 5 min. Further small aliquots of HCl were then added with careful stirring to bring the pH to 4.2 or less, and the suspension was stirred for 5 min. If the pH fell no farther during this period, it was brought to 4.0, again with caution; otherwise no more HCl was added. The suspension was centrifuged 20 min at 27,000 g and at 4° and the pellet was immediately covered with high-salt buffer while the supernatant was returned to pH 8.6 by addition of 1 M NaOH. The pellet was then suspended in high-salt buffer (half the volume of the crude extract) and returned to pH 8.6. Both fractions were dialysed overnight at 4° against 2 litres of high-salt buffer. Undissolved material in the pellet fraction was discarded.

2.2.4.3. Ammonium sulphate purification of the acid precipitate fraction

The precipitate fraction from the pH 4 step (containing the RNA-dependent enzyme contaminated by a small amount of the DNA-dependent enzyme) was further purified by ammonium sulphate fractionation. Salt was removed by dialysis against 10 mM tris-HCl buffer, pH 8.6. No solid salt was added after

dialysis. A single fractionation step was performed as described above (s. 2.2.4.2.1.), the concentration of $(NH_4)_2SO_4$ being raised to 80% of saturation. The precipitate was discarded and the supernatant dialysed overnight at $4^o$ against high-salt buffer (50 vol). The precipitated salt was removed and the dialysis continued for 2 h against a further 50 vol of high-salt buffer.

2.2.4.4. P-60 gel filtration

Both activities were further purified by gel filtration. All operations were carried out at $4^o$. Samples were concentrated by dialysis against Ficoll (50%, w/v, in high-salt buffer) to about 0.025 of the gel bed volume. Gel columns (Bio-Gel P-60, 2.5 cm*, 5 cm or 10 cm x 85-90 cm) were prepared and run as described below (s. 2.2.10.); high-salt buffer was used as eluant. Fractions (4 ml, 10 ml or 20 ml) were collected and the $E_{280}$ of the effluent was monitored, if desired, by a recording monitor (LKB-Produkter AB, Stockholm, Sweden). Active fractions were pooled and concentrated to a final protein content of about 100 µg/ml, either by dialysis against Ficoll or by membrane ultrafiltration.

Particularly in the case of the DNA-dependent enzyme, the purification was continued to this stage with the least possible delay to avoid loss of activity. Henceforward

* In the early stages of the work only. The larger columns gave far better purification since the 2.5 cm columns had to be overloaded to yield usable quantities of enzyme.

both enzymes were stable in high-salt buffer provided the protein concentration exceeded 50 µg/ml.

2.2.4.5. Zinc hydroxide gel adsorption

After chromatography, the RNA-dependent enzyme was purified to homogeneity by adsorption of the remaining contaminants onto $Zn(OH)_2$ gel prepared by a modification (Y.P. See, personal communication) of the method of Mahler (1955). 28.76 g of $ZnSO_4$ were dissolved in 1 litre of distilled water and 100 ml of 1 M NaOH were added with stirring. The gel was washed by decantation four times with 1.5 litres of water and twice with 1 litre of 10 mM tris-HCl, pH 7.4. The suspension was stored overnight in the dark and centrifuged 10 min at 8000 g, and the pellet was resuspended in the Tris buffer to a final concentration of 20 mg/ml (determined by drying an aliquot of the suspension on a weighed planchette). The gel was stored in the dark for five days and then 0.5 vol of the suspension was added to the 100 µg/ml solution of RNA-dependent RNA polymerase. The mixture was stirred thoroughly and allowed to stand for 20 min, the solid was removed by centrifugation for 5 min in a clinical centrifuge and the supernatant was dialysed at $4^{o}$ against two changes (100 vol each) of high-salt buffer for a total of 4 h. This fraction was electrophoretically

homogeneous on polyacrylamide gels (s. 2.2.12.).

2.2.4.6. Chromatography and batchwise adsorption on hydroxylapatite

The DNA-dependent enzyme fraction obtained by P-60 gel filtration was resolved into its two component proteins by differential adsorption on hydroxylapatite at $4^{o}$. 5 g of Bio-Rad HTP powder were suspended in 25 ml of high-salt buffer and washed by decantation and resuspension in 18 ml of the buffer. The slurry was poured into a Sephadex K9/30 column (Pharmacia) and allowed to settle under gravity. The bed was washed by downward flow (5 ml/h) with 50 ml of high-salt buffer. The concentrated P-60 fraction was applied and the column was washed with high-salt buffer (20 ml), 2 ml fractions being collected. During this washing step, two protein peaks were obtained. The second contained one of the polymerase subunits (protein β) and the corresponding fractions were concentrated by Ficoll/high-salt buffer dialysis (s. 2.2.4.4.) to 1 ml. The other subunit (protein α) was then eluted with 0.1 M sodium phosphate in high-salt buffer; the pooled fractions containing protein α were concentrated to 1 ml by Ficoll/high-salt buffer dialysis. The concentrated protein solution was dialysed against high-salt buffer to remove phosphate.

Protein α obtained by the above procedure was electrophoretically homogeneous in polyacrylamide gels (s. 2.2.12.), but protein β usually contained a minor contaminant. The latter was then removed by batchwise treatment with hydroxylapatite. The solution of protein β (1 ml) was diluted to 4 ml with high-salt buffer and hydroxylapatite powder (1 g) was added. The suspension was stirred for 30 min and then centrifuged for 5 min at 2,000 g. The supernatant was discarded and the pellet was resuspended in 0.1 M sodium phosphate in high-salt buffer (1 ml). (The buffer was readjusted to pH 8.6 after addition of the sodium phosphate.) The hydroxylapatite was removed by centrifugation and the supernatant, containing electrophoretically homogeneous protein β, was dialysed against high-salt buffer to remove phosphate.

### 2.2.5. Isolation of H. cutirubrum nucleic acids

A new procedure was developed for the isolation of both DNA and RNA from H. cutirubrum cells. The method combines part of the DNA isolation method of Marmur (1961) with an agarose gel filtration step (Loeb & Chauveau, 1969). All operations were performed at 0-5°, except where indicated.

The nucleic acids were prepared either from freshly harvested bacteria (s. 2.2.2.) or from a powder obtained by

freeze-drying a suspension of the bacteria in 0.15 M NaCl (2 ml/g wet weight). The freeze-dried powder could be used for this purpose after storage for over 18 months at -20° (although freeze-drying inactivated the RNA polymerases). The bacteria (9 g wet weight), or an equivalent weight of freeze-dried powder, were suspended in a mixture of 0.15 M NaCl, 0.1 M EDTA, pH 8 (75 ml) and 25% (w/v) sodium dodecyl sulphate (6 ml). The suspension was incubated at 75° for 10 min and then cooled to room temperature. 5 M $NaClO_4$ was added to a final $NaClO_4$ concentration of 1 M and the mixture was divided into three equal parts. Each sample was swirled for 30 min at 125 rpm on the rotary shaker (see s. 2.2.2.) with an equal volume of 24:1 (v/v) chloroform/ isoamyl alcohol. The mixtures were centrifuged for 5 min at 7,500 g in stainless-steel centrifuge tubes and the aqueous phases were combined. Ethanol (2 vol) was added and the mixture cooled to -20°. The precipitate was collected by centrifugation and dissolved in 15 mM NaCl, 1.5 mM sodium citrate, pH 7 (27 ml), and 3 ml of 3 M sodium acetate, 1 mM EDTA, pH 7, were added. The nucleic acids were separated by dropwise addition of 16.2 ml of isopropanol to the rapidly stirred solution. The precipitated DNA was collected by centrifugation, washed with ethanol and stored at -20°. The supernatant was mixed with ethanol (2 vol), cooled to -20°

and then centrifuged. The RNA (about 10 mg) was washed with ethanol and dissolved in 0.15 M NaCl, 10 mM tris-HCl buffer, pH 7.5 (2 ml) and stored at 4°.

A column (5 cm x 35 cm) was packed (s. 2.2.10.) with Sepharose 4B agarose gel. The eluant was 2 M NaCl. A portion (20 mg) of the DNA from the isopropanol precipitation step was dissolved in 15 mM NaCl, 1.5 mM sodium citrate, pH 7 (10-15 ml). The solution was mixed with an equal volume of 4 M NaCl and the sample was applied to the column. The DNA emerged as the first peak on the UV monitor. Fractions (10 ml) were collected and their $E_{260}$ determined with the Zeiss PMQ II spectrophotometer. The fractions containing DNA were combined and the DNA was precipitated with ethanol (2 vol). The precipitate was stored at -20°. For use, it was dissolved in 0.15 M NaCl, 10 mM tris-HCl buffer, pH 7.5, and the solution (2 mg/ml) was stored at 4°.

### 2.2.6. Liquid-scintillation counting

The measurement of radioactivity forms the basis of most of the work to be described. Samples were counted on solid supports (thin-layer chromatographic medium or paper) immersed in toluene-based scintillation cocktails. Data reduction was performed with the aid of a programmable calculator (Programma 101, Olivetti-Underwood Ltd., Ottawa,

Ont., Canada), and included quench correction and spillover correction where appropriate.

2.2.6.1. Instruments, cocktails and counting

Four different scintillation counters were used in the course of this work. The scintillation cocktail used was adapted to the detector tubes of the individual counters, according to manufacturers' recommendations. The counters and cocktails were:

Nuclear-Chicago Mark I and Unilux II (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.), 0.4% (w/v) PPO, 0.01% (w/v) dimethyl POPOP in reagent grade (the use of special scintillation grade was unnecessary) toluene;

Beckman LS-133 (Beckman Instruments, Inc., Fullerton, Calif., U.S.A.), 0.5% (w/v) PPO in toluene;

Beckman LS-230, 0.8% (w/v) PPO in toluene.

Scintillation vials contained 10 ml of cocktail for paper strips and thin-layer media, and 5 ml for paper discs. Samples were normally counted to 2% ($10^4$ counts).

2.2.6.2. Data reduction

2.2.6.2.1. Quench correction

Counting low levels of weak β activity on solid supports is subject to inaccuracy, whichever of the two

commonly available methods of quench correction is employed. The channels ratio method (Baillie, 1960; Bush, 1963) is relatively indifferent to sample geometry, but depends for its accuracy on a high sample count rate (Peng, 1970), to achieve which would have necessitated a prodigious expenditure of radioactivity in many of our experiments, whereas the external standardization method (Fleishman & Glazunov, 1962) is said to be highly dependent on sample geometry and inherently inaccurate due to differences between the pulse-height spectrum of the Compton recoil electrons arising from $\gamma$-irradiation and the spectra of weak $\beta$ particles under measurement (Peng, 1970). Empirically, however, we have not found significant differences in the results obtained with the two methods, nor have we observed any important dependence upon sample geometry within the limits of variation of the quenching encountered in our work. This has been the case even in double-labelling experiments, where it has been reported (Bransome & Grower, 1970) that self-absorption on solid supports can result in severe inaccuracies in quenching and spillover factor determinations. It has therefore been our practice to use whichever method is most convenient in determining quenching. All measurements in the LS-133 were performed using external standardization, as were all

double-labelling experiments, but the channels ratio method was used for single-isotope counting in the other counters. Commercial standards (Amersham/Searle) were used to determine $^{3}H$ and $^{14}C$ quench correction curves.

2.2.6.2.2. Spillover correction

Quenched $^{14}C$ and $^{32}P$ standards (the latter obtained by adding aliquots of chloroform to standard $^{32}P$ samples in counting vials) were counted in the $^{3}H$ or $^{14}C$ channels used in double-labelling experiments in order to determine spillover.

2.2.6.2.3. Mathematics of data reduction

The graphs of both efficiency and spillover factor against external standardization ratio or channels ratio are nonlinear, so that straight-line approximations may be used only when the efficiency varies over a limited range. However, a second-order polynomial in the ratio gives good approximation to the efficiency or spillover factor over the whole curve. For example, a $^{14}C$ external standardization quench curve determined on the LS-230 counter was approximated within 1% from R = 0.062 to R = 0.734 (E = 0.626 to E = 0.946), where R is the ratio and E the efficiency, by fitting to the data the equation

$E = aR^2 + bR + c$, by the method of least squares, viz.,

$$a = \frac{\frac{(\Sigma R^2 - \frac{(\Sigma R)^2}{n})(\Sigma R^2 E - \frac{\Sigma R^2 \Sigma E}{n})}{\Sigma R^3 - \frac{\Sigma R \Sigma R^2}{n}} - (\Sigma RE - \frac{\Sigma R \Sigma E}{n})}{\frac{(\Sigma R^2 - \frac{(\Sigma R)^2}{n})(\Sigma R^4 - \frac{(\Sigma R^2)^2}{n})}{\Sigma R^3 - \frac{\Sigma R \Sigma R^2}{n}} - (\Sigma R^3 - \frac{\Sigma R \Sigma R^2}{n})}$$

$$b = \frac{\frac{(\Sigma R^4 - \frac{(\Sigma R^2)^2}{n})(\Sigma RE - \frac{\Sigma R \Sigma E}{n})}{\Sigma R^3 - \frac{\Sigma R \Sigma R^2}{n}} - (\Sigma RE - \frac{\Sigma R \Sigma E}{n})}{\frac{(\Sigma R^2 - \frac{(\Sigma R)^2}{n})(\Sigma R^4 - \frac{(\Sigma R^2)^2}{n})}{\Sigma R^3 - \frac{\Sigma R \Sigma R^2}{n}} - (\Sigma R^3 - \frac{\Sigma R \Sigma R^2}{n})}$$

$$c = \frac{\Sigma E - a\Sigma R^2 - b\Sigma R}{n}$$

where n is the number of quenched standards counted. These equations were derived from information in chapters 13 and 14 of the book by Snedecor (1946). The calculator programme used to fit the curve will be found among the appendices (s. 6.1.1.).

A second calculator programme was then employed to perform the conversion from cpm counted to whole-sample dpm according to the equation

$$dpm = \frac{cpm}{fE} = \frac{cpm}{f(aR^2 + bR + c)} \qquad \text{(s. 6.1.2.)}$$

for single-isotope counting, or, if the equation for a spillover factor S be $S = xR^2 + yR + z$,

$$dpm_1 = \frac{cpm_1 - S(cpm_h)}{fE} = \frac{cpm_1 - cpm_h(xR^2 + yR + z)}{f(aR^2 + bR + c)} \qquad \text{(s. 6.1.3.)}$$

where the subscript 1 represents the counts of lower pulse height and the subscript h those of higher pulse height. In both equations f represents the fraction of the sample applied to the support for counting.

2.2.7. Enzyme assays

2.2.7.1. H. cutirubrum DNA-dependent RNA polymerase

The DNA-dependent enzyme was assayed by measuring the incorporation of labelled nucleoside triphosphate into an acid-insoluble product in the presence of ATP, GTP, UTP, CTP and DNA template (Hurwitz, Furth, Anders, Ortiz & August, 1961; Stevens, 1961; Krakow & Ochoa, 1963; Nakamoto, Fox & Weiss, 1964). The incubated samples were

processed by a modification of the Bollum paper technique (Bollum, 1959, 1966, 1968). The standard assay medium (0.1 ml) contained: tris-HCl buffer, pH 8.6, glycine-NaOH buffer, pH 9.5 or TES-NaOH buffer, pH 8.6 (s. 6.2.), 10 µmol; native DNA, 30 µg; one $^{14}C$- or $\alpha$-$^{32}P$-labelled nucleoside triphosphate, 5 nmol (10,000-50,000 dpm/nmol); the other three unlabelled nucleoside triphosphates, 15 nmol each; $MgCl_2$, 10 µmol; $MnCl_2$, 1 µmol; KCl, 150 µmol; NaCl, 60 µmol; enzyme, up to 3 units. The divalent metal ions were added immediately prior to the start of incubation. After 1 h at 37°, a sample (up to 85 µl) of the mixture was streaked on a numbered strip of Whatman no. 3 (not to be confused with 3 MM, which is finer in texture and slightly lighter in weight) chromatography paper. The strips (2 cm x 3 cm) were collected in cold 7% (w/v) $HClO_4$ (10 ml/strip) and the suspension was allowed to stand for a few minutes and then swirled for at least 7 min on a rotary shaker (New Brunswick Scientific Co.) at a speed sufficient to keep the strips barely in motion. The strips were allowed to settle and the fluid was removed by aspiration. The washing procedure was repeated successively with similar volumes of cold 7% (w/v) $HClO_4$, 1% (w/v) $HClO_4$ and ethanol. The washed strips were air-dried, transferred

to individual vials and counted by liquid scintillation (s. 2.2.6.). A unit was defined as the amount of enzyme catalysing the incorporation of 1 nmol of labelled NTP into acid-insoluble material per hour in these conditions. Crude extracts and pH 4 and ammonium sulphate fractions were diluted up to 100-fold to maintain linearity in the assay, it being found that these crude preparations displayed enzyme saturation maxima.

2.2.7.2. H. cutirubrum RNA-dependent RNA polymerase

The assay was essentially similar in design to that described above. The medium contained: tris-HCl buffer, TAPS-NaOH buffer or CAPS-NaOH buffer (s. 6.2.), pH 9.5, 10 µmol; high-molecular-weight RNA, 75 µg; one $^{14}C$- or $\alpha$-$^{32}P$-labelled nucleoside triphosphate, 5 nmol (10,000-50,000 dpm/nmol); the other three unlabelled nucleoside triphosphates, 20 nmol each; $MgCl_2$, 10 µmol; $MnCl_2$, 1 µmol; KCl, 150 µmol; NaCl, 60 µmol; enzyme, up to 3 units. The divalent metal ions were added immediately prior to the start of incubation. Incubation and workup were the same as for the DNA-dependent enzyme, and the enzyme unit was defined as above (s. 2.2.7.1.).

2.2.7.3. Initiation assays

The rate of initiation of new RNA chains was

measured by the method of Travers & Burgess (1969), which is based on the principle that the first nucleotide is incorporated intact into the growing chain (Maitra, Novogrodsky, Baltimore & Hurwitz, 1965). One or more $\gamma$-$^{32}$P-labelled nucleoside triphosphates were included in an assay mixture (s. 2.2.7.1. or 2.2.7.2.) containing $^{14}$C-ATP and 15 or 20 nmol, as specified, of each of the four nucleoside triphosphates. After normal incubation and workup it could be assumed that 1 pmol of $^{32}$P-NTP would be present per pmol of new RNA chains initiated.

2.2.7.4. Controls in the polymerase assays

No washing procedure removed all of the excess labelled substrate from the paper strips containing product RNA. The proportion remaining varied with the assay conditions, and also from one batch to the next, but was reasonably constant within a given batch of identical assay mixtures (but see s. 6.2.). In the standard polymerase assays, which contained $Mn^{2+}$, 0.5-1% of the input radioactivity was usually retained non-specifically on the paper; within a given batch of assays, the variation in the blank rarely exceeded 0.1% of the input. The non-specific component was determined by including in each batch of assays a mixture lacking one essential component, usually the enzyme, or containing an inhibitor

(e.g., rifampicin or ethanol). Unless the contrary is stated, blanks lacking enzyme were used in all of the experiments involving polymerase assays, because the results so corrected most closely approximated those obtained by isolating the radioactive product (s. 2.2.14.), a procedure which gave blanks with radioactivity approaching background, but which was too time-consuming for routine use.

2.2.7.5. Nuclease assays

Deoxyribonuclease and ribonuclease activities in samples of H. cutirubrum RNA polymerases were determined by measuring the liberation of acid-soluble products from the appropriate nucleic acid. The assay medium was as described above for the polymerase assays (s. 2.2.7.1. and 2.2.7.2.) with the following modifications: labelled and unlabelled nucleoside triphosphates were omitted and either DNA (30 µg; DNase assay) or RNA (30 µg; RNase assay) was included. The assays were performed both with and without 1.5 M KCl, 0.6 M NaCl in the medium. After 1 h at 37°, the reactions were stopped with an equal volume of 0.5% (w/v) uranyl acetate in 7% (w/v) $HClO_4$. The mixtures were kept at 0° for 10 min. Carrier nucleic acid (10 µg: DNA or RNA, as appropriate) was added and the

suspensions were kept at $0^{o}$ for a further 5 min. They were then diluted to 1 ml with water and centrifuged. The $E_{260}$ of the supernatant was measured in the Zeiss PMQ II spectrophotometer, with a similarly treated incubation mixture that lacked the substrate as blank. (Controls without enzyme were also performed and treated as above; no hydrolysis of the nucleic acid occurred in these control incubations.)

### 2.2.8. Protein assays

Enzyme protein was assayed spectrophotometrically (Warburg & Christian, 1942).

Protein in nucleic acid preparations was determined by the micro-biuret method (Zamenhof, 1957), with α-chymotrypsinogen A as standard. The stable biuret reagent was prepared by adding 40 ml of 1% (w/v) $CuSO_4$ to 150 ml of 10 M NaOH, dropwise and with stirring, and filtering the suspension through paper. The sample (2 parts) was mixed with 1 part biuret reagent and the mixture read against a reagent blank at 310 and 390 nm. The procedure was repeated with 8 M NaOH instead of biuret reagent to correct for nucleic acid in the sample. The difference in the two readings with NaOH alone was subtracted from the difference in the readings with biuret

reagent and the result was applied to a standard curve (determined at the time of measurement using dilutions of the standard α-chymotrypsinogen solution) to find the protein concentration.

## 2.2.9. Nucleic acid assays

The reading produced by DNA in the orcinol reaction is roughly 25% of that resulting from an equivalent weight of RNA (Dische, 1955); the reaction is thus unsuited for the determination of RNA in the presence of a great excess of DNA. A less sensitive but much more selective procedure is that of Dische (1955), in which the extinction due to DNA peaks at 490 nm, while that due to RNA peaks at 390 nm. The result is that at 390 nm the extinction due to DNA is less than 1.5% of that due to RNA. A rapid assay of DNA, unaffected as to accuracy by RNA in hundredfold excess, was achieved by Stumpf (1947) using the same reaction under slightly different conditions:

### 2.2.9.1. DNA assay (Stumpf, 1947; Dische, 1955)

A solution of DNA was diluted to a concentration between 5 and 200 µg/ml. To 0.5 ml was added 0.05 ml of freshly dissolved 5% (w/v) cysteine hydrochloride and 5 ml of 75% (v/v) $H_2SO_4$ with thorough mixing and no cooling.

The mixture was allowed to stand at room temperature for exactly 15 min and read at 490 nm against a reagent blank. The mixture could not be scaled down. DNA standard solutions were read for calibration.

2.2.9.2. RNA assay (Dische, 1955)

A solution of RNA was diluted to a concentration between 5 and 500 µg/ml. To 0.25 ml was added 1 ml conc. $H_2SO_4$ with tap water cooling and thorough mixing. The mixture was allowed to stand at room temperature for 1 h and then 0.06 ml of freshly dissolved 5% (w/v) cysteine hydrochloride was added. The mixture was stirred and allowed to stand for a further 15 min, then read at 390 nm against a reagent blank. DNA and RNA standard solutions were read for correction and calibration.

2.2.10. Gel filtration

Modifications in swelling, packing and running procedures are necessary when gel filtration is performed in the presence of large amounts of salt. The gel takes longer to equilibrate with the salt solution and tends to compress readily (Peterkin & Fitt, 1971; Peterkin, 1970). The procedure described has been found to yield stable gel beds capable of repeated use; the larger columns could be

used over 6 months before repacking became necessary.

2.2.10.1. Swelling the gel

Sufficient dry gel to give the required bed height was added slowly with stirring to 2.5 times the expected bed volume of eluant buffer or, in the case of agarose gel, the gel slurry was diluted with 1 litre of 4 M NaCl per litre of water in the slurry. $NaN_3$ was added to 0.02% (w/v) and the gel suspension was stored for not less than 28 days at $4^{o}$. Fines were removed by repeated decantation and resuspension.

2.2.10.2. Packing and stabilization

Sephadex columns K15/30, K25/45, K25/100, K50/100 and K100/100 (Pharmacia), the latter four equipped with flow adaptors, were used for dextran and polyacrylamide gel filtration. Agarose gel filtration was performed in a 5 cm x 60 cm column with a fritted glass disc at the lower end (O.H. Johns Glass Co., Toronto, Ont., Canada). The column to be packed was cooled to 2-$5^{o}$ in a cold room or by a coolant circulator and mounted vertically by spirit level, and the bed support was installed and covered to a depth of 4-6 cm with eluant solution. Air bubbles were removed from the bed support by suction through a long glass tube. Column extending devices were

fitted and sufficient gel slurry for the complete bed was added all at once. The gel was allowed to settle under gravity for at least 20 min, the downward flow of eluant was started and 1-2 bed volumes of eluant were passed through the bed at hydrostatic pressures not exceeding 10 cm for agarose, 15 cm for P-60 or 2 m for G-50 or P-2. Columns so equipped were fitted with the upper flow adaptors at this point. A further bed volume of eluant was run downward through the soft gels (agarose or P-60). Flow was then reversed if upward flow was possible, and the hard gels were run at 0.5 m for a final bed volume. Peristaltic pumps were used to maintain flow rates of 1 ml/cm$^2$h for agarose, 1.5-3 ml/cm$^2$h for P-60 and 4 ml/cm$^2$h for G-50 or P-2. Hydrostatic pressure was held at about 2 cm for the remainder of the experiment.

2.2.10.3. Sample application

Samples were applied at running speed and chased with 0.4 ml/cm$^2$ of 10% sucrose in eluant solution. Columns run by upward flow were supplied through distribution valves in order to minimize interruptions in flow during loading. In other cases the liquid level was run down to the level of the upper bed surface, the sample was applied with the pump stationary, the liquid level was

again run down to the bed surface and the sucrose solution was applied in the same way. Eluant was allowed to fill the column to the original level and the system was closed to the outside air. The pump was started and the sample was run through the column without further interruption.

Details of sample composition, fraction collection and effluent monitoring varied with the use to which the column was put, and will be found in sections describing the individual experiments.

2.2.10.4. Void volume determination

The first sample run through any column consisted of 0.4 ml/cm$^2$ of Blue Dextran, 0.2% (w/v) in eluant solution. Adenosine (10 µmol/ml) was also included when the column was to be standardized for molecular weight determination. The solution was centrifuged briefly at 27,000 g before application to remove undissolved material. Fractions (0.5-1% of bed volume) were collected and their $E_{260}$ determined in the Zeiss PMQ II spectrophotometer. Elution volume was taken to be the volume of eluant collected between the start of loading and the elution of the top of a given peak (Fischer, 1969); since Blue Dextran was excluded from all

of the gels used, its elution volume was the void volume of the gel bed. That of adenosine was taken to be equal to the sum of the void and internal volumes.

2.2.10.5. Molecular weight determination by gel filtration

Molecular weight determinations were performed in 2.5 cm x 37 cm and 2.5 cm x 90 cm columns equipped with flow adaptors and run by upward flow. The $E_{280}$ of the effluent was continuously monitored and 4 ml fractions were collected. The extinctions of the fractions were accurately determined with the Zeiss PMQ II at (i) 405 nm for cytochrome c, (ii) 280 nm for other proteins and Blue Dextran and (iii) 257 nm for adenosine.

Standard proteins were dissolved in high-salt buffer and turbid solutions were cleared by a brief centrifugation at 27,000 g. The standards were applied individually to the columns in samples containing 7-10 mg in 0.5-1.5 ml. The size and concentration of samples of enzymes (each applied individually) were determined by their availability: amounts adequate for detection by the standard assays were necessarily used, but larger samples were loaded where possible to permit continuous monitoring of the $E_{280}$ of the effluent and thus facilitate detection of the peaks of activity.

For each standard, the average partition coefficient $K_{av}$ was calculated from the equation $K_{av} = (V_e-V_o)/(V_t-V_o)$ (Fischer, 1969). These values were plotted against the logarithm of the corresponding molecular weights (Determann, 1967; Fischer, 1969) and a straight line was fitted to the linear portion of the curve by the method of least squares (Snedecor, 1946). The molecular weights of the enzymes were then calculated from their $K_{av}$ values by applying the fitted equation.

### 2.2.11. Sucrose-density-gradient centrifugation

Molecular weight determination, confirmation of homogeneity and RNA product size investigations were carried out by sucrose-density-gradient centrifugation according to Martin & Ames (1961), or by a modification of their method in which the gradients were formed in high-salt buffer.

#### 2.2.11.1. Molecular weight of proteins

Linear sucrose gradients (5-20%, w/v) in 10 mM tris-HCl buffer, pH 8.6, or high-salt buffer, were prepared with a Buchler gradient former (Buchler Instruments, Inc., Fort Lee, N.J., U.S.A.). It should be noted that the gradients prepared in high-salt buffer had an average

homogeneous density of 1.187 g/ml, which approaches the safe limit for Beckman rotors, so these salt concentrations should not be exceeded.

4.3 ml gradients were formed in 1.1 cm x 6.0 cm Beckman polyallomer tubes. Samples (0.2 ml) were layered on top of each gradient. Each protein was run in a separate tube. The sample concentration was 7-10 mg/ml for the standard proteins; enzyme concentration was maintained well above the limit of detectability in the standard assay system. The tubes were centrifuged in the SW-56 rotor of a Beckman L2-65B preparative ultracentrifuge for 15.5 h at 308,000 g. All runs were carried out at 5-7$^{o}$ (lower temperatures led to the co-precipitation of salt and protein in the high-salt gradients). The tubes were pierced and 30-32 fractions per tube (about 140 µl per fraction) were collected. Protein concentration was determined by diluting 0.05 ml samples of each fraction to 0.7 ml with water and measuring the $E_{280}$ ($E_{405}$ for cytochrome c) in the Zeiss PMQ II. The distance of the fraction containing the highest concentration of a substance from the rotor centre at the end of the run, referred to as $r_s$, was calculated for each fraction and each enzyme.

A constant k was defined as being that fraction of the gradient through which the substance moves during a

run, i.e. $k = (r_s - r_{min})/(r_{max} - r_{min})$ where $r_{max}$ is the radius to the bottom of the gradient and $r_{min}$ that to the top. The k values thus calculated were plotted against the logarithm of the molecular weight and the enzyme molecular weights were estimated as described above for gel filtration experiments (s. 2.2.10.5.). Similar results were obtained by plotting log k against log (mol. wt.), due to the low molecular weights of the protein involved.

2.2.11.2. Homogeneity of enzyme preparations

A sucrose gradient containing high-salt buffer was run under conditions identical to those described in the previous section. The sample consisted of 20 µg of enzyme protein in 0.2 ml of high-salt buffer. Protein concentration was determined as above and the fractions were assayed for enzyme activity. The recovery of protein and of enzyme units was calculated.

2.2.11.3. Size of RNA molecules

Linear sucrose gradients (5-20%, w/v) in distilled water were prepared with the Buchler gradient former. 4.2 ml gradients were formed in 1.1 cm x 6.0 cm Beckman polyallomer tubes. Samples (150 µl) of unlabelled RNA (0.5 mg/ml) or labelled RNA, the product of a standard

incubation (s. 2.2.7.1. or 2.2.7.2.; the product was isolated as described in s. 2.2.14.), were layered onto the gradients. After centrifugation for 135 min at 297,000 g in the SW-56 rotor, the tubes were pierced and 8-drop fractions were collected, giving 28-31 fractions per gradient. $E_{260}$ of 1:7 aqueous dilutions was read against a water blank. Radioactivity was determined as in the standard assay by streaking 0.1 ml of each fraction on paper strips, drying the strips and counting them by liquid-scintillation.

2.2.12. Micro-disc polyacrylamide gel electrophoresis

To conserve the highly purified proteins α and β and RNA-dependent enzyme, polyacrylamide gel electrophoresis was performed by the micro-disc technique of Neuhoff (Neuhoff, 1968, 1970; Neuhoff, Schill & Sternbach, 1968, 1970), in 5 µl capillary tubes with a precision bore of 0.450 mm diameter (Drummond Microcaps, Arthur H. Thomas Co.).

Separating gels containing 8, 10 and 12% (w/v) polyacrylamide, and 5% (w/v) stacking gels, were prepared from the following stock solutions: (A) 10 ml of 10 mM $MgCl_2$, 20 mM $NH_4Cl$, 10 mM tris-acetate buffer, pH 7.0, 8.0 or 9.4 plus N,N,N',N'-tetramethylethylenediamine

(63 µl); (B) Cyanogum 41 (20 g), $K_3Fe(CN)_6$ (3.75 mg), water to 37.5 ml; (C) ammonium persulphate (7 mg), 4% (v/v) Triton X-100 (2.5 ml), water (2.5 ml); (D) tris (5.98 g) in water (50 ml), 1 M $H_3PO_4$ to pH 6.7, N,N,N',N'-tetramethylethylenediamine (0.46 ml), water to 100 ml; (E) ammonium persulphate (10 mg), water (0.5 ml), 4% (v/v) Triton X-100 (0.5 ml); (F) tris (2.85 g) in water (30 ml), 1 M $H_3PO_4$ to pH 6.7, water to 50 ml.

The separating gels were produced as follows: 0.5 ml of A was mixed with (i) B (0.6 ml) and water (0.9 ml) for 8% gels; or (ii) B (0.75 ml) and water (0.75 ml) for 10% gels; or (iii) B (0.9 ml) and water (0.6 ml) for 12% gels. Finally, 1 ml of the resulting solution was replaced with 1 ml of C. The capillaries were filled with the appropriate separating gel mixtures to a maximum of two-thirds of their length by capillary action and stuck in a plasticine block to seal the lower ends. The upper ends were filled with water by means of a finely drawn Pasteur pipette and the gels were left for not less than 10 h nor more than 3 days in a moist chamber at room temperature.

Immediately before use, a layer of 5% (w/v) stacking gel, pH 6.7, was added to the capillaries. A dilute acrylamide mixture was prepared: B (0.5 ml) was mixed with

D (0.5 ml) and water (1 ml); 1 ml of the mixture was replaced with D (1 ml): finally, 1 ml of the second mixture was replaced with water (0.8 ml). Water was removed from the capillaries with the finely drawn Pasteur pipette, and the height of the separating gel was marked with a waterproof, fine-point felt marker. E (0.2 ml) was added to the dilute acrylamide mixture and a 2-3 mm layer of the resulting solution was added to each capillary, care being taken to introduce no air bubbles. The capillaries were refilled with water. When polymerization was complete, the upper surfaces of the 5% gels were rinsed with a 1:8 (v/v) dilution of F and the position of the surfaces was marked as before. The capillaries were filled to the top with the samples (about 100 µg protein/ml in 1:8 F). Proteins stored in high-salt buffer were dialysed for 24 h against salt-free buffer before electrophoresis.

Rubber plugs (Thomas 2319-B, Arthur H. Thomas Co.) were placed in the holes of the upper electrode bath of a 12-gel Buchler Polyanalyst (Buchler Instruments, Inc.). The sharp end of a 17 ga. hypodermic needle, the Luer end of which had been blocked with silver solder, was inserted through one of the rubber plugs and a capillary dropped into the bore of the needle, the length of which was such

that one-fifth of the capillary projected above its tip. When the needle was withdrawn from the plug, the rubber gripped the capillary, which remained in place. When all the capillaries had been installed, the chambers were filled with electrode buffer (9 g of tris and 43.2 g of glycine in 1500 ml of water) and Bromophenol Blue (2% w/v), 0.5 ml) was added to the buffer in the upper (cathode) chamber. Electrophoresis was performed at 80 V (starting current about 100 µA per gel), with a constant-voltage power supply capable of regulation in the range 0-100 V (Model LL905, Lambda Electronics Corp., Melville, L.I., N.Y., U.S.A.), until the tracking dye had traversed 4 mm of the separating gel. The gels were extruded from the tubes by water pressure into the staining solution. The protein bands were stained with either 0.5% (w/v) Amido Black 10B in 7.5% (w/v) acetic acid or 0.1% (w/v) Coomassie Brilliant Blue R250 in 12.5% (w/v) trichloroacetic acid, and the gels were destained in the appropriate dilute acid lacking the dye.

The method of Swank and Munkres (1971) was subsequently found to give more reliable destaining and better final backgrounds than either of the staining methods discussed above. Gels were immersed for 20 min in a

solution prepared by mixing 1.25 g of Coomassie Blue with 454 ml 50% (v/v) methanol and 46 ml glacial acetic acid and filtering through Whatman no. 1 paper. The staining solution was decanted and the gels were rinsed once with water and destained in methanol-acetic acid-water, 10:3:27 (v/v).

2.2.13. Amino acid analysis of H. cutirubrum polymerase proteins

A sample of each protein, the product of one preparation, was hydrolysed in 6 M HCl at 110° for 22 h and the hydrolysate was chromatographed on a Beckman Model 120C amino acid analyser. Peaks were manually integrated by the half-height approximation and compared with the results obtained from standard mixtures of amino acids. No correction was made for amide N, for unhydrolysed oligopeptides, for cysteine or for tryptophan; the amount of material available did not suffice for tryptophan or cysteine analysis or for additional, longer hydrolyses.

2.2.14. Isolation of labelled RNA produced by the H. cutirubrum polymerases

For further characterization, either by centrifugation or by hydrolysis, the RNA produced in standard incubation mixtures (0.1 ml or 0.5 ml) by the DNA-dependent or the

RNA-dependent enzyme was isolated by phenol extraction according to Bolton (1966). To 0.475 ml of assay mixture were added 0.5 ml of a solution containing 2 mg/ml of carrier polynucleotide and 1 ml of phenol previously equilibrated with 0.02 M sodium phosphate, 0.5% (w/v) sodium dodecyl sulphate, pH 7. The mixture was shaken vigorously and then centrifuged at 1,000 g for 8 min. The phenol and interface precipitate were discarded and the extraction was twice repeated. The aqueous phase was then shaken five times with diethyl ether (1 ml) and centrifuged as above. Nitrogen was bubbled through the aqueous phase until frothing indicated the complete removal of ether. 0.1 ml of 1 M NaCl was added, followed by 2.5 vol of cold ethanol. The precipitate was centrifuged and washed three times with ethanol saturated with unlabelled ATP.

2.2.15. Nearest-neighbour analysis

Product RNA was labelled with $^{32}P$ by including an $\alpha$-$^{32}P$-NTP in the assay mix and was isolated as above. The RNA was dissolved in 0.1 ml of 0.2 M KOH and the solution incubated for 14 h at 37°. The products of the hydrolysis were analysed by descending chromatography on Whatman no. 1 paper in 0.1 M sodium phosphate, pH 6.8-saturated ammonium sulphate-n-propanol (100:60:2) (Litvak,

Carré & Chapeville, 1970). The spots were identified under UV light by comparison with standards and were cut out and counted by liquid-scintillation (s. 2.2.6.).

2.2.16. RNA-DNA hybridization

Hybridization was performed by a modification (W.C. Summers, personal communication) of the method of Siegel & Summers (1970) and the conditions were chosen to yield about two-thirds of the input radioactivity as a ribonuclease-resistant hybrid when using radioactive RNA synthesized by the H. cutirubrum DNA-dependent polymerase. The hybridization mixtures (0.1 ml) contained: $T_7$ DNA, r or 1 strand, 1 µg; $^{14}$C-RNA, 1,000-6,500 cpm; 0.3 M NaCl, 0.03 M sodium citrate, pH 8. Control experiments lacked DNA. The mixtures were incubated for 5 h at 60° and then cooled to room temperature. To each was added 0.45 M NaCl, 0.045 M sodium citrate, pH 8 (2 ml) containing 20 µg/ml of pancreatic ribonuclease, which had previously been incubated at 100° for 5 min to inactivate deoxyribonuclease and then cooled. After 1 h at room temperature, the mixtures were diluted to 10 ml with the same solution lacking RNase and filtered slowly through nitrocellulose membrane filters (2 min; BAC-T-FLEX membrane filters, type B-6, Schleicher & Schuell, Inc., Keene, N.H., U.S.A.).

Each filter was washed with 50 ml of the saline-citrate solution and then dipped in a beaker containing 50 ml more as a final rinse. The discs were blotted and air-dried, and the radioactivity was determined by liquid-scintillation counting (s. 2.2.6.).

## 2.2.17. Base-composition analysis of RNA

The composition of unlabelled RNA was determined by the method of Yoshida and Shibata (1969). A column of Dowex 50-X4, 200-400 mesh, 0.9 cm x 8 cm, was packed and washed with 1 litre of 2 M HCl, with distilled water until the pH of the effluent was above 5, and with 2 litres of 0.05 M HCl. 0.5 mg of RNA was hydrolysed in 0.05 ml of 1 M HCl for 1 h at $100^{o}$. The solution was diluted to 1 ml and loaded onto the Dowex column as for hard gel columns (s. 2.2.10.3.). The column was washed first with 80 ml of 0.05 M HCl and then with 80 ml of 2 M HCl. 5 ml fractions were collected and their $E_{260}$ determined in the Zeiss PMQ II spectrophotometer. Peaks were pooled and identified by their UV spectra, and their purity was checked by $E_{290}/E_{260}$, $E_{280}/E_{260}$ and $E_{250}/E_{260}$ ratios (Volkin & Cohn, 1954). The extinction values determined by Yoshida & Shibata (1969) were used to calculate the base or nucleoside content of each peak as a percentage of the total recovered, which

represented 94% of the material applied to the column.

2.2.18. Purification of $^{3}$H-ApA

Crude $^{3}$H-ApA, tritiated by the procedure of Wilzbach (1957, 1963) was purified by gel filtration. A 2 ml sample containing 0.6 mg of the $^{3}$H-ApA was applied to a column (0.9 cm x 28 cm) of Bio-Gel P-2 (s. 2.2.10.) run by downward flow at 5 ml/h. The ApA was eluted with water, 2 ml fractions being collected, and the $E_{260}$ and radioactivity of the fractions were determined. Purity was verified by analytical thin-layer chromatography on PEI-cellulose; the chromatogram was developed with 0.16 M LiCl (Randerath & Randerath, 1967), the spots were identified under UV light by comparison with standards and the plates were scraped accordingly. The scrapings were counted by liquid-scintillation. The bulk of the purified ApA was freeze-dried and redissolved in water (0.3 mg/ml final concentration).

2.2.19. Check on the purity of labelled nucleoside triphosphates.

NTP labelled with $^{32}$P in the $\gamma$ position were routinely checked for hydrolysis on arrival. DEAE-cellulose thin-layer plates developed in 0.02 M HCl were used for the

analysis (Randerath, 1964). The dried chromatograms were treated as described above (s. 2.2.18.). Other NTP were also checked for purity by this method when partial hydrolysis was suspected.

# 3. RESULTS AND DISCUSSION

## 3.1. Purification of the H. cutirubrum polymerase proteins

### 3.1.1. Separation of DNA-dependent and RNA-dependent activities

The results in Table 3.1. typify the separations achieved by acid and ammonium sulphate precipitations. Precipitation by acid has been generally more reliable than that by ammonium sulphate, although the reproducibility of both appears to depend on a rigid adherence to the prescribed conditions of sonication. In addition to the latter, the maintenance of an exact initial salt concentration proved to be critical to the success of the ammonium sulphate step (Table 3.2.). Removal of all of the KCl and NaCl causes both enzymes to remain in the supernatant at $(NH_4)_2SO_4$ concentrations in excess of 60% of saturation, whereas separation was nearly complete, the DNA-dependent enzyme being entirely precipitated at 25%, when the ammonium sulphate was added to a solution containing 0.087 M KCl, 0.035 M NaCl. [The disintegration of the cell membrane in Halobacteria is progressive with decreasing ionic strength (Stoeckenius & Rowen, 1967; Lanyi, 1971), but the details of the process have not been correlated with precise salt concentrations below 0.5 M,

Table 3.1. Effectiveness and reproducibility of acid and $(NH_4)_2SO_4$ precipitations

| Precipitation by acid | units of activity | | | | | |
|---|---|---|---|---|---|---|
| | Crude Extract | | Supernatant | | Precipitate | |
| | DNA dep. | RNA dep. | DNA dep. | RNA dep. | DNA dep. | RNA dep. |
| 1. | 122 | 106 | 86.4 | 0 | 0 | 49.4 |
| 2. | 1584 | 234 | 1571 | 0 | 6.0 | 228 |
| 3. | 400 | 294.7 | 350.5 | 8.0 | 3.4 | 240.5 |
| 4. | 13,200 | 9,240 | 4,560 | 960 | 8,200 | 6,050 |

| Precipitation by $(NH_4)_2SO_4$ | | | 0 - 25% fraction | | 25 - 35% fraction | | 35 - 75% fraction | | 75% supernatant | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | DNA dep. | RNA dep. | DNA dep. | RNA dep. | DNA dep. | RNA dep. | DNA dep. | RNA dep. |
| 1. | 214 | 384 | 72.8 | 7.3 | 17.8 | 0 | 22.9 | 140 | 16.6 | 508 |
| 2. | 113 | 166 | 104 | 91 | 31 | 33 | 321 | 306 | 382 | 522 |
| 3. | 130 | 22.8 | 127 | 15.6 | 6.0 | 0 | 0 | 0 | 0 | 41.4 |

Table 3.2. Effect of initial salt concentration on separation of polymerases by $(NH_4)_2SO_4$ precipitation

Aliquots (10 ml) of crude extract were subjected to ammonium sulphate fractionation (s. 2.2.4.2.1.). Total salt concentration was determined by chloride analysis.

| Total salt concentration before $(NH_4)_2SO_4$ addition | Units of activity | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0-25% fraction | | 25-35% fraction | | 35-75% fraction | | 75% supernatant | |
| | DNA dep. | RNA dep. | DNA dep. | RNA dep. | DNA dep. | RNA dep. | DNA dep. | RNA dep. |
| 0 | 0 | 0 | 0 | 0 | 12.2 | 0 | 133.6 | 71.1 |
| 0.122 | 127.4 | 15.6 | 0 | 6.0 | 0 | 0 | 0 | 41.4 |
| 0.131 | 134.0 | 76.0 | 0 | 6.5 | 0 | 12.0 | 0 | 2.4 |

at which level 30% of the bacterial protein is still bound to material sedimenting at 15,000 g (Lanyi, 1971). Probably one of the last stages in the disintegration process affects this differential precipitability.] This additional critical factor, combined with the instability of the β protein in the absence of salt (s. 3.5.1.), renders the ammonium sulphate step decidedly inferior to acid precipitation, in favour of which it was abandoned.

A preliminary experiment has indicated that the two enzymes can be separated on hydroxylapatite at the last stage of the purification. If a suitable means of removing membrane fragments and some protein from the crude extract, without fractionating the polymerase activities, were introduced before the gel filtration, it might be possible to avoid the difficulties inherent in the present separation procedure. The introduction of such a step is in any case a prerequisite to increasing the scale of the purification, since the gel columns would otherwise be overloaded.

### 3.1.2. Isolation of the pure subunits of H. cutirubrum DNA-dependent RNA polymerase

Table 3.3. shows the results of a typical purification performed as described (s. 2.2.4.1., 2.2.4.2.2., 2.2.4.4.,

Table 3.3. Purification of H. cutirubrum DNA-dependent RNA polymerase

The enzyme was purified from 40 g (wet wt.) of bacteria as described in the Experimental section. Numbers in parentheses represent units of RNA-dependent RNA polymerase.

| Fraction | Activity (units) | Sp. activity (units/mg) |
|---|---|---|
| Crude extract | 2123 (319) | 2.8 (0.4) |
| pH 4 supernatant | 2041 (0) | 11.7 (0) |
| P-60 concentrated pool | 1910 (0) | 2760 (0) |
| Hydroxylapatite column<br>Protein α, electrophoretically homogeneous | | |
| Hydroxylapatite adsorption<br>Protein β, electrophoretically homogeneous | | |
| α + β, equimolar | 1820 (0) | 14440 (0) |

2.2.4.6.) in the Experimental section. The final product was purified 5,157-fold in 86% yield. Similar results were obtained on other occasions; for example, the material used for the preliminary amino acid analyses (s. 3.4.) was purified 5,060-fold in 84% yield. Over-sonication once led to a specific activity in the crude extract of half the usual figure (1.4 units/mg instead of 2.5-3); the pure enzyme had a 13,000-fold greater specific activity and 79% of the original units was recovered.

The purified enzyme displayed no DNase, RNase or polynucleotide phosphorylase activity in standard assay conditions, nor could deoxynucleotide substrates be substituted for the ribonucleotides.

The α and β proteins thus obtained were electrophoretically pure. Polyacrylamide gel electrophoresis was performed, after removal of salt by dialysis, by the micro-disc method of Neuhoff (s. 2.2.12.) at pH 7.0, 8.0 and 9.4 in 8%, 10% and 12% (w/v) polyacrylamide separating gels. Single bands were observed in each of the nine gels run with each protein. About 200 ng of protein was run, and the limit of detection by Coomassie blue staining and visual inspection was about 4-6 ng per band, so the two subunits were at least 97-98% pure with respect to

other proteins. The $E_{280}/E_{260}$ ratio was 1.16 for the P-60 fraction of Table 3.3., indicating a nucleic acid content of not more than 2% (w/w).

The α and β proteins were also homogeneous in sucrose density gradients after centrifugation (s. 2.2.11.2.), as shown in Fig. 3.1.

### 3.1.3. Isolation of pure H. cutirubrum RNA-dependent RNA polymerase

Table 3.4. shows the results of a typical purification of the RNA-dependent enzyme by the methods described (s. 2.2.4.1., 2.2.4.2.2., 2.2.4.3., 2.2.4.4., 2.2.4.5.) in the Experimental section. The small residual DNA-dependent RNA polymerase activity present in the precipitate from the acid precipitation was eliminated at the next stage by adjustment of the starting KCl/NaCl concentration (s. 3.1.1.), and a contaminant of high electrophoretic mobility still present after gel filtration was removed by adsorption to $Zn(OH)_2$ gel. The $Zn(OH)_2$ supernatant was 4,800-fold purified with respect to the crude extract, and contained 74% of the units present in the $(NH_4)_2SO_4$ supernatant (in which a large increase in the total number of units was always observed, possibly due to a removal of degradative enzymes). The final product was nuclease-free

Fig. 3.1. Sucrose-density-gradient centrifugation of the purified RNA-dependent enzyme and α and β proteins

■—■, protein α; ▲—▲, protein β; o—o, RNA-dependent RNA polymerase; ●--●, RNA-dependent activity (included for comparison).

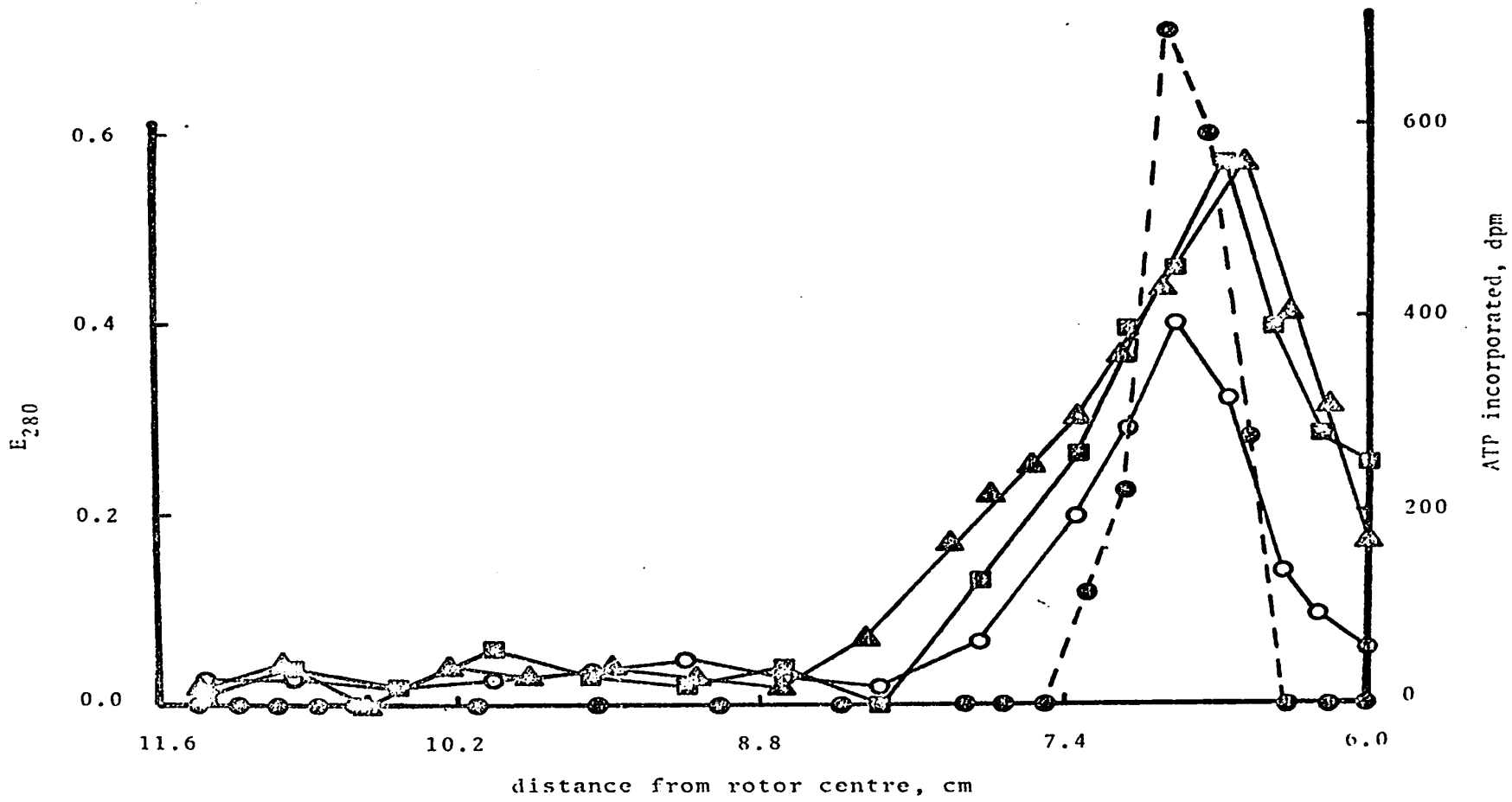

0.6
0.4
0.2
0.0
$E_{280}$
600
400
200
0
ATP incorporated, dpm
11.6
10.2
8.8
7.4
6.0
distance from rotor centre, cm

Table 3.4. Purification of H. cutirubrum RNA-dependent RNA polymerase

The enzyme was purified from 32 g (wet weight) of bacteria as described. Numbers in parentheses represent units of DNA-dependent RNA polymerase.

| Fraction | Activity (units) | Sp. activity (units/mg) |
|---|---|---|
| Crude extract | 234 (1584) | 0.28 |
| pH 4 precipitate | 228 (6) | 0.34 |
| $(NH_4)_2SO_4$ supernatant | 600 (0) | 24.1 |
| Bio-Gel P-60 concentrated pool | 570 (0) | 1294 |
| $Zn(OH)_2$ supernatant | 444 (0) | 1345 |

and displayed no activity when ribonucleoside diphosphates or deoxyribonucleoside triphosphates were substituted for the ribonucleoside triphosphates in the standard assay. It was electrophoretically homogeneous (at least 97% pure) at pH 7.0, 8.0 and 9.4, in each case in 8%, 10% and 12% polyacrylamide gels (cf. previous section), and moved as a single band in sucrose-density-gradient centrifugation (Fig. 3.1.).

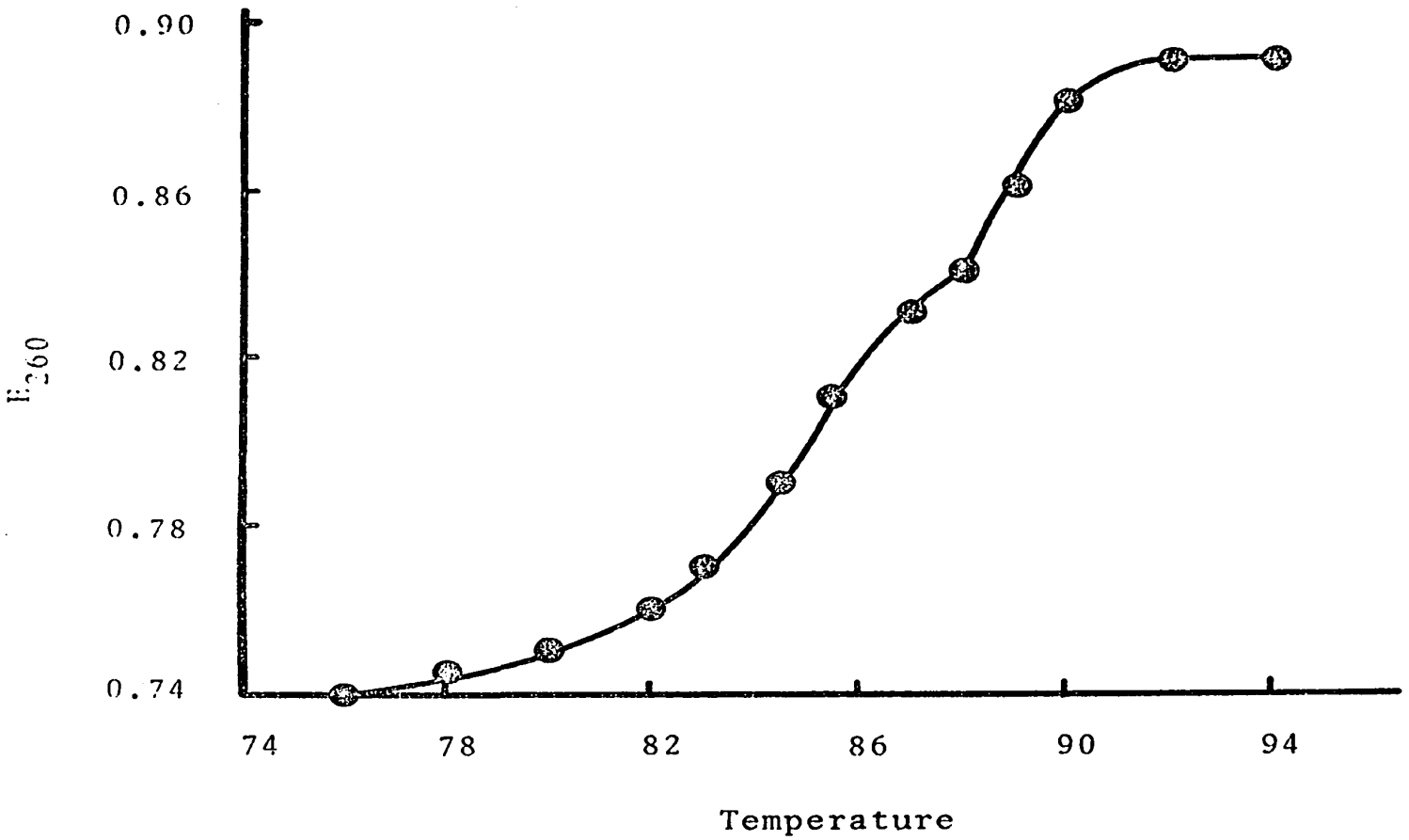
The DNA-dependent and RNA-dependent RNA polymerases of Halobacterium cutirubrum have been purified essentially to homogeneity, under conditions which allow good recovery of enzyme activity. As was discussed in the Introduction (s. 1.3.2.1.), previous attempts at enzyme purification from halophilic organisms met, in the main, with very limited success, owing to the difficulty of working at high salt concentration. It seems probable that the methods used in the present and related work (Peterkin & Fitt, 1971; s. 2.2.4.) for the purification of enzymes from extremely halophilic bacteria will prove applicable to other such enzymes, permitting a more detailed study of their properties, and especially of the effects of salt upon their structure and function, than has hitherto been possible.

## 3.2. Purification of H. cutirubrum nucleic acids

The RNA obtained as described in the Experimental section (s. 2.2.5.) was analysed for DNA and protein (s. 2.2.8., 2.2.9.1.), and found to contain 0.3% (w/w) of DNA; protein was undetectable. The DNA so obtained contained 0.5% (w/w) of RNA (s. 2.2.9.2.) and 0.7% (w/w) of protein; both contaminants could be removed by hydroxylapatite chromatography (Pinck, 1970), but so doing affected neither the template behaviour nor the thermal denaturation curve of the DNA.

Thermal denaturation of H. cutirubrum DNA was performed in a Zeiss PMQ II spectrophotometer fitted with a circulating water bath/temperature programmer, an automatic sample changer, an automatic slit adjusting device, a T-E converter and a chart recorder. Temperature of the circulating water was manually recorded. The temperature was elevated at the rate of $24^{o}$/h and the baseline (E = 0) was automatically reset at 5 min intervals. Covered blank and sample cuvettes contained 0.15 M NaCl, 0.015 M sodium citrate, pH 8; the sample cuvette contained, in addition, 0.73 units of DNA ($E_{260}$ at $25^{o}$). The curve appears as Fig. 3.2. Under these conditions, the $T_m$ values of $88^{o}$ and $84.5^{o}$ are in agreement with the molar percentage of G-C of 66 and 57% in the major and minor components,

Fig. 3.2. Thermal denaturation curve of *H. cutirubrum* DNA


0.90
0.86
0.82
0.78
0.74
$E_{260}$
74
78
82
86
90
94
Temperature

respectively (Moore & McCarthy, 1969a). The curve closely resembles that previously reported for H. salinarium DNA (Joshi, Guild & Handler, 1963).

## 3.3. Molecular weight of the H. cutirubrum polymerase proteins

Sections 2.2.10.5. and 2.2.11.1. describe the determination of molecular weight by gel filtration and sucrose-density-gradient centrifugation. These techniques were employed to determine the molecular weights of the DNA-dependent and RNA-dependent enzymes, and also those of the separated α and β proteins, by comparison with the protein standards shown in Table 3.5.

In the experiment of Fig. 3.3., the RNA-dependent and complete DNA-dependent enzymes were chromatographed on a standardized 2.5 x 37 cm column of Sephadex G-50. In that of Fig. 3.4., the same samples were applied to a 2.5 x 90 cm column of Bio-Gel P-60, through which the same standards had been run. The two enzymes were also subjected to sucrose-density-gradient centrifugation in high-salt buffer (Fig. 3.5.). Molecular weight values of 17,900, 17,800 and 20,900 were obtained for the complete DNA-dependent enzyme in the three experiments, respectively; the greater precision of the gel filtration technique renders an actual size of around 18,000 daltons most

Table 3.5. Standard proteins used in determinations of molecular weight

The proteins shown were used as molecular-weight standards in the experiments described.

| protein | molecular weight | reference |
|---|---|---|
| Cytochrome c (horse heart) | 12,400 | Fish, Mann & Tanford (1969) |
| Myoglobin (sperm whale) | 17,800 | Determann (1967) |
| α-Chymotrypsinogen A (bovine pancreas) | 25,000 | Determann (1967) |
| Ovalbumin | 45,000 | Determann (1967) |
| Bacitracin | 1,450 | Schröder & Lübke (1966) |

Fig. 3.3. Sephadex G-50 gel filtration: DNA-dependent and RNA-dependent enzymes

●, cytochrome c; ▲, myoglobin and H. cutirubrum DNA-dependent RNA polymerase, which had the same $K_{av}$; ▣, α-chymotrypsinogen A; Δ, H. cutirubrum RNA-dependent RNA polymerase; □, H. cutirubrum polynucleotide phosphorylase (included for comparison).

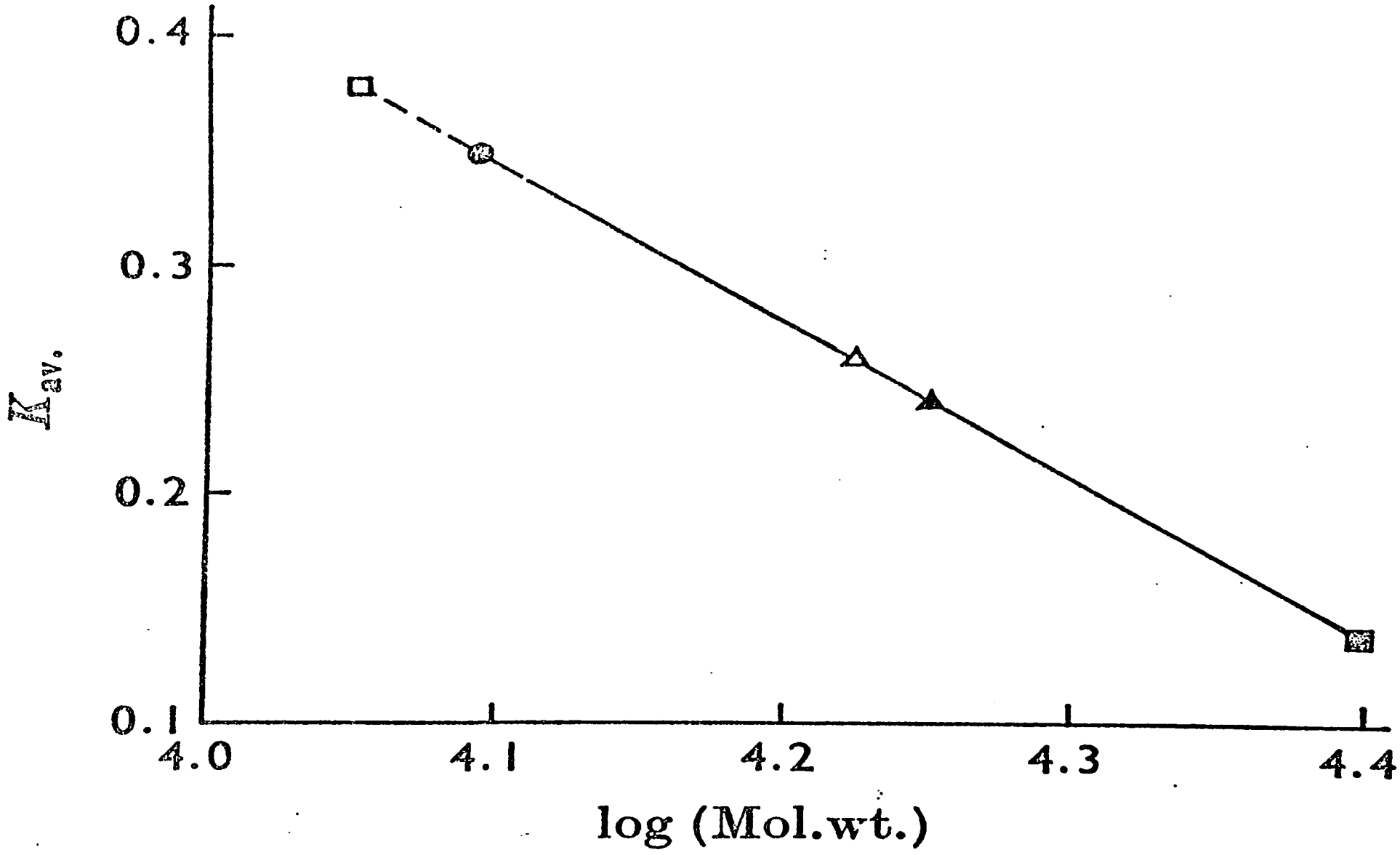


Fig. 3.4. Bio-Gel P-60 gel filtration: DNA-dependent and RNA-dependent enzymes

●, cytochrome c; ■, α-chymotrypsinogen A; ▲, ovalbumin; o, H. cutirubrum DNA-dependent and RNA-dependent RNA polymerases, which had the same $K_{av}$.

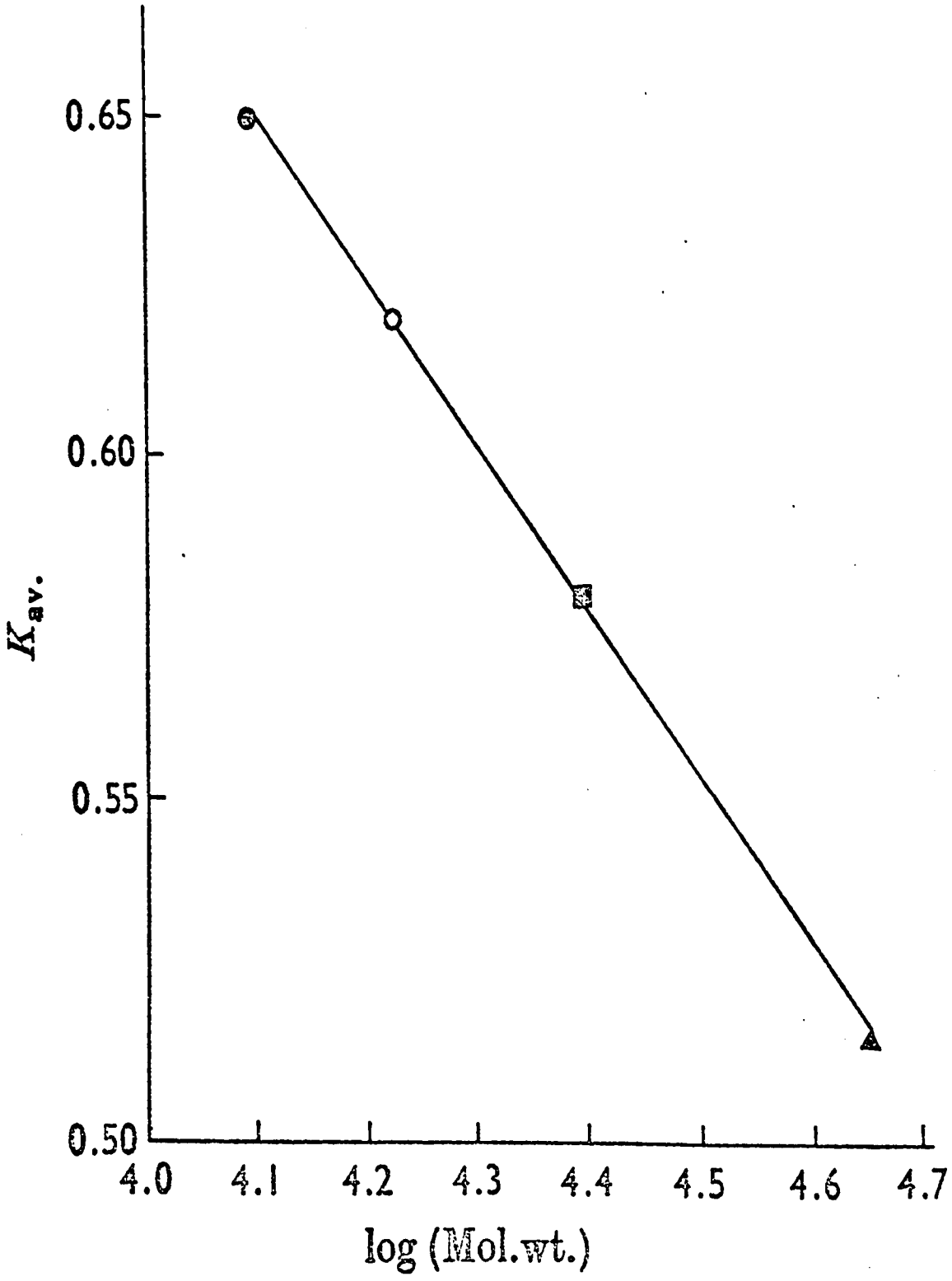
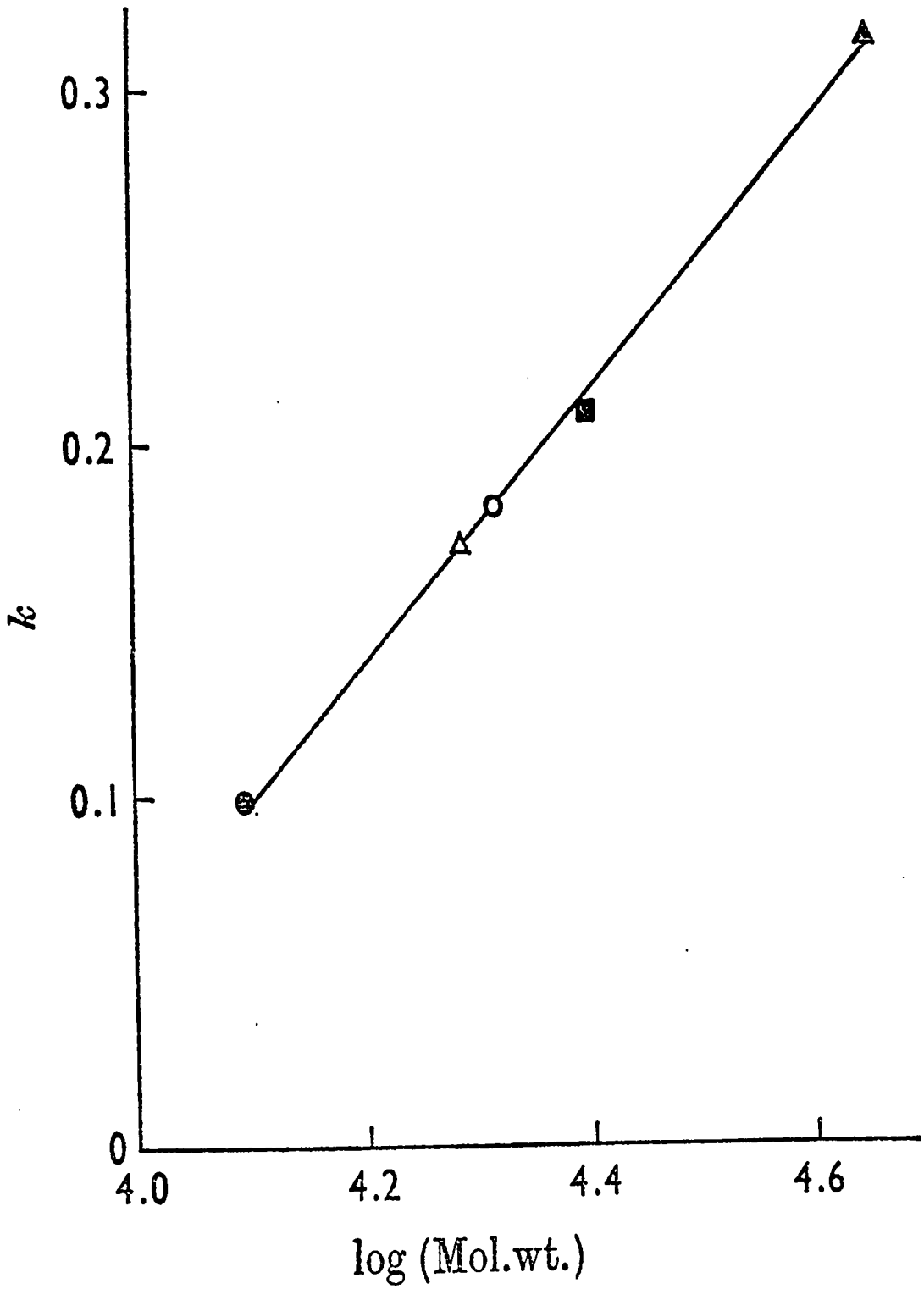

0.65
0.60
0.55
0.50
$K_{av.}$
4.0
4.1
4.2
4.3
4.4
4.5
4.6
4.7
log (Mol.wt.)

Fig. 3.5. Sucrose-density-gradient centrifugation: DNA-dependent and RNA-dependent enzymes

●, cytochrome c; ▣, α-chymotrypsinogen A; ▲, ovalbumin; Δ, H. cutirubrum RNA-dependent RNA polymerase; o, H. cutirubrum DNA-dependent RNA polymerase.

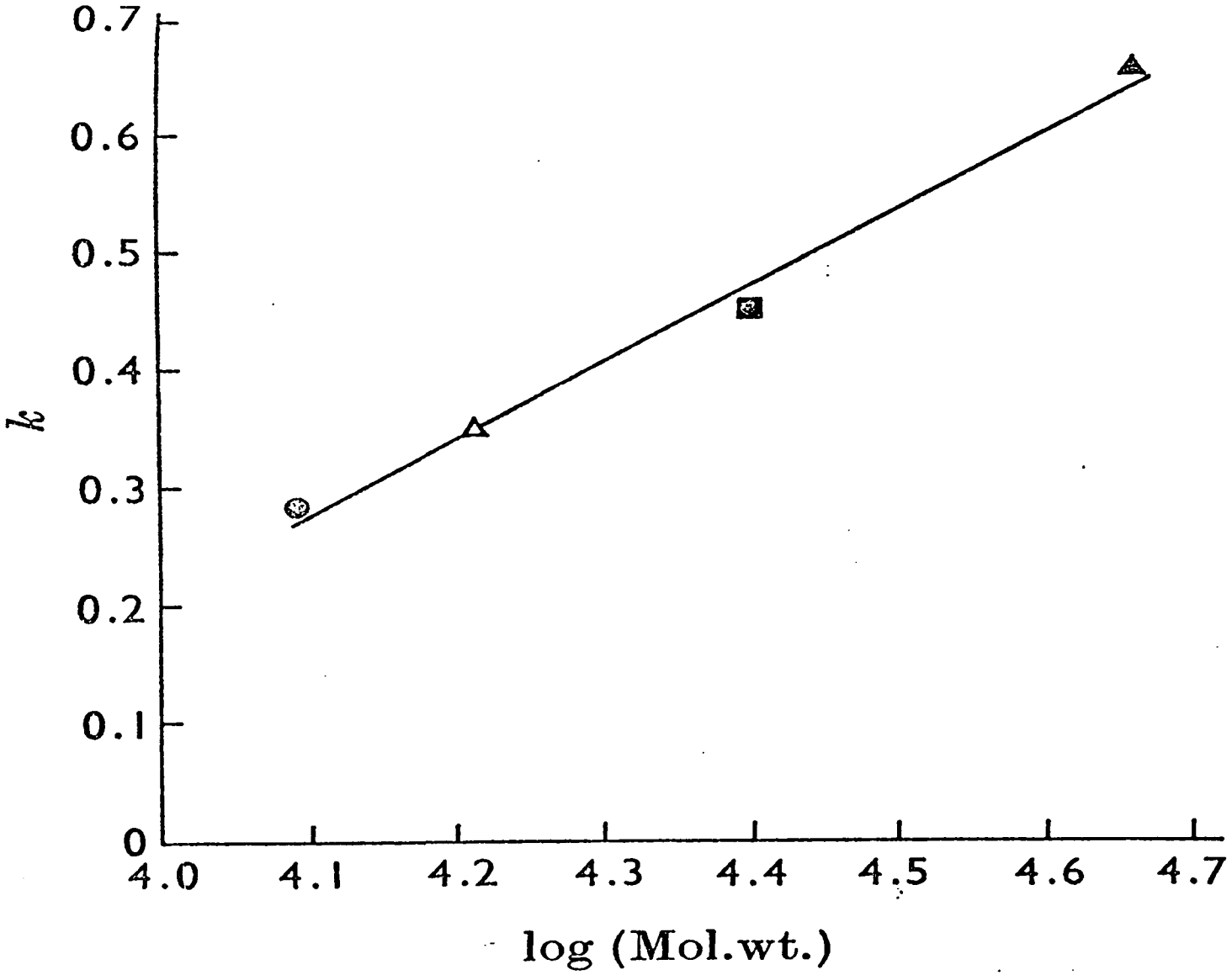

0.3
0.2
0.1
0
k
4.0
4.2
4.4
4.6
log (Mol.wt.)

likely. For the RNA-dependent enzyme the values obtained were 16,800, 17,800 and 18,200 respectively. The relatively high stability of the RNA-dependent polymerase at low salt concentrations (s. 3.6.1.) allowed a further estimate of the molecular weight by sucrose-density-gradient centrifugation in the absence of salt. The classical procedure of Martin and Ames (1961) was followed, and gave a molecular weight value of 16,200 for that enzyme (Fig. 3.6.). The means of the gel filtration and gradient centrifugation estimates thus agreed (17,400 daltons).

Three possible sources of error might account for the fact that the H. cutirubrum enzymes appeared much smaller on gel filtration than do their non-halophile counterparts (v.i.). First, an unusually dense configuration of the H. cutirubrum proteins might lead to abnormal retardation. This is eliminated by the gradient centrifugation results, since unusual density would lead to rapid sedimentation and hence to high, rather than low, molecular weight estimates - and therefore to disagreement with the gel filtration results. Similarly, the possibility of selective adsorption to the dextran or polyacrylamide columns is eliminated by the good agreement of the centrifugation and gel filtration estimates. Finally, the

Fig. 3.6. Sucrose-density-gradient centrifugation in the absence of salt: RNA-dependent enzyme

Symbols are defined in the legend to Fig. 3.5.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
k
4.0
4.1
4.2
4.3
4.4
4.5
4.6
4.7
log (Mol.wt.)

possibility of abnormal behaviour of the protein standards in the high-salt buffer may be rejected because the estimate obtained for the RNA-dependent polymerase by the classical centrifugation procedure agreed closely with those made in the presence of salt. There is therefore little reason to doubt that the low molecular weights observed are in the correct range for the molecules studied.

When the sucrose-density-gradient results were plotted as log k vs log (mol. wt.) (Martin & Ames, 1961), the values obtained for the enzyme proteins were within 7% of those obtained as shown. The method used here is felt to be more accurate in this low molecular weight range, because the k values of the standards were more linearly a function of mol. wt. than were the logarithms of k.

The molecular weights of the α and β proteins of the DNA-dependent enzyme were also determined separately. Both proteins were chromatographed on a calibrated P-60 column (2.5 x 37 cm) and subjected to sucrose-density-gradient centrifugation in high-salt buffer (Figs. 3.7. & 3.8.). The resulting molecular weight estimates were: for protein α, 17,800 and 18,000 respectively, and for protein β, 16,800 and 18,000. It was clear that the two

Fig. 3.7. Bio-Gel P-60 gel filtration: proteins α and β

▼, bacitracin; ●, cytochrome c; ■, α-chymotrypsinogen A; ▲, ovalbumin; o, protein α; □, protein β.

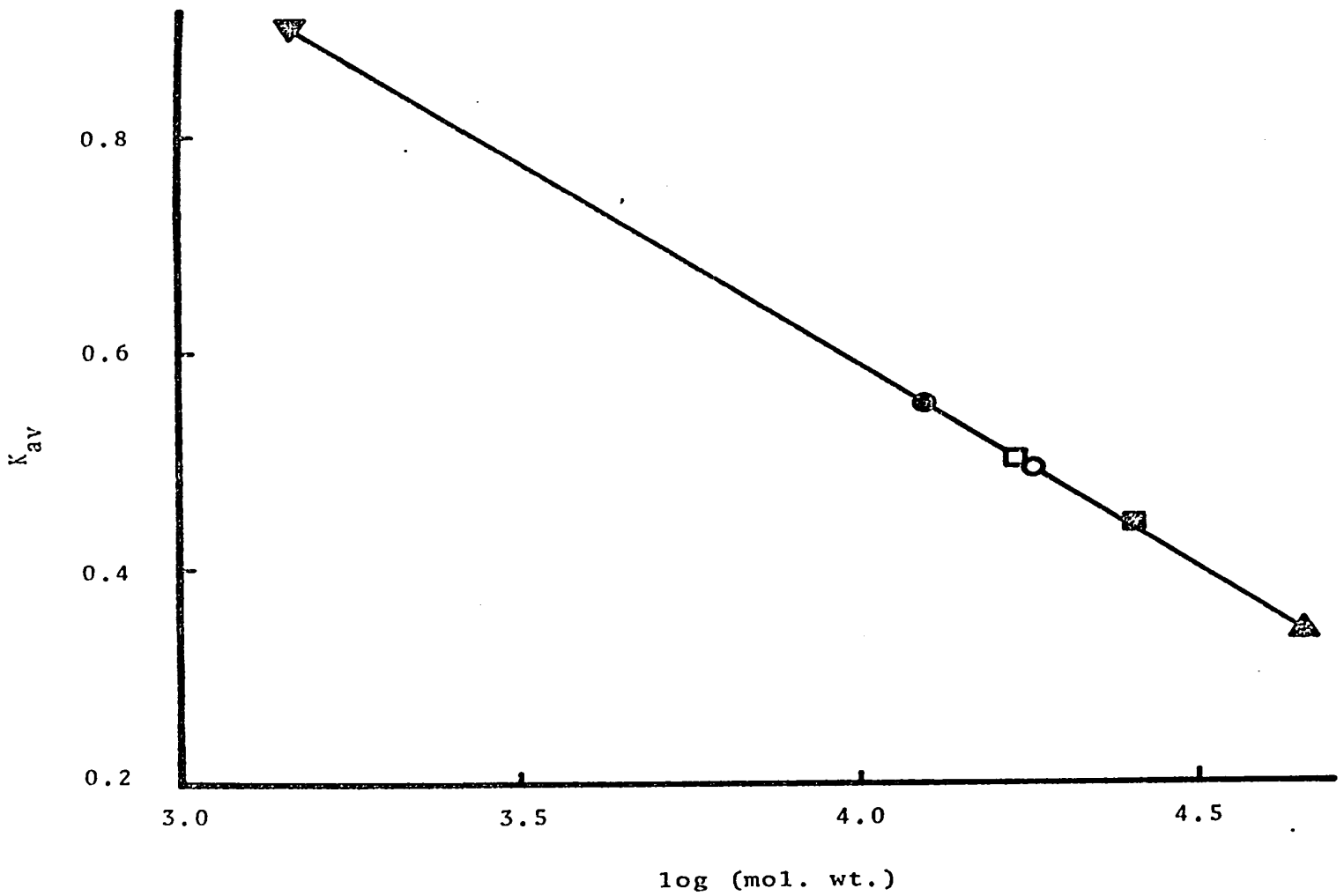
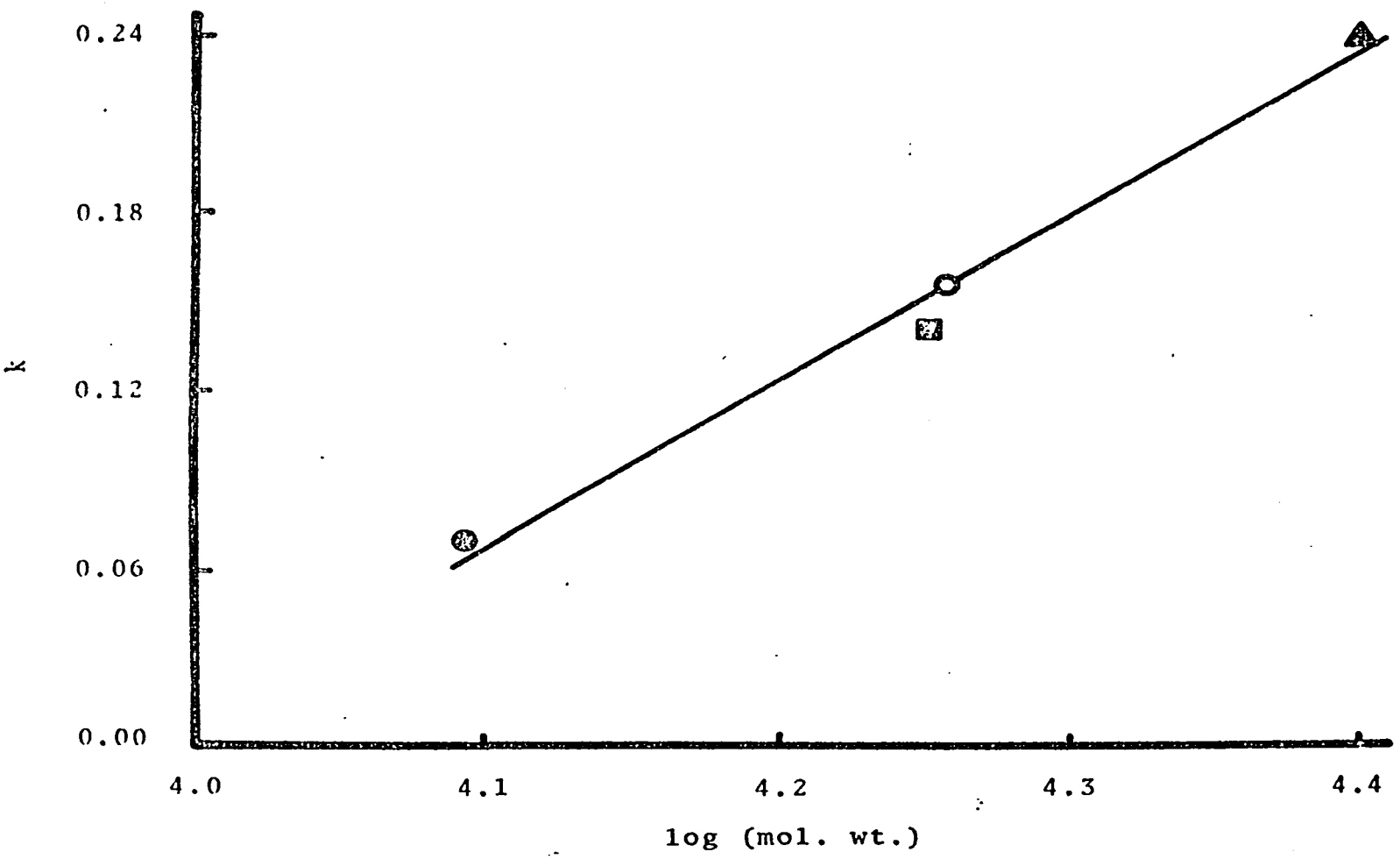

0.8
0.6
0.4
0.2
$K_{av}$
3.0
3.5
4.0
4.5
log (mol. wt.)

Fig. 3.8. Sucrose-density-gradient centrifugation: proteins α and β

●, cytochrome c; ▣, myoglobin; ▲, α-chymotrypsinogen A; o, proteins α and β, which had the same k.


0.24
0.18
0.12
0.06
0.00
K
4.0
4.1
4.2
4.3
4.4
log (mol. wt.)

proteins do not associate in high-salt buffer, since each has the molecular weight displayed by the complete enzyme. When the latter was centrifuged according to the procedure of Martin & Ames (1961), no protein was detected above a k value corresponding to a molecular weight of 23,000; proteins α and β, therefore, also fail to associate in the absence of salt. (The formation of a 1:1 αβ complex in the presence of $Mn^{2+}$ is discussed in s. 3.5.5.).

The molecular weights estimated for the H. cutirubrum polymerase proteins are compared in Table 3.6. with those of similar enzyme proteins from normal or bacteriophage-infected E. coli. The smallest RNA polymerase yet described, other than that from H. cutirubrum, is that of yeast mitochondria (Küntzel & Schäfer, 1971), whose molecular weight is about 64,000. This polymerase, like those induced by $T_3$ and $T_7$ bacteriophage, has a very limited template specificity (Schäfer, Bugge, Grandi & Küntzel, 1971; Dunn, Bautz & Bautz, 1971), unlike the large E. coli enzyme; the H. cutirubrum DNA-dependent RNA polymerase, which in its functional form is slightly more than half the size of the yeast enzyme and less than a tenth that of the E. coli RNA polymerase, resembles the latter rather than the former in function (v.i.), and operates in vivo on a genome of about the same size as the E. coli genome (Moore & McCarthy, 1969b). There

Table 3.6. Molecular Weights of RNA polymerases

| Enzyme | Source | Molecular Weight | Reference |
|---|---|---|---|
| *E. coli* RNA polymerase | *E. coli* | 495,000 | Burgess (1969b) |
| α subunit | | 39,000 | Burgess (1969b) |
| β subunit | | 155,000 | Burgess (1969b) |
| β' subunit | | 165,000 | Burgess (1969b) |
| σ subunit | | 95,000 | Travers & Burgess (1969) |
| *H. cutirubrum* DNA-dependent RNA polymerase | *H. cutirubrum* | 36,000 | s. 3.5.5. |
| α subunit | | 18,000 | s. 3.3. |
| β subunit | | 18,000 | s. 3.3. |
| $T_7$ RNA polymerase | $T_7$-infected *E. coli* | 107,000 | Chamberlin, McGrath & Waskell (1970) |
| $T_3$ RNA polymerase | $T_3$-infected *E. coli* | 110,000 | Dunn, Bautz & Bautz (1971) |
| *Neurospora crassa* | | | |
| mitochondrial RNA polymerase | *N. crassa* mitochondria | 64,000 | Küntzel & Schäfer (1971) |
| Qβ replicase | Qβ-infected *E. coli* | 150,000 | August (1969) |
| *H. cutirubrum* RNA dependent RNA polymerase | *H. cutirubrum* | 17-18,000 | s. 3.3. |

appears to be no relationship, therefore, between size and complexity of function of RNA polymerases. That being so, the H. cutirubrum polymerases should be of great value in the elucidation of structure-function relationships in nucleic acid transcribing enzymes, provided the preparation can be scaled up to yield quantities sufficient for detailed structural analysis.

## 3.4. Preliminary amino acid analyses of the H. cutirubrum polymerase proteins

Inasmuch as no amino acid analysis of an enzyme from an extreme halophile had yet been reported, it was of interest to attempt to determine the composition of the three highly-purified polymerase proteins, despite the fact that the entire product of one preparation barely sufficed for a single hydrolysis and analysis on the most sensitive instrument available to us. Single (22 h) hydrolyses were accordingly performed and the hydrolysates were analysed as described in s. 2.2.13. The results, uncorrected for amide N, cysteine (if any) or tryptophan, appear in Table 3.7.

Amino acid analyses of E. coli RNA polymerase have been published by several authors (Maitra & Hurwitz, 1967; Preiss & Zillig, 1967; Neuhoff, Weise & Sternbach, 1970;

Table 3.7. Amino acid composition of *H. cutirubrum* polymerase proteins

| Amino acid | Amino acid composition (mol/100 mol) | | | |
|---|---|---|---|---|
| | *H. cutirubrum* RNA-dependent RNA-polymerase | *H. cutirubrum* protein α | *H. cutirubrum* protein β | *E. coli* polymerase |
| Asp | 8.2 | 7.0 | 6.3 | 10.2 |
| Thr | 6.7 | 5.3 | 5.0 | 5.3 |
| Ser | 6.9 | 15.9 | 16.1 | 6.7 |
| Glu | 11.0 | 14.2 | 13.4 | 13.6 |
| Pro | 3.7 | 4.2 | 4.1 | 3.4 |
| Gly | 17.6 | 15.6 | 17.8 | 7.4 |
| Ala | 9.9 | 8.0 | 8.2 | 8.8 |
| Val | 4.5 | 3.6 | 3.6 | 6.7 |
| Met | 0.8 | 0.6 | 0.5 | 2.9 |
| Ile | 2.5 | 1.8 | 2.2 | 5.6 |
| Leu | 4.6 | 3.1 | 4.0 | 10.9 |
| Tyr | 2.0 | 1.8 | 1.9 | 2.6 |
| Phe | 2.1 | 1.4 | 1.8 | 2.8 |
| Lys | 12.0 | 11.1 | 9.0 | 5.4 |
| His | 3.8 | 3.1 | 3.4 | 1.6 |
| Arg | 3.6 | 3.2 | 2.7 | 5.8 |

Nicholson, 1971: that of Nicholson appears for comparison in Table 3.7.). Proteins α and β of the H. cutirubrum DNA-dependent polymerase have very similar compositions which differ in detail from that of the E. coli enzyme. In particular, the glycine and serine content of the α and β proteins is much higher than that of the E. coli polymerase.

Proteins of the cell envelope of Halobacteria (Brown, 1963; Kushner & Onishi, 1966; McClare, 1967; Marshall, Wicken & Brown, 1969; Steensland & Larsen, 1969) and of their ribosomes (Bayley, 1966) are somewhat acidic with respect to their non-halophilic counterparts, and the bulk protein of some extreme halophiles is also slightly more acidic than that of some non-halophiles (Reistad, 1970). It has been suggested (Brown, 1963, 1964; Kushner & Bayley, 1963) that the conformation of halophile proteins in strong salt solutions depends on interactions of negatively charged groups in the proteins with cations, and it is known, for example, that removal of salt causes unfolding of H. cutirubrum isocitrate dehydrogenase (Hubbard & Miller, 1969). In this connection it should be noted that the total acidic, total basic and total neutral amino acids of the complete H. cutirubrum (αβ) and the E. coli DNA-dependent RNA polymerases do not differ by more than a few percent. Possibly the suggestion of Lanyi & Stevenson (1970)

that the conformation is determined largely by hydrophobic forces within the molecule more nearly approaches the truth in this case; despite their very similar amino acid compositions, the α and β proteins have markedly different stabilities in the absence of salt (s. 3.5.1.), which probably arise from differences in primary structure leading to a stronger hydrophobic component in the stability of protein α. It seems unlikely that relatively non-specific interaction with cations could be so drastically altered by changes in the locations of the charged groups as to effect the observed differences in stability.

## 3.5. The DNA-dependent RNA polymerase of H. cutirubrum

From this point on, it will be convenient to discuss the DNA-dependent and RNA-dependent enzymes separately. A consideration of their interrelationship will follow the section devoted to the latter.

### 3.5.1. Effect of ionic strength on the stability of proteins α and β

Both the purified subunits of H. cutirubrum DNA-dependent RNA polymerase could be stored in high-salt buffer at 0-5° for at least 60 days with only a slight loss of activity. After 90 days activity had diminished by 32%, and after 5 months only 25% of the activity remained. Removal

of the salt by dialysis caused inactivation of the α and β subunits, but at markedly different rates (the activity of each subunit being measured in the presence of an equimolar amount of the other, which had been stored exclusively in high-salt buffer and so retained full activity). Protein α was fully active after dialysis against salt-free buffer for periods of up to 24 h, under conditions in which the salt concentration was negligible after 4 h, and retained 50% of its original activity after 20 days' storage in the absence of salt. At no time was any change in its electrophoretic mobility observed. Protein β, on the other hand, was quite unstable in the absence of salt, and was thus clearly responsible for the instability of the complete enzyme observed in these conditions.

The inactivation of protein β in the absence of salt was studied by dialysing samples of that protein for 4, 10 and 24 h against salt-free buffer under the conditions described above. Enzyme activity was determined in the presence of an equimolar amount of active α protein and electrophoretic mobility was determined in micro-disc polyacrylamide gels (s. 2.2.12.). A progressive change in mobility, from a slower to a faster-moving form, was observed,

and the activity of the β protein appeared closely related to the amount of protein remaining in the slow-moving band. This slow-moving form, therefore, probably represents the active species. After 24 h, the β protein was irreversibly inactivated and the slow-moving band could no longer be detected in the gels.

The electrophoretic mobility of protein α was less than that of either form of protein β.

### 3.5.2. Requirements for optimum incorporation of nucleoside triphosphates into acid-insoluble material

The conditions for the standard assays of H. cutirubrum RNA polymerase activity were chosen to yield the greatest possible incorporation of ribonucleoside triphosphates into acid-insoluble material without regard to the nature of the polymerase reaction or to that of the product RNA. It will later be seen that in these conditions the enzymes faithfully transcribe their templates. The DNA-dependent enzyme will be shown to display specificity of initiation similar to that of the E. coli polymerase, but to produce a large number of short RNA chains. Detailed studies of the effect of changes in pH, monovalent cation concentration and $K^+/Na^+$ ratio, divalent cation concentration and $Mg^{2+}/Mn^{2+}$ ratio were carried out, but no improvement in the average

chain length could be obtained by such variations; the standard assay conditions are, therefore, those yielding optimum enzyme activity in every sense.

3.5.2.1. pH

The DNA-dependent enzyme exhibits a sharp pH optimum at 8.6-8.8, and is only 50% active at pH 8.4 or pH 9.2.

Glycine buffer of pH 9.5 was used in the standard assay. The weak buffering capacity of glycine as compared to Tris or to the Good's buffers resulted in a pH within the optimum range in the complete assay mixture. This situation was tolerated because the use of glycine buffer gave the most reliably low blank incorporation of the buffers tried (see s. 6.2.), and because at higher glycine concentrations the mild chelating effect of the amino acid interfered with the interaction between the enzyme proteins and $Mn^{2+}$ to the detriment of incorporation activity.

The stability of the DNA-dependent enzyme did not appear to vary with the pH of storage in the range 7.5-10.

3.5.2.2. Substrate concentration

The DNA-dependent enzyme had a sharp substrate optimum at 150 nmol/ml of each nucleoside triphosphate.

The effect of substrate concentration on the activity of the enzyme is shown in Fig. 3.9. The activity was determined by the standard assay with the indicated concentrations of nucleoside triphosphates (all four NTP being present at the concentrations shown).

In the standard assay, the concentration of ATP was reduced from 150 to 50 nmol/ml and the specific radioactivity trebled with respect to that used in the experiment of Fig. 3.9. Since in these conditions the activity was only slightly reduced, the sensitivity of the assay was greatly improved.

The substrate concentration was unaffected by changes in the salt concentration or template used in the assay.

3.5.2.3. Divalent cations

The DNA-dependent polymerase had an absolute requirement for both $Mg^{2+}$ and $Mn^{2+}$ in the assay medium. If either or both of the cations were omitted, no incorporation took place. In the presence of 100 µmol/ml of $Mg^{2+}$ there was a pronounced $Mn^{2+}$ optimum between 8 and 10 µmol/ml (Fig. 3.10.a); however, the activity increased linearly with increasing $Mg^{2+}$ concentration in the range tested with a $Mn^{2+}$ concentration of 10 µmol/ml was used (Fig. 3.10.b). Higher

Fig. 3.9. Substrate saturation curve for the DNA-dependent enzyme

All four nucleoside triphosphates were present at the concentrations shown. Each point represents the average of two determinations that differed by less than 10%.

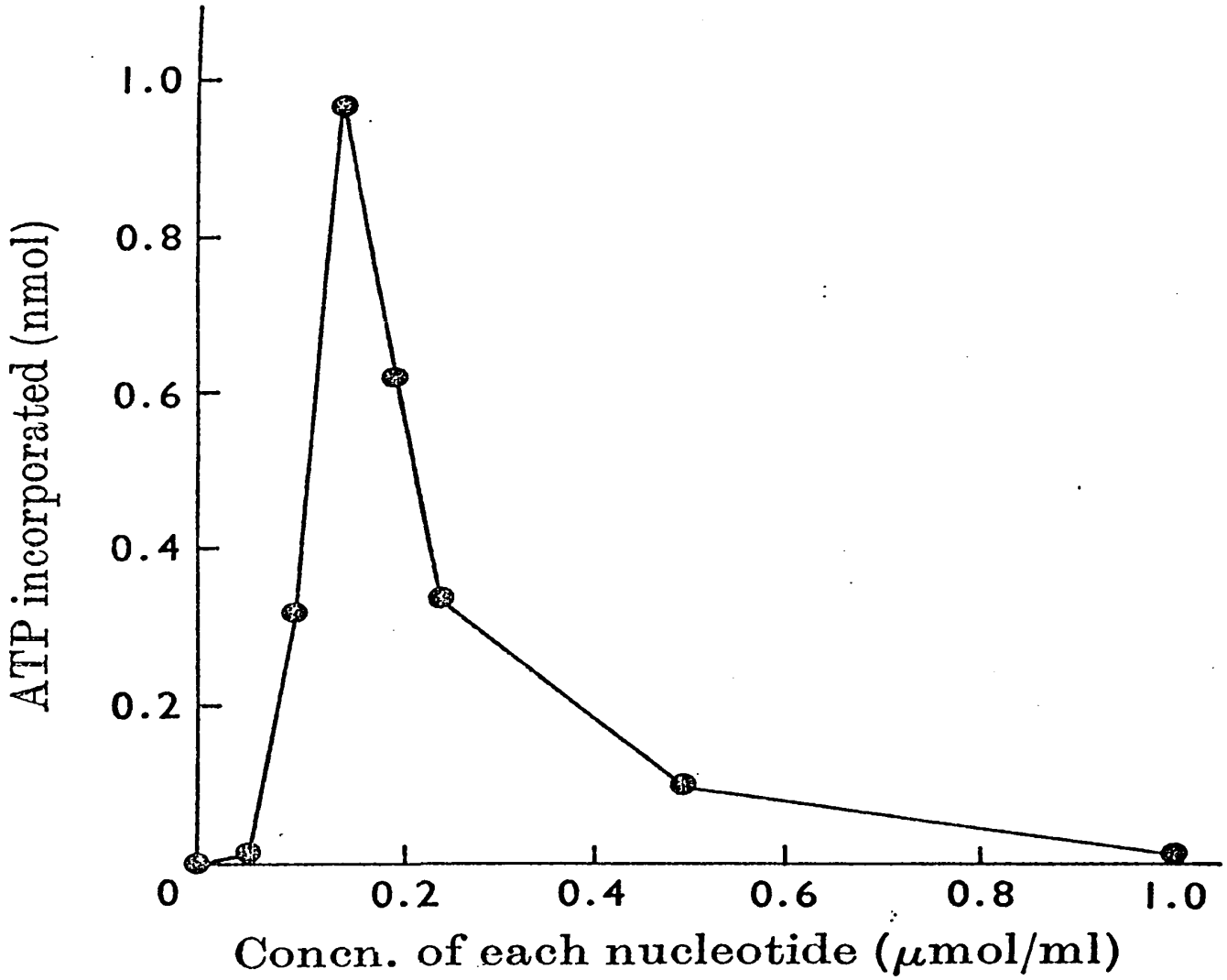
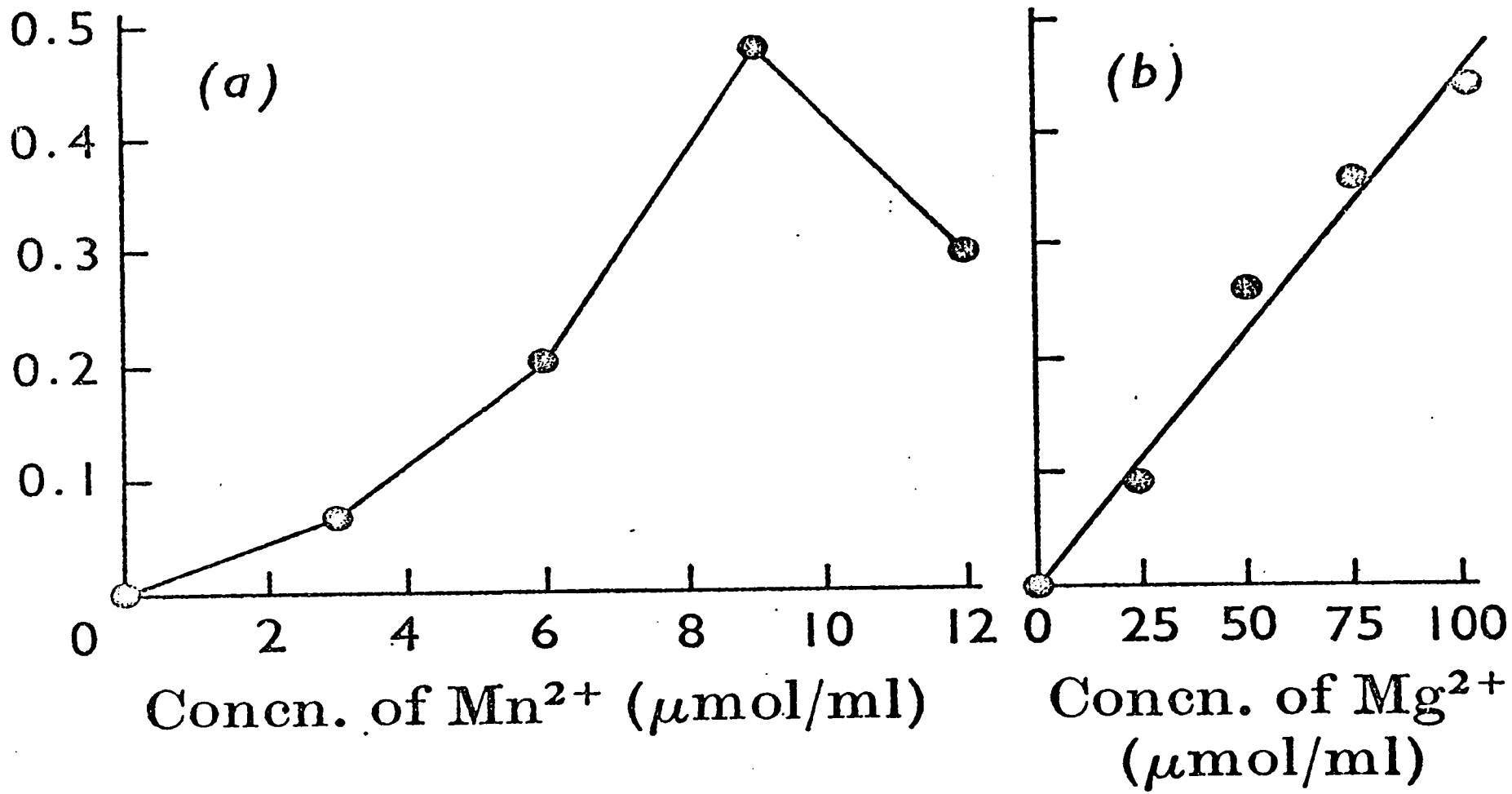

ATP incorporated (nmol)
1.0
0.8
0.6
0.4
0.2
0
0.2
0.4
0.6
0.8
1.0
Concn. of each nucleotide (μmol/ml)

Fig. 3.10. Effect of $Mg^{2+}$ and $Mn^{2+}$ on the activity of the DNA-dependent enzyme

a) $Mn^{2+}$ concentration was varied in the presence of $MgCl_2$ (100 µmol/ml)

b) $Mg^{2+}$ concentration was varied in the presence of $MnCl_2$ (10 µmol/ml)

In both cases the ordinate represents ATP incorporated (nmol). Each point is the average of two results that differed by less than 10%.


(a)
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
Concn. of Mn2+ (μmol/ml)
(b)
0
25
50
75
100
Concn. of Mg2+
(μmol/ml)

concentrations of $Mg^{2+}$ could not be tested in the presence of the optimum amount of $Mn^{2+}$, because they led to the formation of brown precipitates and to high, variable blank values.

The requirement for metal ions was independent both of the salt concentration and of the nature of the DNA template.

3.5.2.4. Polybasic compounds and sulphydryl reagents

Assay systems for RNA polymerase frequently include polyamines, which have in some cases been found to stimulate activity (Krakow, 1963; Fox & Weiss, 1964; Maitra & Hurwitz, 1967) and thiols such as 2-mercaptoethanol (Maitra & Hurwitz, 1965, 1967; Frederick, Maitra & Hurwitz, 1969), dithiothreitol (Lee-Huang & Warner, 1969; Küntzel & Schäfer, 1971) or mercaptoethylamine (Krakow & Ochoa, 1963; von der Helm & Krakow, 1972). Putrescine and spermidine were found to inhibit the H. cutirubrum DNA-dependent enzyme, while 2-mercaptoethanol affected neither the activity nor the stability of the enzyme in concentrations of up to 10 mM.

3.5.2.5. Template requirement and the effect of salt

The purified DNA-dependent RNA polymerase had an absolute requirement for native DNA as template. Single-

stranded DNA and high-molecular-weight RNA failed to stimulate incorporation. Sonication of the native DNA had no significant effect on its template activity. In the experiment of Table 3.8., these effects were demonstrated both in the presence of 2.1 M salt, using H. cutirubrum DNA, and in its absence, using calf thymus DNA. (The activities in the presence and absence of salt should not be compared, because the data were obtained on different occasions using different enzyme preparations.)

The effect of template concentration upon enzyme activity is shown in Fig. 3.11. Activity was determined in the absence of salt with calf thymus DNA as template. In the standard assay, with H. cutirubrum DNA as template, the plateau is reached at the same DNA concentration (300 µg/ml).

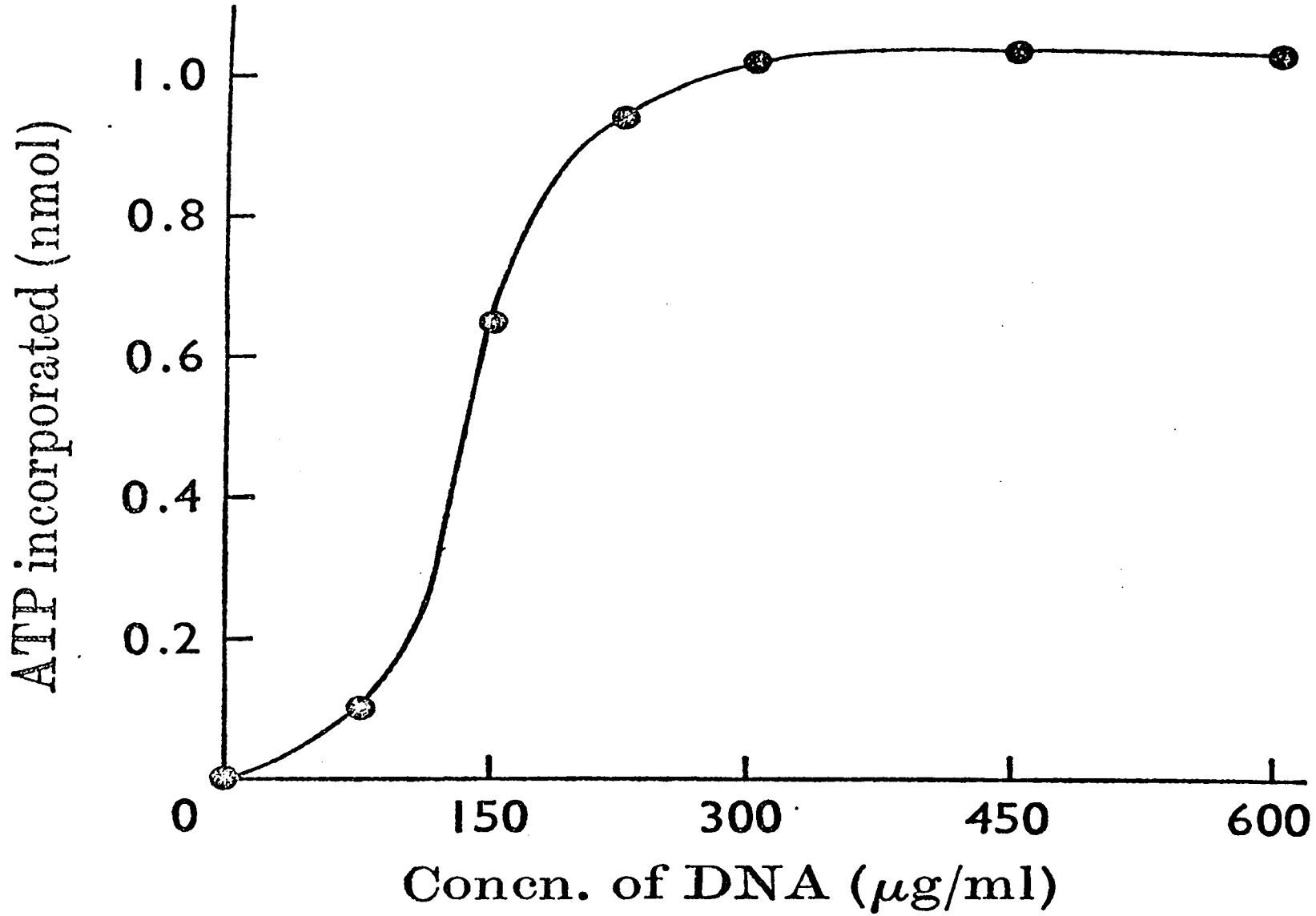
The RNA polymerases of Micrococcus lysodeikticus (luteus) (Fox, Robinson, Haselkorn & Weiss, 1964), E. coli (Gomatos, Krug & Tamm, 1964) and Azotobacter vinelandii (agilis) (Krakow & Ochoa, 1963) catalyse both DNA- and RNA-directed synthesis of RNA. The purified DNA-dependent RNA polymerase of H. cutirubrum displays no RNA-dependent activity, and it may be presumed that any in vivo function requiring RNA-directed RNA synthesis employs the RNA-dependent enzyme exclusively. Indeed, the existence of the latter strongly suggests that some such function may be of

Table 3.8. Template requirement of the DNA-dependent enzyme

Sonicated DNA was prepared by sonication for 30 sec of native calf thymus or H. cutirubrum DNA (2 mg/ml) with a Bronwill Biosonik III apparatus at 40% of maximum intensity. Portions of the native DNA solutions were heated at 100° for 10 min and then cooled in ice to obtain heat-denatured DNA.

| Template | ATP incorporated (nmol) | |
|---|---|---|
| | low salt (calf thymus DNA) | high salt (H. cutirubrum DNA) |
| None | 0 | 0 |
| Native DNA (300 µg/ml) | 1.02 | 1.56 |
| Sonicated DNA (300 µg/ml) | 1.08 | 1.52 |
| Heat-denatured DNA (300 µg/ml) | 0.04 | 0 |
| High-mol.-wt. RNA (750 µg/ml) | 0.01 | 0.02 |

Fig. 3.11. Effect of DNA concentration on the activity of the DNA-dependent enzyme


ATP incorporated (nmol)
1.0
0.8
0.6
0.4
0.2
0
150
300
450
600
Concn. of DNA (μg/ml)

importance in the life of the cell; the matter should certainly be investigated.

All three of the non-halophile RNA polymerases mentioned above will transcribe heat-denatured DNA to some extent (Fox & Weiss, 1964; Furth, Hurwitz & Goldmann, 1961a; Burma, Kröger, Ochoa, Warner & Weill, 1961), although only the E. coli enzyme appears to be equally able to accept single- or double-stranded DNA templates. (The enzyme from Saccharomyces cerevisiae, by contrast, preferentially transcribes denatured DNA; Frederick, Maitra & Hurwitz, 1969.) The H. cutirubrum polymerases have no activity with denatured DNA at either high or low salt concentration. By the argument used above in connection with the RNA-directed RNA synthesis, it might be suggested that the ability to use single-stranded DNA may be of little functional importance in vivo, but the negative finding is less cogent in this regard, particularly in view of the increasing evidence that RNA synthesis may be involved in DNA replication (Chang & Bollum, 1972; Brewin, 1972; Karkas, Stavrianopoulos & Chargaff, 1972; Clewell, Evenchik & Cranston, 1972; Wickner, Brutlag, Schekman & Kornberg, 1972; Lark, 1972). No search has been made for a 'denatured-DNA-dependent' RNA polymerase activity in H. cutirubrum.

It was surprising to find, during the early work on the H. cutirubrum DNA-dependent RNA polymerase, that the enzyme was inhibited by salt when calf thymus DNA was used as template. One would naturally have expected activity in the presence of salt, and possibly salt-dependence, of any enzyme from an extremely halophilic organism. A detailed study of the template specificity, and the effect thereon of the salt concentration, was therefore undertaken. The results appear in Table 3.9. and in Fig. 3.12. All of the DNA species tested were templates in 2.1 M salt and not at low ionic strength, except for those from calf thymus and salmon sperm, where the reverse is true; for poly d(A-T), which was a template both with and without salt; and for $T_4$ coliphage DNA, which was not a template at all. The result for poly d(A-T), together with those for Bacillus subtilis and salmon sperm DNA, both of which contain 44% G-C, shows clearly that the G-C content of the DNA does not determine its template activity. (The values for mole % G-C in Table 3.9. are from the following sources: H. cutirubrum and H. halobium DNA, major and minor components, Moore & McCarthy, 1969a; $T_4$ DNA, Millette, Trotter, Herrlich & Schweiger, 1970; all other DNA species, Davidson, 1969. Activity was determined in the standard assay using 30 µg of the indicated DNA.) The failure of $T_4$ DNA to serve as a template may be

Table 3.9. Template specificity of the DNA-dependent enzyme

| DNA | G + C (%) | | ATP incorporated (nmol) | |
|---|---|---|---|---|
| | major | minor | +salt | -salt |
| *H. cutirubrum* | 66 | 57 (11%) | 1.25 | 0.05 |
| *H. halobium* | 67 | 59 (30%) | 0.56 | 0.04 |
| *E. coli* | 51 | - | 1.19 | 0.10 |
| *B. subtilis* | 44 | - | 1.34 | 0.08 |
| $T_7$ coliphage | 41 | - | 0.92 | 0.06 |
| Calf thymus | 40 | - | 0.16 | 0.73 |
| Salmon sperm | 44 | - | 0.10 | 0.69 |
| poly d(A-T) | 0 | - | 0.89 | 0.56 |
| $T_4$ coliphage | 67 | - | 0 | 0 |

due to the fact that cytosine in that DNA is replaced by 100% glucosylated hydroxymethyl cytosine (Davidson, 1969), which could result in a failure of recognition by the H. cutirubrum polymerase.

The effect of salt concentration on the template activity of calf thymus and H. cutirubrum DNA is shown in Fig. 3.12. A salt concentration above 2 M is necessary for highest activity with H. cutirubrum DNA, and the activity falls off rapidly as the salt concentration is decreased below 2 M. With calf thymus DNA the enzyme is barely active at salt concentrations of 0.5 M and above. These effects are indifferent to whether the salt used is KCl, NaCl or a mixture of the two. (Total salt concentrations higher than 2.1 M cannot be used conveniently in the standard assay, requiring as they do the weighing of solid salt into each individual assay tube.)

3.5.2.6. Requirement for equimolar amounts of the α and β proteins

The effect of varying the amount of either protein in the presence of a constant amount of the other was studied using H. cutirubrum DNA as template in the standard assay. Chain initiation (s. 3.5.4.3.) was measured (s. 2.2.7.3.) simultaneously with total synthesis. As shown in Table 3.10,,

Fig. 3.12. Effect of salt concentration on the template activity of calf thymus and H. cutirubrum DNA in the standard assay for DNA-directed polymerization

Each point represents the average of two results that differed by less than 10%. ● and o, KCl; ■ and □, NaCl; ● and ■, H. cutirubrum DNA; o and □, calf thymus DNA.

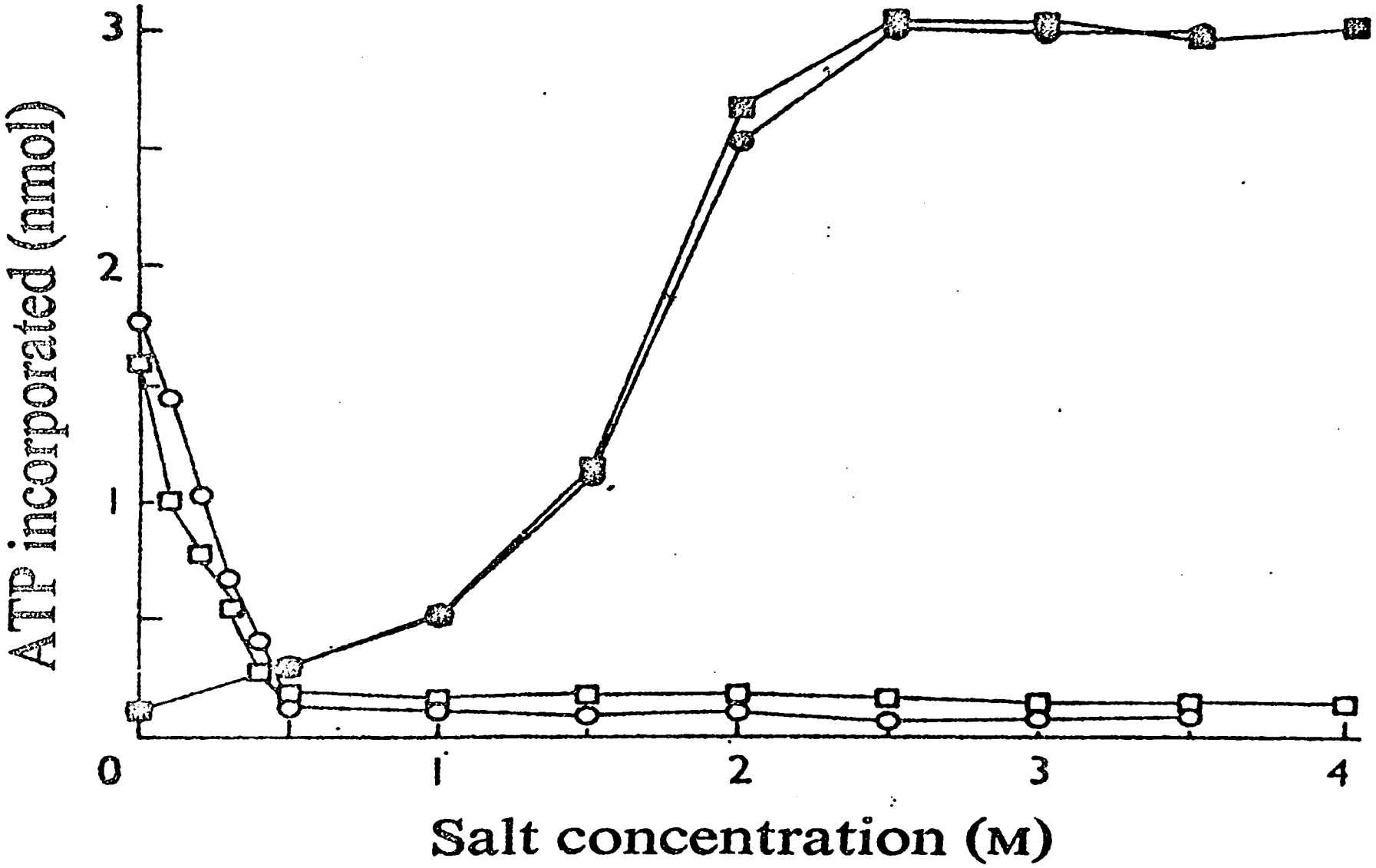

ATP incorporated (nmol)
3
2
1
0
1
2
3
4
Salt concentration (M)

Table 3.10. Effect of changes in relative concentration of proteins α and β on DNA-dependent RNA polymerase activity

Chain elongation ($^{14}$C-ATP incorporation) and initiation ($\gamma$-$^{32}$P-ATP and -GTP incorporation) were measured in the standard assay system using H. cutirubrum DNA as template in the presence of 1.5 M KCl, 0.6 M NaCl and the indicated amounts of proteins α and β.

| Protein (ng) | | Incorporation (nmol) | | Ratio |
|---|---|---|---|---|
| α | β | $^{14}$C-ATP | $\gamma$-$^{32}$P-ATP and -GTP | $^{14}$C/$^{32}$P |
| Expt. 1 | | | | |
| 40 | 0 | 0 | 0 | - |
| 40 | 16 | 0.351 | 0.020 | 17.5 |
| 40 | 32 | 0.672 | 0.037 | 18.1 |
| 40 | 40 | 0.722 | 0.040 | 18.0 |
| 40 | 48 | 0.730 | 0.041 | 17.8 |
| 40 | 80 | 0.711 | 0.040 | 17.8 |
| Expt. 2 | | | | |
| 0 | 40 | 0 | 0 | - |
| 16 | 40 | 0.370 | 0.020 | 18.5 |
| 32 | 40 | 0.784 | 0.040 | 18.4 |
| 40 | 40 | 0.900 | 0.050 | 18.0 |
| 48 | 40 | 0.910 | 0.050 | 18.2 |
| 80 | 40 | 0.920 | 0.050 | 18.4 |

a plateau of activity was attained when the weight (and hence molar) ratio of the two proteins was 1:1, while the ratio of chain elongation to chain initiation remained constant for all molar ratios tested, provided significant incorporation was detected.

### 3.5.3. Time course of polymerization

With the highly-purified enzyme (αβ), incorporation was linear for 2 h in the standard assay conditions, but after 2 h the rate of incorporation fell rapidly to zero.

### 3.5.4. Identification of the polymerase

DNA-dependent RNA polymerase (E.C. 2.7.7.6.) is identified according to the following criteria: (1) a DNA template is required for activity. (2) All of the common ribonucleoside triphosphates corresponding to the deoxyribonucleotides present in the template must be present before incorporation will take place. (3) The product of the reaction consists of new polyribonucleotide chains. (4) The RNA formed is complementary to the DNA template. (5) Under appropriate conditions, the enzyme recognizes and transcribes specific sites on the template DNA. The data presented in the next four subsections show conclusively that the DNA-dependent enzyme from H. cutirubrum meets these five criteria.

3.5.4.1. Requirement for all four ribonucleoside triphosphates

The enzyme required the presence of all four ribonucleoside triphosphates when a natural DNA was used as template. In the experiment of Table 3.11., the activity was determined using calf thymus DNA in the absence of salt. Omission of any nucleotide decreased the incorporation by at least 90%. In the presence of a single nucleoside triphosphate no incorporation took place, except to a limited extent with CTP.

3.5.4.2. Effect of nucleases on the DNA-dependent enzyme assay.

The addition of pancreatic deoxyribonuclease to a preincubated assay mixture stops the reaction after a short time (Table 3.12.), presumably because the nuclease destroys the template. This confirms the requirement for DNA already demonstrated in s. 3.5.2.5. The product of the reaction, as would be expected, is not DNase-sensitive but is destroyed by ribonuclease, so that the addition of RNase decreased the measured incorporation to a low value. Such a result indicates that the product of the reaction is a polyribonucleotide.

3.5.4.3. Initiation of new RNA chains

Table 3.11. Substrate requirements of the DNA-dependent enzyme

Enzymic activity of the purified enzyme was determined by the standard assay with the indicated nucleotides (150 nmol/ml in all cases, including the radioactive nucleotide) as substrates.

| Nucleotides | Radioactive nucleotide | $^{14}C$- or $^{3}H$-labelled nucleotide incorporated (nmol) |
|---|---|---|
| (1) ATP, CTP, UTP, GTP | $^{14}C$-ATP | 1.11 |
| (2) CTP, UTP, GTP | $^{3}H$-CTP | 0 |
| (3) ATP, UTP, GTP | $^{14}C$-ATP | 0.08 |
| (4) ATP, CTP, GTP | $^{3}H$-CTP | 0.10 |
| (5) ATP, CTP, UTP | $^{14}C$-ATP | 0.08 |
| (6) ATP, CTP | $^{14}C$-ATP | 0 |
| (7) ATP, UTP | $^{14}C$-ATP | 0.09 |
| (8) ATP, GTP | $^{14}C$-ATP | 0 |
| (9) CTP, UTP | $^{3}H$-UTP | 0.05 |
| (10) CTP, GTP | $^{3}H$-CTP | 0 |
| (11) UTP, GTP | $^{3}H$-UTP | 0.01 |
| (12) ATP | $^{14}C$-ATP | 0 |
| (13) CTP | $^{3}H$-CTP | 0.06 |
| (14) UTP | $^{3}H$-UTP | 0 |
| (15) GTP | $^{3}H$-GTP | 0 |
| (16) dATP, dCTP, dTTP, dGTP | $^{14}C$-dATP | 0 |
| (17) ADP, CDP, UDP, GDP | $^{14}C$-ADP | 0 |

Table 3.12. Effect of nucleases on polymerization of ribonucleoside triphosphates by the DNA-dependent enzyme

Standard assay mixtures containing the purified enzyme were incubated for 60 min at 37°C. Water, deoxyribonuclease or ribonuclease were then added as indicated, The 60 min control was prepared immediately by the usual method and the remaining three solutions were incubated for a further 30 min before being processed.

| Addition after 60 min | $^{14}$C-ATP incorporated (nmol) |
|---|---|
| (1) Water, 20 µl (60 min control) | 1.25 |
| (2) Water, 20 µl (90 min control) | 2.20 |
| (3) Deoxyribonuclease (0.5 mg/ml), 20 µl | 1.44 |
| (4) Ribonuclease (0.5 mg/ml), 20 µl | 0.18 |

Chain initiation was measured as described in s. 2.2.7.3. Both ATP and GTP are equally effective as chain initiators (Table 3.13.), whereas the two pyrimidine nucleotides are not incorporated at the beginning of the polymer chain. In this respect the H. cutirubrum DNA-dependent enzyme resembles the E. coli (Maitra & Hurwitz, 1965) and A. agilis (Krakow & Horsley, 1967) RNA polymerases. The elongation/initiation ratio was 37 with ATP and 39 with GTP, in agreement with the value of about 18 measured when both γ-labelled purine nucleotides were present in the assay mixture. Both elongation and chain initiation were linear for two hours in these conditions, so the elongation/initiation ratio was constant, in contrast with the tenfold increase over a period of one hour observed by Maitra & Hurwitz (1965) with the E. coli enzyme. It is possible that this difference is due to the low elongation/initiation ratio of the H. cutirubrum enzyme, since a ratio of 18 indicates an average chain length of 72 nucleotides when both γ-labelled purines are used. A sucrose-density-gradient centrifugation study of the product length was performed by isolation of the product from a standard assay (s. 2.2.14.) followed by centrifugation (s. 2.2.11.3.) in 5-20% sucrose gradients. E. coli 16 S and 23 S ribosomal RNA were included as markers. The gradient profile

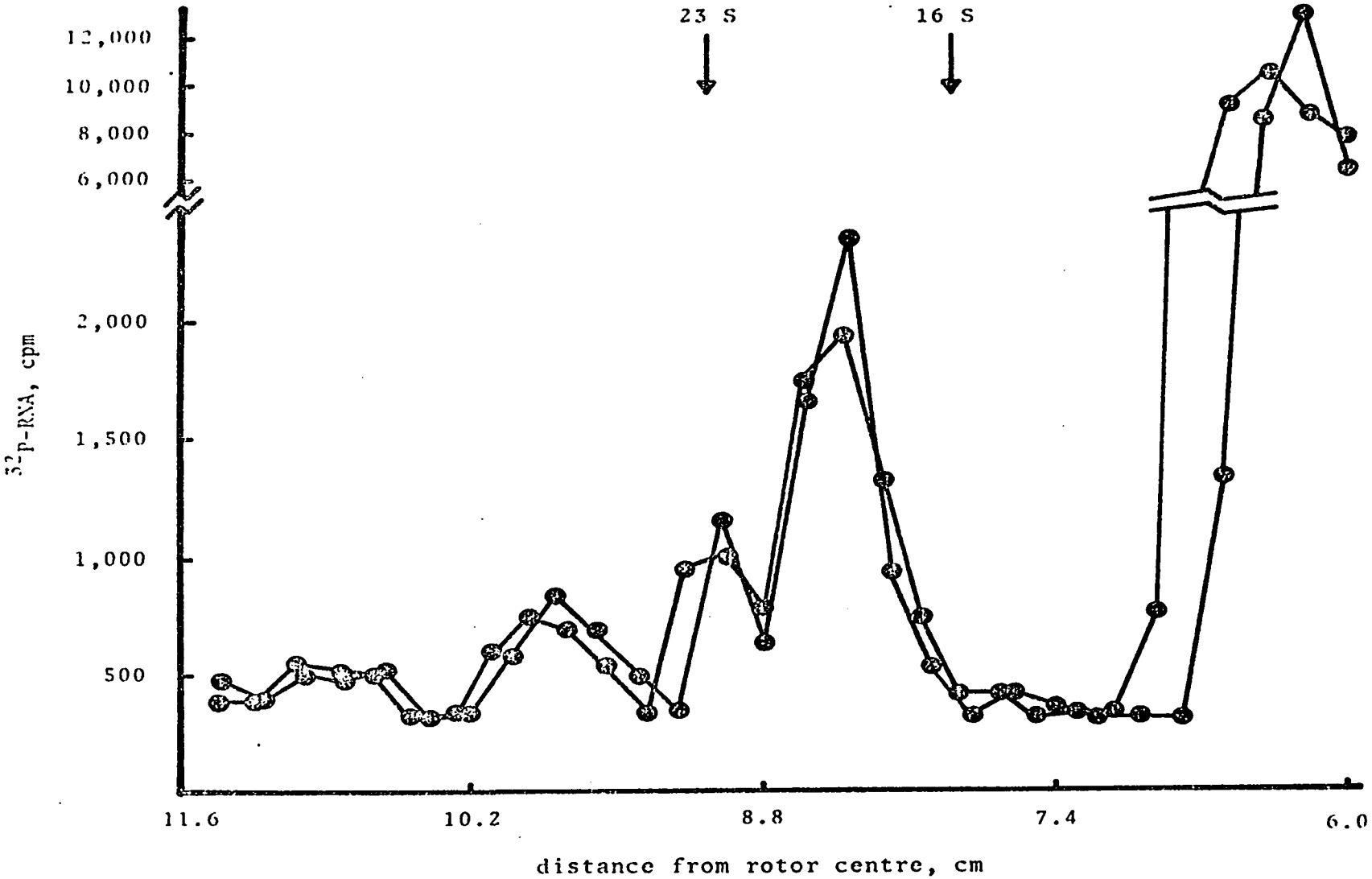
Table 3.13. Incorporation of $\gamma$-$^{32}$P-labelled nucleoside triphosphates into RNA

| $\gamma$-$^{32}$P-NTP | Chain elongation ($^{14}$C-ATP incorporation) (nmol) | Chain initiation ($^{32}$P-ATP incorporation) (nmol) | $^{14}C/^{32}P$ ratio |
|---|---|---|---|
| ATP | 1.29 | 0.039 | 37 |
| GTP | 1.26 | 0.032 | 39 |
| CTP | 1.47 | 0.005 | - |
| UTP | 1.46 | 0.005 | - |

(Fig. 3.13.) shows that the average chain length is composed from several small peaks of RNA of considerable size and one very large peak of very small RNA. The latter (as measured by the initiation assay of s. 2.2.7.3.) could not be reduced or eliminated by changing the pH of the reaction between 6 and 10, by altering the $Mg^{2+}$ concentration between 10 mM and 1 M or the $Mn^{2+}$ concentration between 1 mM and 10 mM, by altering the $Mg^{2+}/Mn^{2+}$ ratio between 1 and 1000, or by varying the KCl and NaCl concentrations and ratio over the entire solubility range. The *in vitro* synthesis has been demonstrated of a class of RNA molecules which are terminated very shortly after their initiation, so that the resulting product does not contribute significantly to the total weight of synthesized RNA in the *E. coli* system (Bremer, Konrad, Gaines & Stent, 1965). The polymerase molecules catalysing the synthesis of this class of RNA had been termed 'early quitters' (Bremer, Konrad & Bruner, 1966), and had been thought to lack factors which *in vivo* promote chain elongation and determine the correct termination of RNA molecules. It is possible that the *H. cutirubrum* DNA-dependent enzyme preparation contains a high proportion of such 'early quitter' polymerase molecules, so high that the small RNA they produce contributes appreciably to the total weight of synthesized RNA and to the average chain length. It is

Fig. 3.13. Sucrose-density-gradient centrifugation of RNA synthesized by the complete enzyme ($\alpha\beta$)

Duplicate experiments are shown. The arrows indicate the positions of *E. coli* 16 S and 23 S RNA in an identical gradient.


23 S
16 S
12,000
10,000
8,000
6,000
2,000
1,500
1,000
500
$^{32}$P-RNA, cpm
11.6
10.2
8.8
7.4
6.0
distance from rotor centre, cm

unlikely that extensive nicking of the DNA templates could be responsible for the production of the short RNA molecules, because the $T_7$ DNA gave an elongation/initiation ratio of 22, not much larger than that obtained with DNA from H. cutirubrum (see Table 3.21.), and yet had less than two nicks per molecule (W.C. Summers, personal communication).

### 3.5.4.4. Fidelity and asymmetry of transcription

The specificity of poly d(A-T) as a template for RNA polymerase has been described by Furth, Hurwitz & Goldmann (1961b). With poly d(A-T) as primer, only AMP and UMP were incorporated. GMP and CMP were not incorporated and their addition or omission had no appreciable effect, whereas AMP incorporation was dependent on the presence of UTP and UMP incorporation on the presence of ATP. The results in Table 3.14. show that the H. cutirubrum enzyme follows this pattern. When all four nucleotides were present, equal amounts of AMP and UMP were incorporated and very little incorporation of either CMP or GMP occurred. When only ATP and UTP were present, the reaction rate was the same as that observed in the presence of all four nucleotides, and equal amounts of the two nucleotides were incorporated. However, with CTP or GTP in place of UTP, the reaction rate was reduced to 11% of the maximum value.

Table 3.14. Transcription of poly d(A-T) by *H. cutirubrum* DNA-dependent RNA polymerase

The ratio of the incorporation of the indicated $^{3}$H-NTP to the incorporation of $^{14}$C-ATP in the standard assay (see the Experimental section) with poly d(A-T) as template was determined: (i) with all four nucleotides present (15 nmol of each) (Expt. A); (ii) with only the two radioactive nucleotides present (Expt. B). The values in parentheses in the last column refer to the expected ratio of incorporation of the two isotopes.

| Expt. | $^{3}$H-NTP | $^{14}$C-ATP incorporation (nmol) | $^{3}$H-NTP incorporation (nmol) | $^{3}$H/$^{14}$C ratio |
|---|---|---|---|---|
| A | CTP | 3.38 | 0.46 | 0.13 (0) |
| | GTP | 3.37 | 0.41 | 0.12 (0) |
| | UTP | 3.00 | 2.98 | 0.99 (1) |
| B | CTP | 0.34 | 0.05 | 0.14 (0) |
| | GTP | 0.34 | 0.06 | 0.18 (0) |
| | UTP | 2.99 | 2.86 | 0.96 (1) |

Since poly d(A-T) is composed of alternating units of deoxyadenylate and deoxythymidylate (Schachman, Adler, Radding, Lehman & Kornberg, 1960), its use allowed a simple nearest-neighbour analysis. Alkaline hydrolysis of the RNA product results in transfer of $^{32}P\text{-}PO_4$ from the 5' position of the original nucleotide to the 3' or 2' position of the adjacent nucleotide. The data in Table 3.15. show that nearly every $^{32}P$-adenylate was adjacent to a uridylate residue in the RNA synthesized by the H. cutirubrum DNA-dependent RNA polymerase in the standard assay conditions using poly d(A-T) as template, indicating that the RNA consisted of AMP and UMP units in alternating sequence, and thus that the template was accurately transcribed.

The H. cutirubrum DNA-dependent RNA polymerase recognizes the same initiation signals as the E. coli polymerase, and catalyses asymmetric transcription of a native, double-stranded DNA template. The DNA from coliphage $T_7$ provides a suitable system for proving this, as the two strands are separable and only one, the r strand, is transcribed by E. coli RNA polymerase (Summers & Szybalski, 1968). Further, the genome of $T_7$ coliphage is divided into early and late genes, in order of transcription following infection, and one of the former, gene 1, codes

Table 3.15. Nearest-neighbour analysis of the product of poly d(A-T)-directed RNA synthesis by the DNA-dependent enzyme

The analysis was carried out as described in s. 2.2.14. and 2.2.15.

| | |
|---|---|
| Total incorporation of 5'-$^{32}$P-AMP units | 0.639 nmol (100%) |
| Recovery of 2'(3')-$^{32}$P-AMP | 0.020 nmol (3.2%) |
| Recovery of 2'(3')-$^{32}$P-UMP | 0.609 nmol (95.1%) |
| Total recovery of 2'(3')-$^{32}$P-AMP and -UMP | 0.629 nmol (98.3%) |

for a $T_7$-specific RNA polymerase which transcribes the late genes (Chamberlin, McGrath & Waskell, 1970), the transcription of the early genes being carried out by the E. coli enzyme (Summers & Siegel, 1969). As the transcription of the late genes is blocked by addition of chloramphenicol to the medium containing the $T_7$-infected E. coli (Summers & Siegel, 1969), it is possible to obtain by this means early-gene-specific RNA for hybridization competition experiments. It was thus possible to demonstrate in this system that the H. cutirubrum polymerase catalyses asymmetric transcription of the early genes selectively.

The hybridizations were carried out as described in s. 2.2.16. In the experiment of Table 3.16., $^{14}$C-RNA synthesized by the H. cutirubrum enzyme in the presence of native $T_7$ DNA, together with $^{14}$C-RNA from untreated ($T_7^+$ RNA) and chloramphenicol-treated ($T_7$CM RNA), $T_7$-infected E. coli, was hybridized with the separated strands of $T_7$ DNA. (The labelled $T_7$CM RNA was of low specific activity, and the sensitivity of the experiment in which it was used was accordingly decreased with respect to that of the $T_7^+$ species.) The polymerase product (HC RNA) hybridized exclusively with the r strand of $T_7$ DNA, proving that the H. cutirubrum DNA-dependent RNA polymerase catalyses asymmetric transcription of double-stranded $T_7$ DNA.

Table 3.16. Hybridization efficiency of RNA species with $T_7$ DNA strands

| | $^{14}$C-RNA added | | |
|---|---|---|---|
| Source... | H. cutirubrum | $T_7^+$ | $T_7$CM |
| c.p.m. added | 1064 | 6120 | 4033 |
| DNA | $^{14}$C-RNA hybridized (c.p.m.) | | |
| None | 0 | 0 | 0 |
| l strand | 0 | 0.2 | 4.3 |
| r strand | 702.7 | 2761.3 | 262.0 |
| % of total radioactivity hybridized with r strand | 66.2 | 45.4 | 6.5 |

The results of hybridization competition experiments are shown in Table 3.17. The addition of unlabelled RNA to preliminary control experiments lacking DNA did not increase nonspecific binding of labelled RNA to the membrane filters. Unlabelled whole genome ($T_7^+$) RNA competed effectively for r strand in all cases, as expected. The pattern of competition displayed by the RNA from chloramphenicol-treated, infected bacteria, however, varied with the labelled RNA: $T_7^+$ RNA was relatively unaffected by the presence of $T_7$CM RNA, whereas the unlabelled $T_7$CM RNA displaced the labelled $T_7$CM RNA, as expected. The RNA synthesized by H. cutirubrum polymerase was also displaced very efficiently by unlabelled $T_7$CM RNA, indicating that this enzyme transcribed the early genes of $T_7$ coliphage preferentially, as is the case with E. coli RNA polymerase (v.s.).

The results reported in the previous sections (s. 3.5.4.1. to 3.5.4.4.) clearly prove that the DNA-dependent enzyme from H. cutirubrum is an RNA polymerase by the criteria stated in s. 3.5.4. (introduction). To summarize, the enzyme requires native DNA and all four ribonucleoside triphosphates in order to synthesize RNA complementary to the template, and the RNA consists of new, separate polyribonucleotide chains. In appropriate conditions, it recognizes specific initiation

Table 3.17. Hybridization competition

The hybridization mixtures contained $T_7$DNA r strand and the labelled and unlabelled RNA species indicated.

| Unlabelled RNA | $^{14}$C-RNA ... | Hybridization (%) | | |
|---|---|---|---|---|
| | | H. cutirubrum | $T_7^+$ | $T_7$CM |
| None | | 100 | 100 | 100 |
| $T_7^+$, 5 µg | | 85.3 | 85.2 | 80.6 |
| $T_7^+$, 10 µg | | 71.7 | 79.5 | 83.6 |
| $T_7^+$, 25 µg | | 50.1 | 53.2 | 71.8 |
| $T_7^+$, 50 µg | | 28.7 | 34.6 | 35.7 |
| $T_7$CM, 5 µg | | 57.6 | 107.0 | 68.1 |
| $T_7$CM, 10 µg | | 46.8 | 82.0 | 61.4 |
| $T_7$CM, 25 µg | | 27.9 | 93.9 | 35.6 |
| $T_7$CM, 50 µg | | 4.1 | 86.1 | 6.4 |

signals and transcribes particular portions of the template.

3.5.5. Functional association of the H. cutirubrum polymerase component proteins

It was shown in s. 3.5.2.3. that $Mg^{2+}$ and $Mn^{2+}$ (at optimum concentrations of 100 mM and 10 mM respectively) are required by H. cutirubrum DNA-dependent RNA polymerase. No reaction took place if either cation was omitted from the standard assay. The molecular-weight studies described in s. 3.3. had established that proteins α and β do not associate in high-salt buffer, and yet the two are required in equimolar amounts for optimum activity (s. 3.5.2.6.). Since some association must occur, between the two proteins (forming a complex) and DNA or between each protein and DNA separately, in the conditions of the standard assay, an investigation was made of the effects of $Mg^{2+}$ and $Mn^{2+}$ on the binding of the two proteins to each other or to the template. The proteins were chromatographed on polyacrylamide gel in high-salt buffer (s. 2.2.10.) in the presence and absence of the divalent cations and of native H. cutirubrum DNA. A column of Bio-Gel P-60 (1.5 x 27 cm) was packed as described (s. 2.2.10.2.) without reversing the flow of buffer through the bed. Samples (1 ml) were applied directly to the upper bed surface and rinsed onto the column

with 1 ml of the appropriate buffer, care being taken not to exceed a hydrostatic pressure of 15 cm. Eluant buffer changes were kept to a minimum, and experiments involving $Mn^{2+}$ were performed last. After each change of eluant, 30 ml of buffer was run through the column before the next sample was loaded. Eluant buffer and sample compositions are shown in Tables 3.18. to 3.20. Fractions (1 ml) were collected and their protein and nucleic acid contents determined from their $E_{260}$ and $E_{280}$ values. The average partition coefficient ($K_{av}$) was calculated for each peak as previously described (s. 2.2.10.5.).

Table 3.18. shows the effects of $Mg^{2+}$ and $Mn^{2+}$ on the migration of proteins α and β on the gel column in the absence of DNA. A $K_{av}$ of 0.62 on Bio-Gel P-60 in high-salt buffer represents a molecular weight of about 18,000. The two proteins did not associate either in high-salt buffer alone or in the presence of 100 mM $MgCl_2$, but were eluted in the presence of 10 mM $MnCl_2$ at a $K_{av}$ corresponding to molecular weight 36,000. As neither protein dimerizes in these conditions (v.i.), this result indicates the formation of a 1:1 complex between α and β.

The two polymerase proteins were then chromatographed singly in the presence of DNA (Table 3.19.). The DNA invariably appeared at the void volume, unaccompanied by

Table 3.18. Association of α and β in presence of $Mn^{2+}$

| Sample | Divalent Cations | Protein Peak | |
|---|---|---|---|
| | | $K_{av}$ | Recovery (±5%) |
| α | - | 0.62 | 100% |
| β | - | 0.62 | 100% |
| α + β | - | 0.61 | 90% |
| α + β | $Mg^{2+}$ | 0.62 | 90% |
| α + β | $Mn^{2+}$ | 0.53 | 100% |
| α + β | $Mn^{2+}$ + $Mg^{2+}$ | 0.53 | 100% |

Table 3.19. Absence of dimerization or binding to DNA by α alone or β alone

| Sample | Divalent Cations | Nucleic Acid Peak | | Protein Peak | |
|---|---|---|---|---|---|
| | | $K_{av}$ | Recovery (±5%) | $K_{av}$ | Recovery (±5%) |
| α + DNA | - | 0.00 | 95% | 0.62 | 90% |
| α + DNA | $Mg^{2+}$ | 0.00 | 100% | 0.62 | 90% |
| α + DNA | $Mn^{2+}$ | 0.00 | 100% | 0.62 | 100% |
| α + DNA | $Mg^{2+}$ + $Mn^{2+}$ | 0.00 | 95% | 0.62 | 100% |
| β + DNA | - | 0.00 | 100% | 0.62 | 100% |
| β + DNA | $Mg^{2+}$ | 0.00 | 90% | 0.62 | 100% |
| β + DNA | $Mn^{2+}$ | 0.00 | 95% | 0.62 | 90% |
| β + DNA | $Mg^{2+}$ + $Mn^{2+}$ | 0.00 | 95% | 0.62 | 90% |

protein, while the protein was eluted at the $K_{av}$ corresponding to its monomer molecular weight. The metal ions thus had no influence, either on self-association of the proteins or on binding of either alone to DNA.

Finally, the binding of the αβ complex to DNA was studied by chromatography of samples containing the nucleic acid and both subunits (Table 3.20.). In the absence of divalent cations, or in the presence of 100 mM $MgCl_2$ alone, the DNA was eluted at the void volume and the proteins emerged at $K_{av}$ 0.62, corresponding to the monomer molecular weight. When chromatographed with 10 mM $MnCl_2$ alone the DNA still appeared at the void volume without any protein, while the protein emerged at $K_{av}$ 0.53 (mol. wt. 36,000), indicating the formation of the αβ complex. Binding between DNA and protein occurred only if both divalent cations were included in the eluant buffer, when the entire sample appeared at the void volume. Since the protein does not form a large aggregate in the presence of the two cations (see Table 3.18.), a $Mg^{2+}$-mediated binding of the $Mn^{2+}$-dependent αβ complex to DNA is clearly demonstrated by this result.

The requirement for $Mn^{2+}$ to mediate subunit association markedly differentiates the H. cutirubrum DNA-dependent RNA

Table 3.20. Binding of the αβ complex to DNA

| Sample | Divalent Cations | Nucleic Acid Peak | | Protein Peak | |
|---|---|---|---|---|---|
| | | $K_{av}$ | Recovery (±5%) | $K_{av}$ | Recovery (±5%) |
| α + β + DNA | - | 0.00 | 100% | 0.62 | 90% |
| α + β + DNA | $Mg^{2+}$ | 0.00 | 95% | 0.62 | 100% |
| α + β + DNA | $Mn^{2+}$ | 0.00 | 100% | 0.53 | 100% |
| α + β + DNA | $Mn^{2+}$ + $Mg^{2+}$ | 0.00 | * | 0.00 | * |

* Approximately 95% recovery of UV-Absorbing material

polymerase from that of E. coli. The H. cutirubrum enzyme is active both at low and at high ionic strength, but the component proteins do not associate in the absence of $Mn^{2+}$, either at low or at high ionic strength. Although the E. coli polymerase subunits do dissociate at high ionic strength, the dissociation is accompanied by complete inactivation, and the ionic strength must be lowered and the subunits reassociated in order to restore activity (Lill & Hartmann, 1970; Heil & Zillig, 1970). Apparently the process of evolutionary adaptation to life at high salt concentrations introduced the requirement for metal-ion-mediated association both internally and externally, since $Mg^{2+}$, though required by the E. coli polymerase for full function, is unnecessary for DNA binding (Richardson, 1969) or subunit association.

### 3.5.6. Role of the α and β proteins in RNA chain initiation and elongation

The functionally distinguishable steps of transcription by E. coli RNA polymerase, viz., binding, initiation, polymerization, termination and release, have been described in the Introduction (s. 1.1.4.4.), together with the progress so far made in assigning to specific subunits involvement in particular steps. The simplicity of the

complete H. cutirubrum polymerase, together with its obvious similarity of function to the E. coli enzyme, gives reason to suppose that elucidation of the subunit functions may be less difficult in the H. cutirubrum system than in that of E. coli. Some progress has already been made in this direction, as will be apparent from the following subsections.

### 3.5.6.1. Effect of template on the chain length of the RNA synthesized

The effect of changing the template on the elongation/initiation ratio is negligible (Table 3.21.); although not every possible template was tried, those chosen represent the three classes of templates observed, defined by their transcription at high salt concentration only (H. cutirubrum DNA), low salt concentration only (calf thymus DNA) or both [poly d(A-T)]. Since the elongation/initiation ratio also remained constant for all molar ratios of proteins α and β (Table 3.10.), and the optimum α/β ratio was 1:1, the tentative conclusion was drawn that the two proteins are essential subunits and that the αβ complex is the active species, neither protein being an independent initiation factor similar to the σ factor of other bacterial polymerases (s. 1.1.1.).

Table 3.21. Effect of template on the elongation/initiation ratio

Chain elongation ($^{14}$C-ATP incorporation) and initiation ($\gamma$-$^{32}$P-ATP and -GTP incorporation) were measured in the standard assay with and without 1.5 M KCl, 0.6 M NaCl; 160 ng each of both protein α and protein β were used in all cases. Abbreviations: CT, calf thymus; d(AT), polydeoxy(adenylate-thymidylate) copolymer; HC, H. cutirubrum.

| | | Incorporation (nmol) | | |
|---|---|---|---|---|
| DNA | Salt | $^{14}$C-ATP | $\gamma$-$^{32}$P-ATP and -GTP | Ratio $^{14}C/^{32}P$ |
| HC | + | 3.03 | 0.164 | 18.8 |
| HC | - | 0.01 | 0.004 | - |
| CT | + | 0.20 | 0.013 | 15.4 |
| CT | - | 1.77 | 0.098 | 18.1 |
| d(AT) | + | 1.56 | 0.070 | 22.3 |
| d(AT) | - | 1.59 | 0.071 | 22.4 |

### 3.5.6.2. Inhibition of chain initiation by rifampicin

The antibiotics rifampicin and streptolydigin both inhibit RNA polymerase activity in prokaryotes (see discussion s. 1.1.4.9.); rifampicin blocks chain initiation but allows the elongation, termination and release of pre-initiated chains, while streptolydigin blocks only elongation at low concentrations, although it inhibits initiation as well when large amounts of the drug are present. The effects of these two antibiotics on the activity of the H. cutirubrum DNA-dependent RNA polymerase were therefore examined.

An experiment to test the effect of rifampicin on chain initiation is shown in Table 3.22. Complete standard assays, containing equimolar amounts of proteins α and β, were preincubated to permit the initiation of new RNA chains to occur. Rifampicin was then added to the test solution, together with $^{14}$C-ATP, γ-$^{32}$P-ATP and γ-$^{32}$P-GTP. The control received the radioactive substrates but no antibiotic. Although a significant rate of chain elongation was observed in the presence of rifampicin, very little γ-$^{32}$P was incorporated and the chain elongation/chain initiation ratio ($^{14}$C/$^{32}$P) was 40 times that measured in the absence of the antibiotic. The reduction of the rate of chain elongation

Table 3.22. Inhibition of chain initiation by rifampicin

| Experiment | $^{14}C$ incorporated (nmol) (elongation) | $^{32}P$ incorporated (nmol) (initiation) | Ratio $^{14}C/^{32}P$ |
|---|---|---|---|
| -Rifampicin | 0.68 | 0.057 | 12.0 |
| +Rifampicin | 0.19 | 0.00041 | 473 |

by rifampicin is probably due to the fact that the bulk of chains are terminated shortly after initiation (s. 3.5.4.3.), so that enzyme molecules which would re-initiate in the standard assay remain idle in the presence of rifampicin, ceasing to contribute to the elongation rate. Detectable RNA synthesis thus ceases within 20 min of the addition of the antibiotic to the incubation mixture.

Rifampicin added at the beginning of the incubation inhibits the reaction completely (Table 3.23.). The effect of the antibiotic is uninfluenced by the presence of a large excess of the α protein, but the inhibition is completely relieved by an excess of β. In the experiment described in the table, 8000 ng of protein α or β represents a 2.2-fold molar excess over rifampicin. The relief of inhibition by protein β implicates β in the initiation process and indicates that the rifampicin binding site must be on that subunit.

The possibility that β might be an initiation enzyme, capable of initiating RNA chains entirely in the absence of protein α, was tested by the following experiment (Table 3.24.): Standard assay mixtures containing either protein α or protein β were preincubated for 30 min before addition of rifampicin and the other subunit. No elongation could be measured in either case, indicating that

Table 3.23. Effect of excess α or β on rifampicin inhibition

| Protein (ng) | | | $^{14}C$-ATP incorporated (nmol) | $^{32}P$-NTP incorporated (nmol) | Ratio $^{14}C/^{32}P$ |
|---|---|---|---|---|---|
| α | β | Rifampicin | | | |
| 40 | 40 | - | 1.01 | 0.061 | 16.7 |
| 40 | 40 | + | 0 | 0 | — |
| 8000 | 40 | - | 1.00 | 0.060 | 16.7 |
| 8000 | 40 | + | 0 | 0 | — |
| 40 | 8000 | - | 1.00 | 0.057 | 17.8 |
| 40 | 8000 | + | 0.94 | 0.061 | 15.4 |

Table 3.24. Inability of α and β singly to initiate new RNA chains

| Polymerase component in standard assay | addition after 30 min incubation | $^{14}$C-ATP incorporation (nmol) |
|---|---|---|
| α | β + 1.16 nmol of rifampicin | 0 |
| β | α + 1.16 nmol of rifampicin | 0 |
| α + β | 1.16 nmol of rifampicin | 2.8 |

no chain initiation had taken place during the preincubation. (The control assay, in which the expected high rate of incorporation was obtained, contained both subunits from the start of incubation.) Neither subunit was capable of supplying by itself dinucleotides as primers for chain elongation; it would appear, therefore, that initiation is a function requiring the participation of both.

The effect of streptolydigin on the activity of the complete enzyme (αβ) was also examined. With 0.2 mM streptolydigin, double the concentration that inhibited the E. coli RNA polymerase completely (Cassani, Burgess, Goodman & Gold, 1971), there was no effect on the H. cutirubrum enzyme, as is the case with the $T_3$ and $T_7$ RNA polymerases (Dunn, Bautz & Bautz, 1971). (The latter, however, are also insensitive to rifampicin.)

### 3.5.6.3. Primer-dependent RNA synthesis catalysed by protein α alone

Although no activity could be measured in the standard assay conditions in the presence of either protein α or protein β alone (Tables 3.10. & 3.24.), the inclusion of one of the dinucleoside monophosphates ApA, ApU or GpU in a standard assay mixture containing only protein α resulted in RNA synthesis at a rate comparable to that obtained in the

standard assay when both component proteins were present (Table 3.25.). The dinucleotide apparently acted as a primer (chain initiator): no incorporation of γ-labelled purines was detected in the primed reaction, and the 3'-blocked dinucleotide ApA>p failed to stimulate RNA synthesis, while $^{3}$H-ApA was incorporated into the product formed when α alone catalysed the reaction, and not when both α and β were present (Table 3.26.). The primed reaction required a DNA template, but was unlike that catalysed by the complete enzyme (αβ) in being unaffected by rifampicin (Table 3.25.). When the product of ApA-primed, poly d(A-T)-directed synthesis catalysed by protein α alone was hydrolysed and the hydrolysate chromatographed on paper (s. 2.2.14. & 2.2.15.), the transfer of essentially all of the $^{32}$P label from AMP to UMP (Table 3.27.) indicated accurate copying of the template by the α protein (cf. s. 3.5.4.4.). No polymerization occurred in the presence of protein β alone, and the dinucleoside phosphates CpC, CpU and CpG were not primers, in agreement with the observation (Table 3.13.) that the initiating nucleotide in RNA chains synthesized by the complete enzyme must contain a purine base.

The effect of variation in the length of the oligonucleotide primer was also investigated (Table 3.25.), using

Table 3.25. Effect of oligonucleotides on RNA synthesis catalysed by protein α alone

Incorporation of $^{14}$C-ATP into an acid-insoluble form was measured by the standard assay with protein α (40 ng) as enzyme together with the indicated oligonucleotides (5 nmol).

| Oligonucleotide / Template ... | ATP incorporation (nmol) | |
|---|---|---|
| | H. cutirubrum DNA | Poly d(A-T) |
| None | 0 | 0 |
| ApA | 1.30 | 1.33 |
| $(Ap)_2A$ | 1.15 | 1.18 |
| $(Ap)_3A$ | 0.73 | 1.30 |
| $(Ap)_4A$ | 0.29 | 1.24 |
| $(Ap)_5A$ | 0.098 | 1.33 |
| ApU | 1.69 | — |
| GpU | 1.66 | — |
| CpC, CpU or CpG | 0 | — |
| ApA + rifampicin (1.16 nmol) | 1.28 | — |
| ApA>p | 0 | — |

Table 3.26. Incorporation of $^{3}$H-ApA into RNA in the presence of protein α alone

Standard assay mixtures containing 5 nmol of $^{3}$H-ApA in addition to α-$^{32}$P-ATP were incubated in the presence of the indicated enzyme proteins.

| Enzyme | α-$^{32}$P-ATP incorporated (nmol) | $^{3}$H-ApA incorporated (pmol) | $^{32}$P/$^{3}$H ratio |
|---|---|---|---|
| α + β (40 ng each) | 0.67 | 0 | - |
| α (40 ng) | 0.89 | 8.8 | 101 |

Table 3.27. Nearest-neighbour analysis of the product of ApA-primed poly (A-U) synthesis catalysed by protein α

The conditions of the experiment appear in the text.

| | (nmol) | |
|---|---|---|
| Total incorporation of 5'$^{32}$P-AMP units | 1.61 | (100%) |
| Recovery of 2'(3')-$^{32}$P-UMP | 1.42 | (88.1%) |
| Recovery of 2'(3')-$^{32}$P-AMP | 0.0013 | (0.1%) |
| Total recovery | 1.421 | (88.2%) |

the series $(Ap)_nA$, n = 1-5, with either the very regular alternating copolymer poly d(A-T) or the more natural H. cutirubrum DNA as template. The length of the primer did not alter its effectiveness significantly with poly d(A-T) as template, within the range studied; but with H. cutirubrum DNA the priming ability of the oligonucleotides decreased markedly with increasing primer length. Possibly the priming effect of oligoadenylates on poly d(A-T) involves some kind of slippage mechanism which renders the primer length unimportant, while on the natural template there might be a paucity of sites where slippage would occur, so that the $(Ap)_nA$ primer could only act at $(Tp)_{m\geq n}T$ sequences.

Template specificity in the primed reaction differed impressively from that displayed by the complete enzyme (Table 3.28.). All four templates examined were effective both in the presence and in the absence of salt (cf. Table 3.9., in which appear data on the salt-dependent template specificity of the reaction catalysed by the complete H. cutirubrum polymerase). It is particularly noteworthy that coliphage $T_4$ DNA, which was not accepted as a template by the complete enzyme, is fully active in the primed reaction at both low and high salt concentrations.

Table 3.28. Lack of template specificity in the primer-dependent reaction

Standard assays (see the Experimental section) were performed with the indicated templates (30 µg), in the presence and absence of 1.5M KCl, 0.6M NaCl. Protein α (40 ng) was used as enzyme and ApA (5 nmol) as primer.

| DNA | Salt | ATP incorporation (nmol) |
|---|---|---|
| None | + | 0.00 |
| None | - | 0.00 |
| H. cutirubrum | + | 1.27 |
| H. cutirubrum | - | 1.07 |
| $T_4$ coliphage | + | 1.25 |
| $T_4$ coliphage | - | 1.20 |
| Calf thymus | + | 1.22 |
| Calf thymus | - | 0.98 |
| Poly d(A-T) | + | 1.27 |
| Poly d(A-T) | - | 1.20 |

These results conclusively prove that the salt-dependent template specificity of the complete enzyme is a property of the β protein alone.

An interesting feature of the primed reaction involving only protein α is the ability of the primer concentration to influence the length of the RNA chains synthesized in the reaction. The ratio of chain elongation (AMP incorporation) to $^{3}$H-ApA chain initiation was followed in a manner analogous to the Travers & Burgess (1969) initiation assay used with the complete enzyme (s. 2.2.7.3.). Table 3.29. shows the effect of decreasing primer concentration on the elongation/initiation ratio so determined. Below a primer concentration of 1 μM, chain length rapidly increased as the amount of primer diminished. Confirmation of this effect was obtained by sucrose-gradient centrifugation studies on the product of ApA-primed RNA synthesis catalysed by α and directed by H. cutirubrum DNA. As shown in Fig. 3.14., reduction of the ApA concentration in the standard assay mixture from 50 μM to 50 nM results in the near-elimination of the lighter of two RNA peaks synthesized at the higher ApA concentration and in considerable enlargement of the heavier one. The diminution of chain length at increased primer concentration may result from competition between the dinucleoside phosphate and the growing RNA chain for the α protein

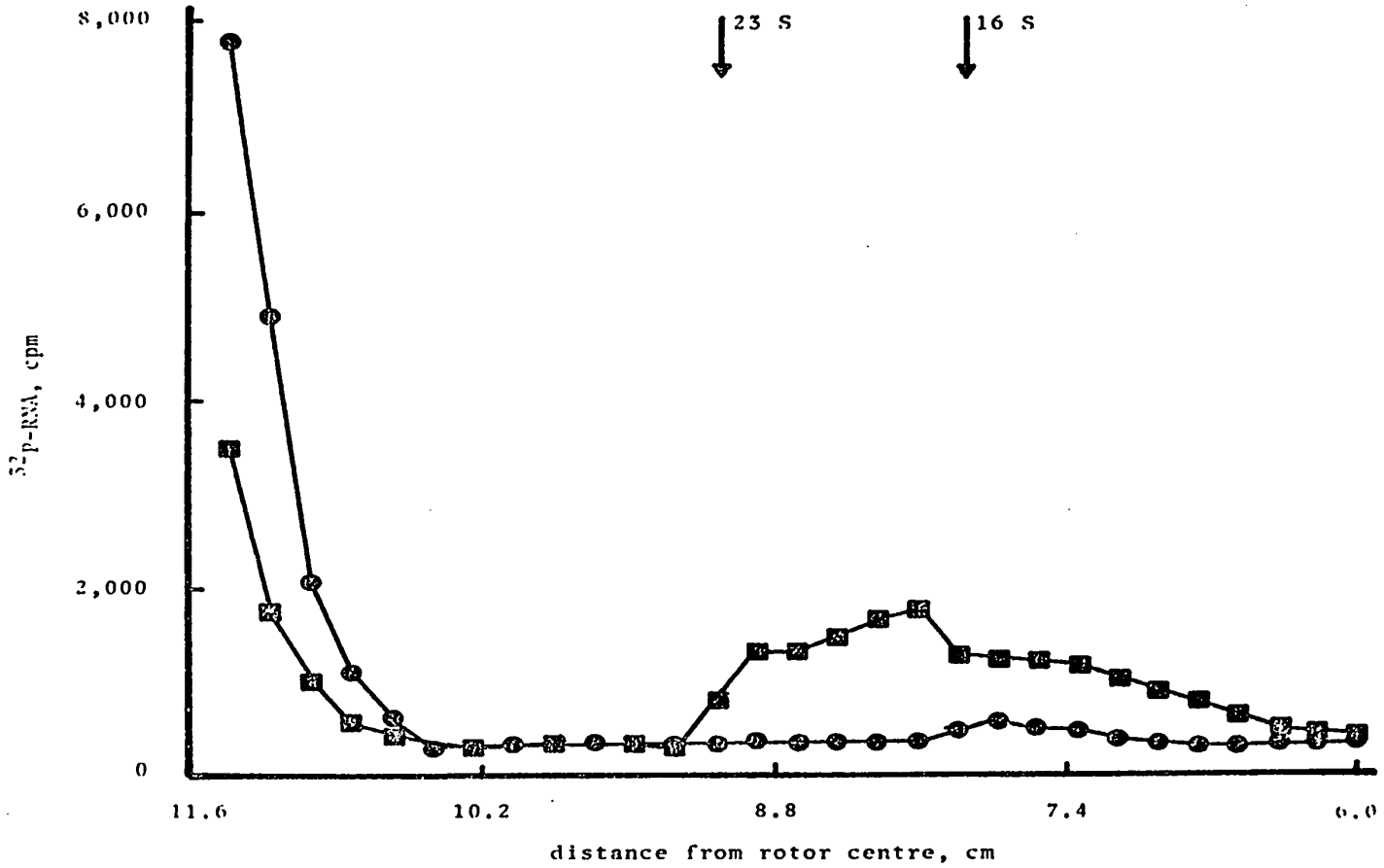
Table 3.29. Effect of primer concentration on product length in the primer-dependent reaction

Standard assay mixture containing α-$^{32}$P-ATP and the indicated concentration of $^{3}$H-ApA were incubated in the presence of protein α (40 ng) alone.

| $^{3}$H-ApA concentration μM | α-$^{32}$P-ATP incorporated (nmol) | $^{3}$H-ApA incorporated (pmol) | $^{32}$P/$^{3}$H ratio |
|---|---|---|---|
| 50 | 0.89 | 8.8 | 101 |
| 10 | 0.84 | 8.5 | 99 |
| 5 | 0.87 | 7.5 | 116 |
| 1 | 0.80 | 7.4 | 108 |
| 0.5 | 0.76 | 4.7 | 162 |
| 0.1 | 0.70 | 1.8 | 378 |
| 0.05 | 0.70 | 1.3 | 538 |
| 0 | 0 | - | - |

Fig. 3.14. Sucrose-density-gradient centrifugation of the product of ApA-primed RNA synthesis catalysed by protein α

●, RNA synthesized in the presence of 50 nM ApA; ■, RNA synthesized in the presence of 50 μM ApA. The arrows indicate the positions of E. coli 16 S and 23 S RNA in an identical gradient.


8,000
6,000
4,000
2,000
0
23 S
16 S
$^{32}$P-RNA, cpm
11.6
10.2
8.8
7.4
6.0
distance from rotor centre, cm

molecules. Since no similar effect is observed in the case of the complete enzyme (s. 3.5.2.6.), it seems unlikely that an in vivo, physiological function of the DNA-dependent polymerase is involved. It is nevertheless interesting to note, in view of the data on chain length obtained with the complete enzyme (s. 3.5.4.3.), that protein α is capable of catalysing the synthesis of RNA molecules with sedimentation coefficients considerably greater than 23 S.

The foregoing results allow several conclusions as to the roles of the two component proteins of H. cutirubrum DNA-dependent RNA polymerase in RNA synthesis. The mere existence of the primed reaction indicates that the α protein is capable of template binding, of substrate binding (except for the initiating nucleotide) and of phosphodiester bond formation, as well as of termination and release. This being admitted, the necessity of protein β for chain initiation in the absence of primer suggests that protein β contains the binding site for the first nucleotide, and the fact that rifampicin inhibition is relieved by excess β confirms the suggestion, since the known effect of rifampicin is to prevent the binding of the initiating nucleotide (Goldthwait, Anthony & Wu, 1970). This assignment of function is also consistent with the finding (Table 3.24.) that neither

protein is by itself capable of chain initiation. Participation of α in the binding is not, however, excluded. The data on template specificity, as already pointed out, indicate that protein β modifies template binding by α, and is probably involved in the recognition of specific initiation sites on the DNA. In this regard it would be of interest to repeat the hybridization experiments (s. 3.5.4.4.) with the primed reaction to see whether the absence of β would obviate the asymmetry of transcription and/or the preferential transcription of the early genes of $T_7$ DNA. Finally, the chain length results suggest that protein β has also a role in chain termination, since in its absence the RNA synthesized is generally longer than that synthesized by the complete enzyme. Further investigation of this point will be necessary to determine why most of the latter RNA molecules are terminated so soon after initiation.

## 3.6. The RNA-dependent RNA polymerase

The foregoing section concluded the presentation of data related specifically to the DNA-dependent RNA polymerase of H. cutirubrum. In the following sections, the characterization and identification of the RNA-dependent enzyme will be reported.

3.6.1. Stability

When stored at 4° in high-salt buffer, the RNA-dependent polymerase lost 27% of its activity in 21 days and 90% in four months. The absence of salt had a less severe effect than on the DNA-dependent enzyme: 38% of RNA-dependent activity remained after 21 days' storage at low ionic strength. (Although the DNA-dependent activity was gone after 24 h without salt; it should be noted that the α protein was still 50% active after 20 days when assayed in the presence of fresh β, so the effect of low-salt storage on the RNA-dependent enzyme resembles that on protein α of the DNA-dependent polymerase.)

3.6.2. Requirements for optimum incorporation of nucleoside triphosphates into acid-insoluble material

3.6.2.1. pH

The RNA-dependent polymerase had a sharp optimum at pH 9.5. At pH 8.6 about 35% of the activity remained, and at pH 10.0, the highest tested, the enzyme had still 85% of the maximum activity. Stability seemed to be unaffected by the pH of storage in the range 7.5-10.

3.6.2.2. Substrate concentration

Both polymerases displayed a sharp substrate optimum.

The nucleotide concentration yielding maximum RNA-dependent activity was 200 nmol/ml (Fig. 3.15.; cf. 150 for the DNA-dependent activity). In the standard assay, greater sensitivity was achieved by quadrupling the specific radioactivity of the labelled nucleotide and reducing its concentration to 50 nmol/ml, since the detrimental effect of the reduction was more than offset by the increase in dpm/nmol.

### 3.6.2.3. Divalent cations

The RNA-dependent enzyme had an absolute requirement for a divalent cation in the assay medium. Both $Mg^{2+}$ and $Mn^{2+}$ stimulated incorporation (Fig. 3.16.), but the latter was significantly more effective. Inclusion of both resulted in roughly additive stimulation effects, suggesting that the metal ions act at separate sites or at two independent steps in the reaction. The roles of divalent cations in RNA-directed RNA synthesis by the H. cutirubrum enzyme have yet to be investigated.

### 3.6.2.4. Template requirement

RNA was essential to the activity of the purified enzyme (Table 3.30.). Substitution of native or denatured calf thymus DNA resulted in negligible incorporation.

Fig. 3.15. Substrate saturation curve for the RNA-dependent enzyme

All four nucleoside triphosphates were present at the concentrations shown. Each point represents the average of two determinations that differed by less than 10%.

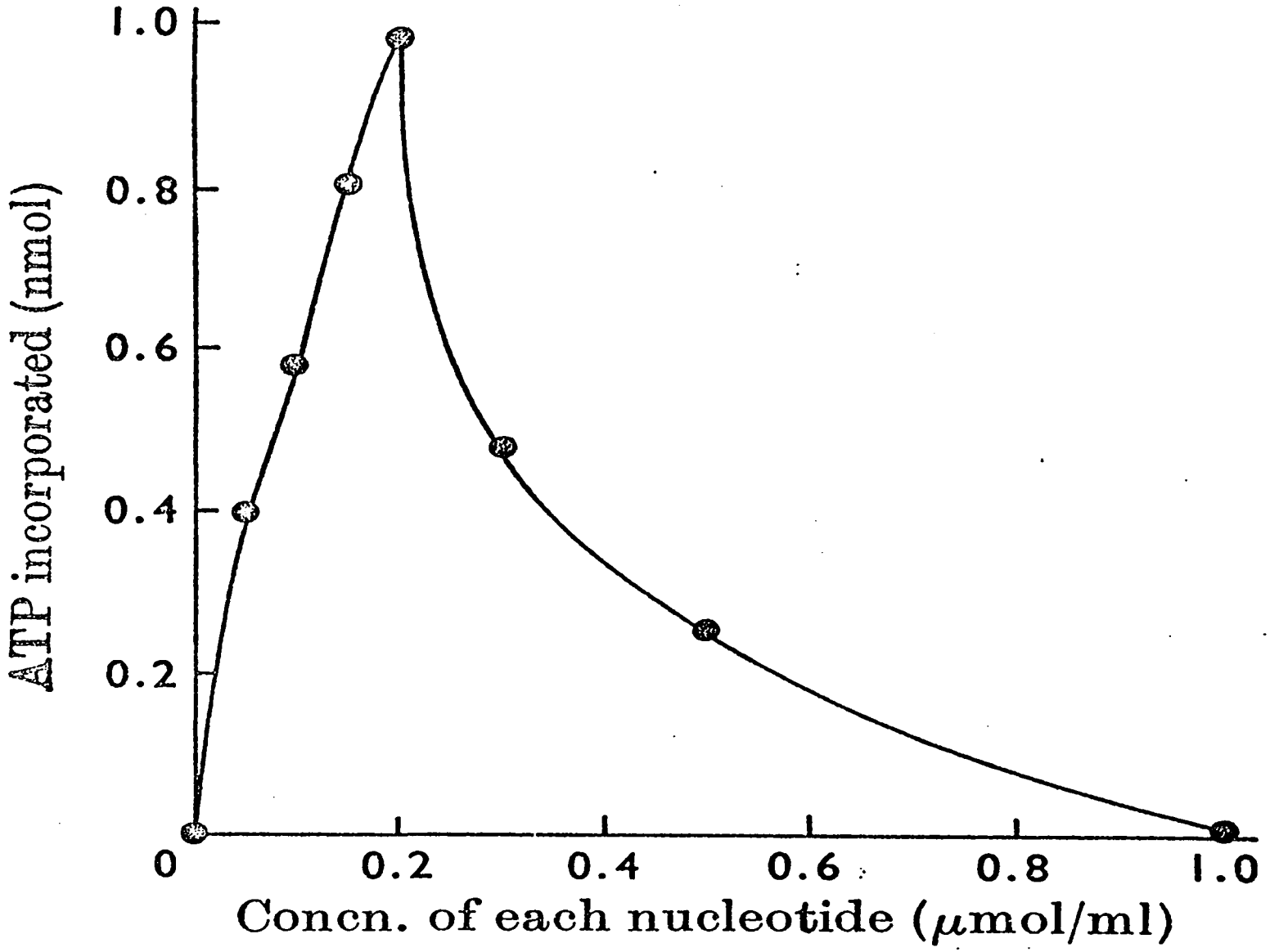

ATP incorporated (nmol)
1.0
0.8
0.6
0.4
0.2
0
0.2
0.4
0.6
0.8
1.0
Concn. of each nucleotide (μmol/ml)

Fig. 3.16. Effect of $Mg^{2+}$ and $Mn^{2+}$ on the activity of the RNA-dependent enzyme

■, $MgCl_2$; ●, $MnCl_2$; □, $MgCl_2$ + 10 µmol/ml $MnCl_2$; ○, $MnCl_2$ + 100 µmol/ml $MgCl_2$. Each point represents the average of two results differing by less than 10%.

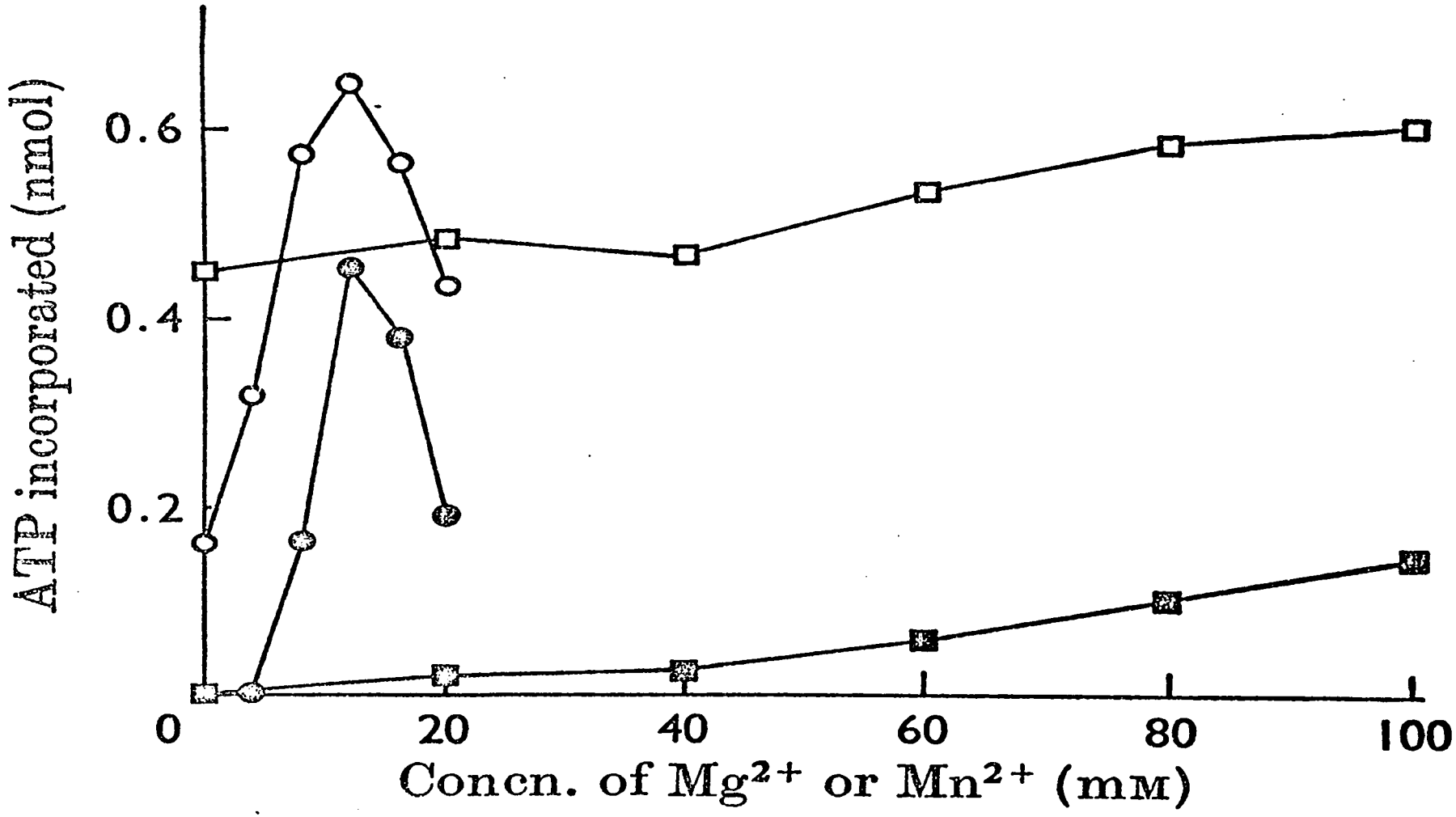

ATP incorporated (nmol)
0.6
0.4
0.2
0
20
40
60
80
100
Concn. of Mg2+ or Mn2+ (mM)

Table 3.30. Template requirement of the RNA-dependent enzyme

Enzyme activity was determined in the standard assay system with the indicated nucleic acid as template. tRNA was treated with venom phosphodiesterase by the method of Preiss, Dieckmann & Berg (1961). The denatured DNA was prepared by heating a solution of native calf thymus DNA (2 mg/ml) at 100°C for 10 min and then cooling it in ice.

| Template | ATP incorporated (nmol) |
|---|---|
| None | 0 |
| High-molecular-weight RNA (0.75 mg/ml) | 0.77 |
| tRNA (2.4 mg/ml) | 0.41 |
| Venom-diesterase-treated tRNA (2.4 mg/ml) | 0.30 |
| Native DNA (0.3 mg/ml) | 0.01 |
| Denatured DNA (0.3 mg/ml) | 0.04 |

High-molecular-weight RNA was much more effective as a template than tRNA; treatment of the latter with venom phosphodiesterase to remove the terminal CCA group (Preiss, Dieckmann & Berg, 1961) decreased its effectiveness yet further, indicating that the enzyme preparation contained no RNA-adenylate(cytidylate) pyrophosphorylase ('CCA-adding') activity. The results shown in Fig. 3.17. confirm that high-molecular-weight RNA was more effective than tRNA even when a large excess of the latter was used.

Qβ replicase and similar virus-induced RNA-dependent RNA polymerases are highly specific for their related viral genomes (see s. 4.3.2.1.). The results in Table 3.31. show that the RNA-dependent enzyme from H. cutirubrum has no such restricted template specificity. The nature of the high-molecular-weight RNA appears to be unimportant provided it is not double-stranded; poly U was a template for ATP incorporation and both ATP and UTP were incorporated in the presence of poly (A,U), but poly A·poly U had no template activity. Poly A was only half as effective a template as was poly U, probably because false-paired initiation must occur before poly A can be transcribed: RNA synthesized by the H. cutirubrum RNA-dependent enzyme must begin with a purine at the 5' end (s. 3.6.5.3.).

Fig. 3.17. Effect of template concentration on the activity of the RNA-dependent enzyme

Activity was determined in the standard assay with the indicated concentrations of high-molecular-weight RNA (▣) or tRNA (●). The ordinate represents ATP incorporated (nmol).

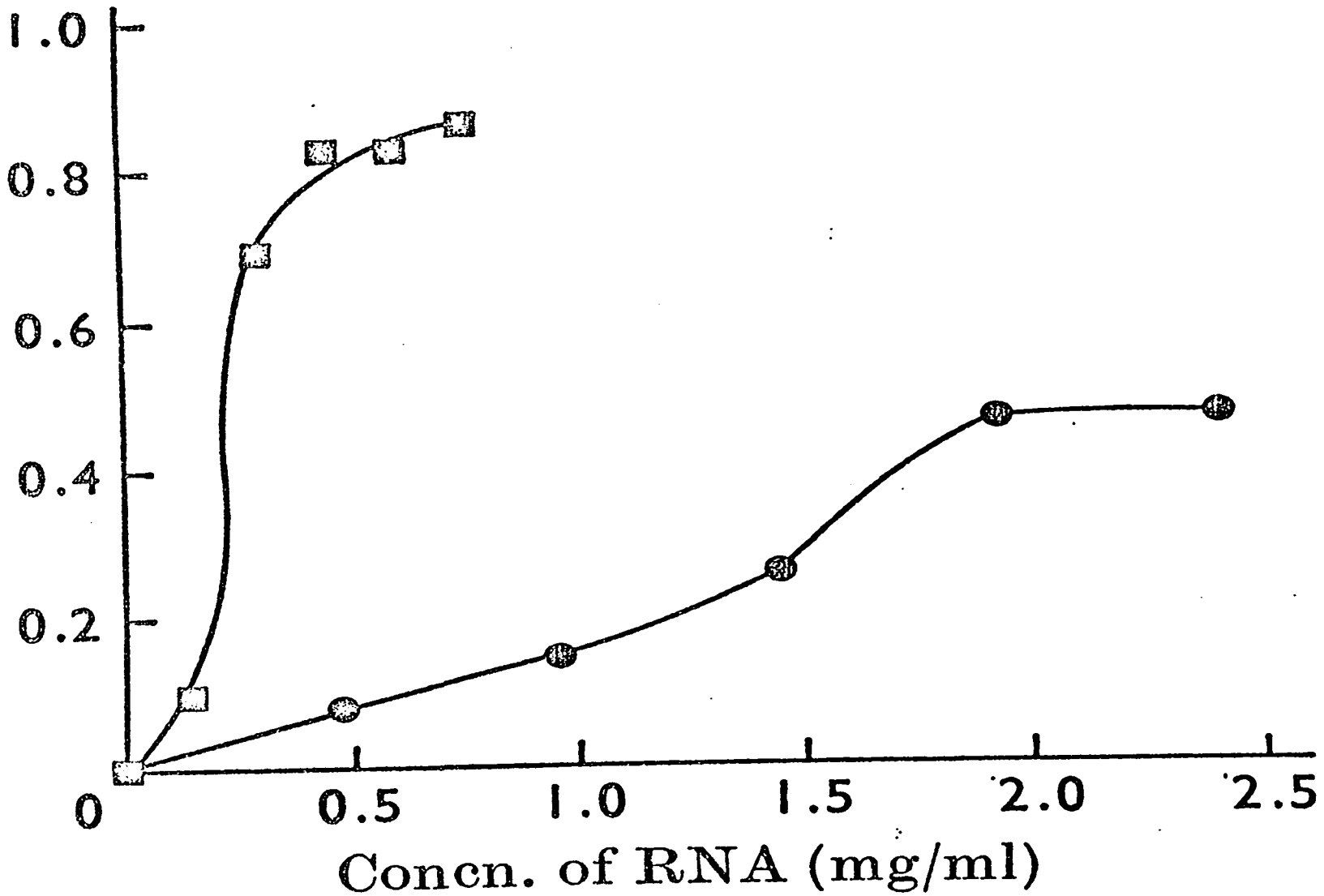

1.0
0.8
0.6
0.4
0.2
0
0.5
1.0
1.5
2.0
2.5
Concn. of RNA (mg/ml)

Table 3.31. Template specificity of the RNA-dependent enzyme

Enzyme activity was measured by the standard assay (see the Experimental section) with the indicated nucleic acid (75 μg) as template. All four nucleoside triphosphates were present in every case.

| Nucleic acid | Labelled nucleoside triphosphate | $^{14}C$- or $^{3}H$-Nucleoside triphosphate incorporated (nmol) |
|---|---|---|
| None | $^{14}C$-ATP | 0.00 |
| Yeast high-molecular-weight RNA | $^{14}C$-ATP | 0.97 |
| Wheat-germ high-molecular-weight RNA | $^{14}C$-ATP | 1.04 |
| Wheat-germ high-molecular-weight RNA + rifampicin (1.16 nmol) | $^{14}C$-ATP | 1.09 |
| *H. cutirubrum* RNA | $^{14}C$-ATP | 1.04 |
| Poly U | $^{14}C$-ATP | 1.06 |
| Poly(A,U) | $^{14}C$-ATP | 1.06 |
| Poly(A,U) | $^{3}H$-UTP | 1.03 |
| Poly A .poly U | $^{14}C$-ATP | 0.00 |
| Poly A .poly U | $^{3}H$-UTP | 0.00 |
| Poly d(A-T) | $^{14}C$-ATP | 0.00 |

3.6.2.5. Monovalent salt concentration

By comparison with the DNA-dependent enzyme, the RNA-dependent RNA polymerase of H. cutirubrum is relatively indifferent to the salt concentration in the reaction mixture. Fig. 3.18. shows that the activity of the enzyme increases slightly with increasing KCl or NaCl concentration up to about 3 M. Greater stimulation is achieved by addition of both salts together, an optimum being reached at 3 M KCl, 1 M NaCl. (As has been mentioned, routine assays cannot conveniently be performed at salt concentrations higher than 2.1 M, because of the necessity of weighing solid salt into the assay tubes.)

3.6.3. Time course of polymerization

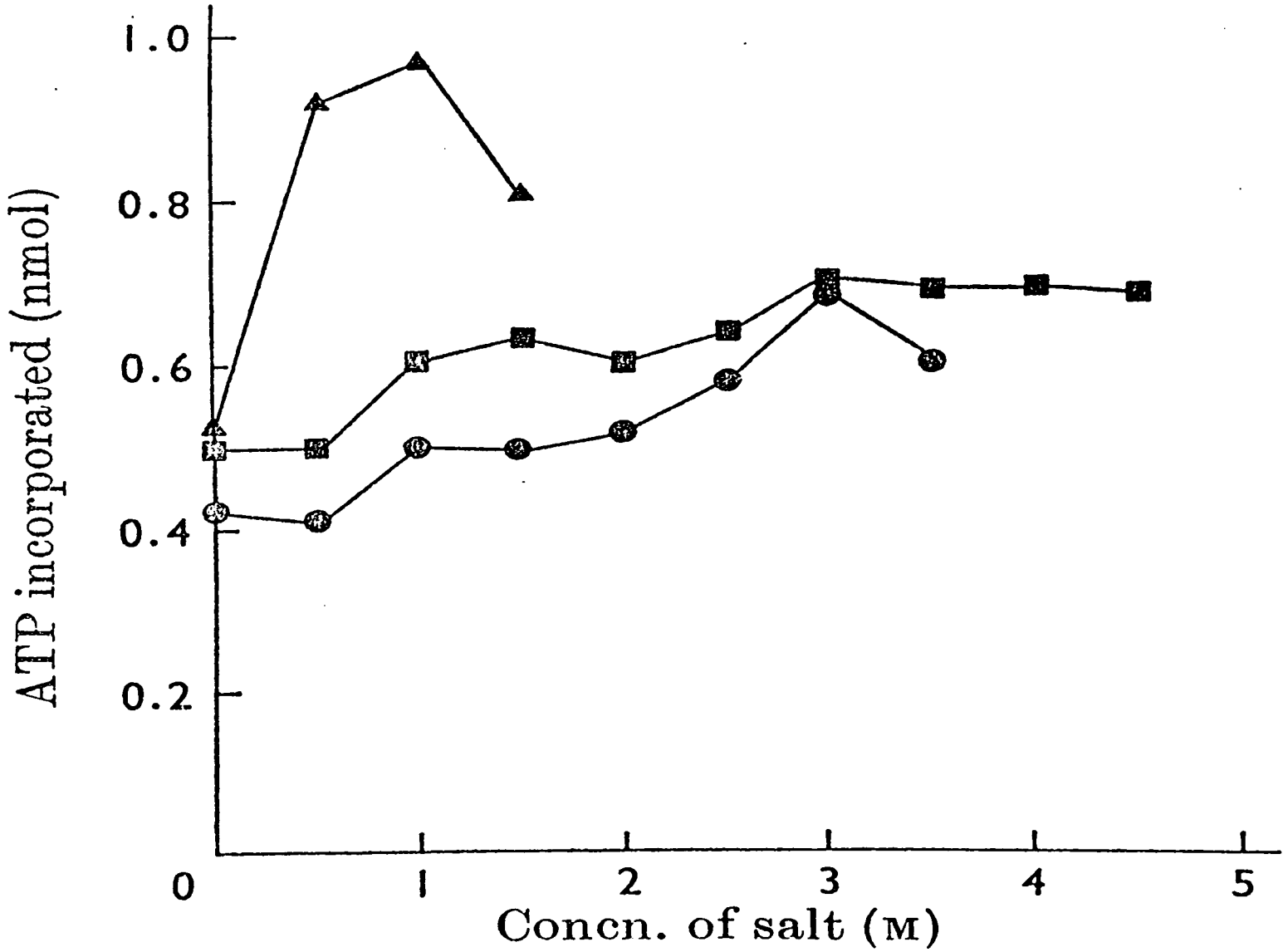
In the standard assay, incorporation was linear with time from 0 to 90 min.

3.6.4. Effect of antibiotics

Both rifampicin and streptolydigin were tested for their effect on polymerization by the RNA-dependent enzyme. Neither affected the rate of incorporation, though the concentration of rifampicin used completely inhibited the DNA-dependent enzyme (s. 3.5.6.2.) and the concentration of streptolydigin was double that required for complete

Fig. 3.18. Effect of KCl and NaCl on the activity of the RNA-dependent enzyme

●, KCl; ■, NaCl; ▲, NaCl in the presence of 3M KCl. Each point represents the average of two results differing by less than 10%.


ATP incorporated (nmol)
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
Concn. of salt (M)

inhibition of E. coli RNA polymerase (Cassani, Burgess, Goodman & Gold, 1971).

### 3.6.5. Identification of the polymerase

The criteria for identification of an RNA polymerase have already been stated (s. 3.5.4.). Briefly recapitulated, they are: the enzyme requires a nucleic acid template and the ribonucleoside triphosphates complementary to those present in the template in order to synthesize new polyribonucleotide chains complementary to the template, which in the case of an RNA-dependent enzyme may be partly or entirely copied. In the following subsections it will be shown that the RNA polymerase from H. cutirubrum satisfies these criteria.

#### 3.6.5.1. Requirement for all four ribonucleoside triphosphates

When the reaction was performed in the presence of wheat-germ high-molecular-weight RNA, all four ribonucleoside triphosphates were required (Table 3.32.), and omission of any one markedly reduced the incorporation of the others. In particular, no detectable incorporation occurred with a mixture of ATP and CTP as substrate, confirming the absence of 'CCA-adding' activity. Further, the rate of incorporation of any one labelled nucleotide in the presence of the other

Table 3.32. Substrate requirements of the RNA-dependent enzyme

Enzymic activity of the purified enzyme was determined by the standard assay with the indicated nucleotides (200 nmol/ml in all cases, including the radioactive nucleotide) as substrates.

| Nucleotides | Radioactive nucleotide | $^{14}C$- or $^{3}H$-labelled nucleotide incorporated (nmol) |
|---|---|---|
| (1) ATP, CTP, UTP, GTP | $^{14}C$-ATP | 0.89 |
| (2) CTP, UTP, GTP | $^{3}H$-CTP | 0.05 |
| (3) ATP, UTP, GTP | $^{14}C$-ATP | 0.04 |
| (4) ATP, CTP, GTP | $^{3}H$-CTP | 0 |
| (5) ATP, CTP, UTP | $^{14}C$-ATP | 0.10 |
| (6) ATP, CTP | $^{14}C$-ATP | 0 |
| (7) ATP, UTP | $^{14}C$-ATP | 0.03 |
| (8) ATP, GTP | $^{14}C$-ATP | 0.12 |
| (9) CTP, UTP | $^{3}H$-CTP | 0 |
| (10) CTP, GTP | $^{3}H$-CTP | 0 |
| (11) UTP, GTP | $^{3}H$-UTP | 0 |
| (12) ATP | $^{14}C$-ATP | 0.12 |
| (13) CTP | $^{3}H$-CTP | 0 |
| (14) UTP | $^{3}H$-UTP | 0.06 |
| (15) GTP | $^{3}H$-GTP | 0 |
| (16) dATP, dCTP, dTTP, dGTP | $^{14}C$-dATP | 0 |

three was independent of which nucleotide was actually labelled (within 5% or so). The failure of deoxynucleotides to act as substrates indicates that the enzyme has no reverse transcriptase (Baltimore, 1970; Temin & Mizutani, 1970) activity.

3.6.5.2. Effect of nucleases on the RNA-dependent enzyme reaction

In the experiment of Table 3.33., standard assay mixtures containing the purified enzyme were preincubated to allow product formation and then incubated in the presence of DNase or RNase (ethanol, which inhibits the enzyme, was added to one mixture for comparison). DNase had no effect on the reaction, whereas RNase destroyed both template and product and reduced incorporation to near zero. These results confirm that DNA is not required and that the product of the reaction is a polyribonucleotide.

3.6.5.3. Initiation of new RNA chains

Chain initiation was measured as described in s. 2.2.7.3. The results appear in Table 3.34. As with the DNA-dependent polymerases of E. coli (Maitra & Hurwitz, 1965), A. agilis (Krakow & Horsley, 1967) and H. cutirubrum (s. 3.5.4.3.), the reaction with the RNA-dependent enzyme

Table 3.33. Effect of nucleases on polymerization of ribonucleoside triphosphates by the RNA-dependent enzyme

Standard assay mixtures containing the purified enzyme were incubated for 60 min at 37°C. Water, ethanol, deoxyribonuclease or ribonuclease were then added as indicated. The 60 min control was treated immediately by the usual method and the remaining four solutions were incubated for a further 30 min before being processed.

| Addition after 60 min | $^{14}$C-ATP incorporated (nmol) |
|---|---|
| (1) Water, 20 µl (60 min control) | 0.93 |
| (2) Water, 20 µl (90 min control) | 1.42 |
| (3) Ethanol, 20 µl (reaction stopped) | 0.91 |
| (4) Deoxyribonuclease (0.5 mg/ml), 20 µl | 1.38 |
| (5) Ribonuclease (0.5 mg/ml), 20 µl | 0.20 |

Table 3.34. Incorporation of $\gamma$-$^{32}$P-labelled nucleoside triphosphates into RNA

| $\gamma$-$^{32}$P-Nucleoside triphosphate | Chain elongation ($^{14}$C-ATP incorporation, nmol) | Chain initiation ($^{32}$P-Nucleoside triphosphate incorporation, pmol) | $^{14}C/^{32}P$ ratio |
|---|---|---|---|
| ATP | 1.04 | 8.0 | 130 |
| CTP | 0.94 | <0.01 | - |
| GTP | 1.04 | 7.3 | 142 |
| UTP | 0.97 | <0.01 | - |

produces chains initiated with purine nucleotides only. The initiation process, however, is not rifampicin-sensitive in this case (s. 3.6.4.).

The chain initiation/elongation ratio shown in Table 3.34. corresponds to a product with an average chain length of 250-300 nucleotide units (only half the initiation was measured, since only one purine nucleotide at a time was γ-labelled). This corresponds to an average molecular weight of about 100,000. Fig. 3.19. shows the results of a detailed study by sucrose-density-gradient centrifugation of the size distribution of the products from two independent experiments in which the template was H. cutirubrum RNA isolated as described in s. 2.2.5. For comparison, the results of duplicate density gradient analyses of the sample of H. cutirubrum RNA are plotted on the same axes. The enzyme appears to copy all the molecular species present in the somewhat heterogeneous RNA sample. (No detailed study of the physical properties of the RNA of extreme halophiles has been reported, so it is uncertain whether the sample used was representative of undegraded H. cutirubrum RNA, although it was an effective template.)

3.6.5.4. Complementarity of product and template in the RNA-directed reaction

Table 3.35. shows the results of a nearest-neighbour

Fig. 3.19. Sucrose-density-gradient centrifugation of product RNA

•, H. cutirubrum RNA template, $E_{260}$; o, radioactive product, cpm. Duplicate gradients are shown in each case. The arrows indicate the positions of E. coli 16 S and 23 S RNA in an identical gradient.

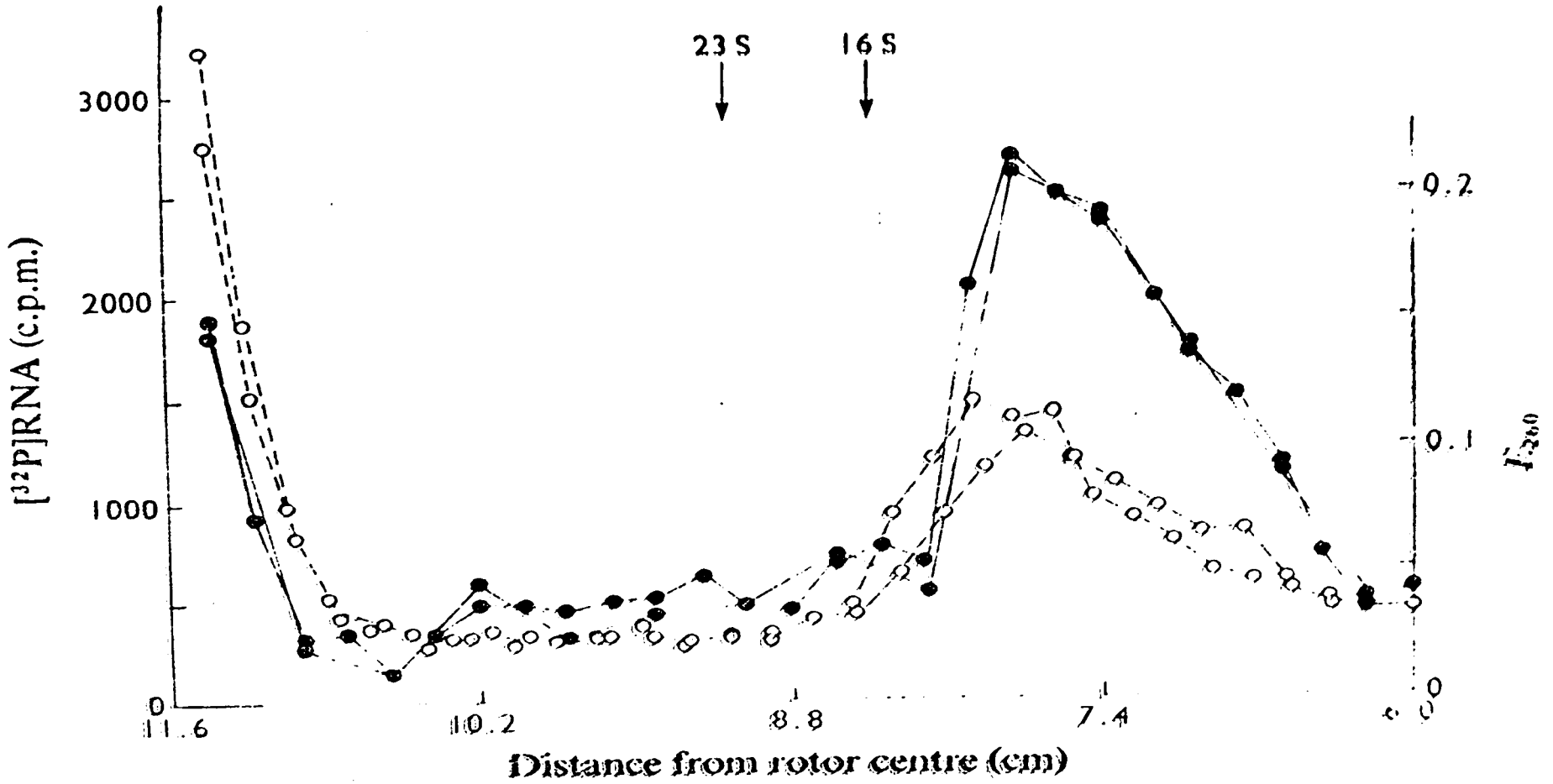

23 S
16 S
3000
2000
1000
0
[32P]RNA (c.p.m.)
0.2
0.1
0
$E_{260}$
11.6
10.2
8.8
7.4
Distance from rotor centre (cm)

Table 3.35. Nearest-neighbour analysis of the product of poly(A,U)-directed RNA synthesis by the RNA-dependent RNA polymerase

| | $^{32}$P-Nucleoside monophosphate incorporated (nmol) | % of total incorporation |
|---|---|---|
| Total incorporation of 5'-$^{32}$P-AMP units | 2.01 | |
| Recovery of 2'(3')-$^{32}$P-AMP | 0.836 | 41.6 |
| Recovery of 2'(3')-$^{32}$P-UMP | 0.886 | 44.1 |
| Total recovery of 2'(3')-$^{32}$P-AMP and 2'(3')-$^{32}$P-UMP | 1.722 | 85.7 |
| Ratio of U:A in product | 1.06 | |

analysis of the product formed with the random 1:1 copolymer poly (A,U) as template. After alkaline hydrolysis, the $^{32}P$ label from the $\alpha$-$^{32}P$-ATP was equally distributed between 2'(3')-UMP and -AMP, as would be expected in view of the random composition of the template.

In the experiment of Table 3.36., H. cutirubrum DNA-dependent RNA polymerase was used to synthesize the unlabelled alternating copolymer poly (A-U) with poly d(A-T) as template. It has been shown (s. 3.5.4.4.) that the product of this reaction is an accurate transcript of the poly d(A-T) and that the DNA-dependent enzyme copies one strand of a DNA template only (so a double-stranded product is unlikely). (Subsequent hybridization might occur but the limited amount of poly (A-U) synthesized would be highly diluted in the reaction mixture so annealing would be slow.) Rifampicin, which inhibits initiation by the DNA-dependent enzyme and thus causes synthesis to come to a halt after 20 min (s. 3.5.6.2.), was added to the reaction mixture after 2 h and the incubation was continued for a further 30 min. The RNA-dependent enzyme, for which poly d(A-T) is not a template (Table 3.30.), and which is unaffected by rifampicin (s. 3.6.4.), was then added, together with $\alpha$-$^{32}P$-ATP. After a further 2 h incubation the product

Table 3.36. Nearest-neighbour analysis of the product formed by the RNA-dependent RNA polymerase on a poly(A-U) template synthesized in situ

The analysis was performed as described in s. 2.2.14 and 2.2.15. A control without RNA-dependent RNA polymerase was also performed and the experimental values were corrected accordingly (incorporation in the control was <0.1% of the experiment values).

| | $^{32}$P-Nucleoside monophosphate incorporation (nmol) | % of total incorporation |
|---|---|---|
| Total incorporation of 5'-$^{32}$P-AMP | 1.930 | |
| Recovery of 2'(3')-$^{32}$P-AMP | 0.001 | 0.05 |
| Recovery of 2'(3')-$^{32}$P-UMP | 1.790 | 92.9 |
| Total recovery of 2'(3')-$^{32}$P-AMP and 2'(3')-$^{32}$P-UMP | 1.791 | 93.0 |

of the reaction was isolated and analysed. Virtually all the $^{32}P$ incorporated was recovered in 2'(3')-UMP, as would be expected if the poly (A-U) synthesized *in situ* had been accurately copied by the RNA-dependent enzyme.

In the experiment of Table 3.37., wheat-germ RNA was used as template and the incorporation of $^{3}H$-UTP, CTP and GTP was measured simultaneously with that of $^{14}C$-ATP. The three ratios thus obtained were used to calculate the base composition of the product RNA, which was compared with that of the template, which was subjected to base-composition analysis as described in s. 2.2.17. The comparison shows clearly that the product was complementary to the template. (The latter was present in great excess, so that significant recopying of the product would not be expected.)

The result in the foregoing subsections show that the RNA-dependent enzyme from *H. cutirubrum* requires high-molecular-weight RNA and all four ribonucleoside triphosphates and synthesizes new RNA chains complementary to the template. These properties identify it as an RNA polymerase.

## 3.7. Relationship between *H. cutirubrum* DNA-dependent and RNA-dependent RNA polymerases

The pure RNA-dependent enzyme could not substitute for

Table 3.37. Base composition of the product of wheat-germ RNA-directed RNA synthesis by the RNA-dependent enzyme

| $^{3}$H-Nucleoside triphosphate | Product | | | Base composition (%) | | |
|---|---|---|---|---|---|---|
| | $^{3}$H-Nucleoside triphosphate incorporated (nmol) | $^{14}$C-ATP incorporated (nmol) | Nucleoside triphosphate/ATP ratio | Base | Product | Template |
| | | | | A | 28.4 | 20.5 |
| UTP | 1.55 | 2.27 | 0.683 | U | 19.4 | 30.0 |
| GTP | 2.53 | 2.32 | 1.09 | G | 30.9 | 22.3 |
| CTP | 1.95 | 2.60 | 0.750 | C | 21.3 | 27.2 |

either protein of the DNA-dependent enzyme, nor had either α or β alone any RNA-directed activity. The presence of the RNA-dependent enzyme had no effect on the activity of the DNA-dependent polymerase in the standard assay for the latter, and neither protein α nor protein β affected RNA-dependent enzyme activity in its standard assay; but in the presence of the complete DNA-dependent enzyme the RNA-dependent activity was slightly reduced. This reduction was probably due to competition for substrates, since it could be obviated by addition of rifampicin, in the presence of which the DNA-dependent enzyme does not bind nucleotides (v.s.).

These results make it unlikely that there is any direct functional relationship between the two enzymes. Further studies will be necessary before anything definitive can be said about the function of the RNA-dependent polymerase *in vivo*, and the question of whether RNA replicative forms can be isolated will be of particular interest in this regard, since the product of RNA-directed synthesis is complementary to and not identical with the template. As a working hypothesis, in view of the broad template specificity of the enzyme, it might be supposed that it acts as a general amplifier of RNA synthesis by the DNA-dependent polymerase.

# 4. CONCLUSIONS

## 4.1. Comparison of the H. cutirubrum RNA polymerases

Inspection of the related sections of the results will quickly show that the RNA-dependent and DNA-dependent RNA polymerases are very similar in their properties. The similarity is particularly great if the former is compared with protein α in the primed reaction. Neither the RNA-directed nor the primed DNA-directed reaction displays the restricted template specificity characteristic of the DNA-directed reaction catalysed by the complete enzyme; both are relatively indifferent to the salt concentration, unlike the αβ reaction; as far as has been studied, the primed reaction retains the metal ion and substrate concentration requirements displayed by the αβ reaction, which are similar to those of the RNA-directed synthesis. Indeed, the similarity of the amino acid compositions suggests that the RNA-dependent polymerase may be a modified α-like protein which retained or acquired the ability to initiate new RNA chains during its evolution. The slightly lower molecular weight of the RNA-dependent enzyme, together with its much lower serine content, would in that case suggest the loss of a serine-rich peptide segment: it

will be interesting to see whether such a segment exists in protein α and whether the sequences of the two are otherwise similar. If so, the identification of the structural features determining the specificity of the one for double-stranded DNA and of the other for single-stranded RNA, and subsequent characterization of the structure-function relationship involved in template binding, might be gratifyingly easy.

## 4.2. The DNA-dependent enzyme as transcriptase

In view of the functional similarity between the H. cutirubrum and E. coli DNA-dependent RNA polymerases, there is little doubt that the former is the genetic transcriptase of H. cutirubrum. It is therefore of interest to compare it with similar transcriptases from other sources.

### 4.2.1. Comparison with other prokaryotic RNA polymerases

The RNA polymerases of E. coli, M. lysodeikticus, A. vinelandii and Pseudomonas putida have all been studied in some detail (see s. 1.). All are similar in size and subunit composition, and display only minor differences in function (for example, all accept denatured DNA and RNA templates, but the efficacies of these templates vary from enzyme to enzyme). To this family must now be added the

H. cutirubrum DNA-dependent polymerase, which is less than a tenth the size of its brethren, yet recognizes the same initiation signals and transcribes its templates with equal accuracy. There are, nevertheless, significant differences other than that of size.

The H. cutirubrum enzyme is quite unable to transcribe denatured DNA or to replicate RNA. It seems likely that both of these functions, which were once thought to be incidental because the rate of synthesis by the polymerases was slower than with native DNA, are of importance in the life of the cell (see s. 3.5.2.5.). Their presence in the big bacterial polymerases may be part of the reason for the size of the latter, but not a large part, since the N. crassa mitochondrial enzyme accepts denatured DNA with 82% of maximum activity and yet has a molecular weight of only 64,000 (Küntzel & Schäfer, 1971).

Up to now the following assignments of function to E. coli subunits have been suggested: the β' subunit is involved in binding to DNA, the β subunit in rifampicin binding and initiation and the σ factor in determining the specificity of initiation. (For the arguments leading to these conclusions see s. 1.1.4.9.) The function of the two α subunits is unclear and it is unknown whether chain elongation is a function of any one or of several subunits.

The α protein of H. cutirubrum is capable of DNA binding, chain elongation, chain termination and product release, since more RNA chains are synthesized in 60 min than there are α molecules in the reaction mixture (about 10 chains per molecule in the presence of 5 nmol ApA). The β protein contains the rifampicin binding site and is involved in chain initiation and in the determination of initiation specificity. Clearly, the functional organization of the two polymerases is not the same, as indeed one might expect from the dramatic difference in size.

Subunit association in the big polymerases is automatic, requiring denaturing conditions for dissociation. By contrast, the H. cutirubrum enzyme requires $Mn^{2+}$ to keep the protein components together. There are several polymerases (v.i.) with divalent cation requirements similar to those of the DNA-dependent polymerase of H. cutirubrum, including the RNA-dependent enzyme, but the involvement of the cations in protein association appears to be unique.

In view of these basic differences, care will certainly be necessary in relating information gained in studies of the H. cutirubrum enzyme to the functioning of the big polymerases; though the same functions are carried out, the mechanisms by which the enzymes work clearly need not be identical. To this extent the broad aim of studying

the template-enzyme-substrate interactions in limiting conditions of ionic strength, with which this work was first planned, promises to be justified: elucidation and comparison of mechanistic differences in similar processes (including the subunit association as well as the various steps of the reaction) should enhance understanding of the processes themselves.

The existence of the primed reaction in the H. cutirubrum system may point the way to a new means of investigating the roles of E. coli and related polymerase subunits. It has been told how limited has been the success of attempts to obtain partial function in separate subunits and subunit groups (s. 1.1.4.9.). The inclusion of dinucleotide primers had not been tested, because there had been no evidence of stimulatory effect on DNA-directed synthesis by the complete enzyme $(\alpha_2\beta\beta')\sigma$, although stimulation of the RNA-directed activity had been reported (Niyogi & Stevens, 1965b). Since the completion of this work, however, a more detailed investigation by Niyogi (1972) has been published, in which it was shown that small oligonucleotides could substitute for σ factor in transcription of synthetic templates by E. coli RNA polymerase. Synthesis of poly dA and poly dT were both stimulated by σ, and to the same extent in the absence of σ by $(Up)_3U$ and $(pA)_5$ respectively. In the

poly U-directed reaction, inclusion of $(Ap)_3A$ resulted in decreased incorporation of $\gamma$-$^{32}$P-ATP both in the absence and in the presence of $\sigma$, indicating that primed synthesis was superseding normal chain initiation. Two significant differences between the E. coli and H. cutirubrum primer-dependent reactions are immediately obvious: first, in the E. coli system, both purine and pyrimidine oligonucleotides were primers, whereas only purine-terminated oligonucleotides stimulated synthesis by H. cutirubrum $\alpha$ protein, and second, the normal initiation mechanism of the complete ($\alpha\beta$) H. cutirubrum enzyme supersedes primed synthesis, whereas the reverse occurs with E. coli polymerase.

The value of the primed reaction as a tool for studying elongation unaffected by initiation rate variations may be considerable. Since the elongation of RNA chains is temporally dependent on initiation, itself a complex process likely to be influenced by changes in the conditions of the reaction, a true 'elongation assay' has not until now been possible in the E. coli system. It would seem that in the reaction catalysed by protein $\alpha$ alone initiation can be held at any desired level merely by altering the primer concentration, so that a nearer approach to the 'elongation assay' should in principle be available.

4.2.2. Comparison with phage-induced RNA polymerases

The related bacteriophages $T_3$ and $T_7$ both induce formation of phage-specific RNA polymerases (Chamberlin, McGrath & Waskell, 1970; Dunn, Bautz & Bautz, 1971; Maitra, 1971) in E. coli. These transcribe the late genes of their respective phage genomes (Summers & Siegel, 1970). In this they differ from the H. cutirubrum enzyme, which, like that of E. coli, copies the early genes selectively (s. 3.5.4.4.). Also unlike the H. cutirubrum enzyme, the $T_3$ and $T_7$ polymerases are highly specific for $T_3$ and $T_7$ DNA respectively; neither can bind to poly d(A-T), nor are $T_4$ or calf thymus DNA transcribed. Both will accept denatured DNA provided it be endogenous. The two phage-specific polymerases are about three times the size of the H. cutirubrum enzyme. Neither is inhibited by rifampicin or streptolydigin, whereas the former prevents initiation by the H. cutirubrum DNA-dependent polymerase. Clearly the latter is unrelated to the $T_3$ and $T_7$ RNA polymerases.

4.2.3. Comparison with eukaryotic RNA polymerases

The bacterial RNA polymerases so far compared with the H. cutirubrum DNA-dependent enzyme have required $Mg^{2+}$ only for optimum activity. The requirement displayed by the H. cutirubrum polymerases for both $Mg^{2+}$ and $Mn^{2+}$ is,

however, by no means uncommon among polymerases from higher organisms. Some of these are also closest in size to the DNA-dependent polymerase from H. cutirubrum; others are apparently bigger still than the E. coli enzyme, but these results may be influenced by the tendency of many RNA polymerases to aggregate.

The smallest RNA polymerase so far described in the literature has been that of Neurospora crassa mitochondria. The enzyme consists of a single polypeptide chain of molecular weight 64,000 (Küntzel & Schäfer, 1971), which does tend to aggregate to form apparently large molecules even at 'high' ionic strength (0.5 M KCl). Optimum activity requires both $Mg^{2+}$ and $Mn^{2+}$ in 10:1 molar ratio; although the absolute levels needed are lower than those producing optimum activity with the H. cutirubrum enzyme, the ratio of 10:1 is the same in the two cases. The enzyme from N. crassa is rifampicin-sensitive.

Yeast mitochondria contain two polymerases of apparent molecular weight 450,000-500,000 (Eccleshall & Criddle, 1972), both of which are reportedly insensitive to rifampicin.

Rat liver mitochondria possess a rifampicin-sensitive RNA polymerase which has been solubilized and partially purified (Reid & Parsons, 1971; Gallerani, Saccone, Cantatore & Gadaleta, 1972) and which also requires $Mg^{2+}$ and $Mn^{+}$ in 10:1

ratio. The mol. wt. is 64,000-68,000.

Quite different results have been obtained with nuclear enzymes, which are rifampicin-insensitive in all cases. Yeast nuclei contain two RNA polymerases (Brogt & Planta, 1972), of which one, polymerase B or II, requires a 13:1 molar excess of $Mn^{2+}$ over $Mg^{2+}$ for activity - just the reverse of the H. cutirubrum requirement. A 10:1 ratio of $Mn^{2+}$ to $Mg^{2+}$ is required by polymerase II from rat liver nuclei (Roeder & Rutter, 1969). Occasional reports of the existence of a third nuclear polymerase have been questioned on the basis of its similarity to the mitochondrial enzymes (Strain, Mullinix & Bogorad, 1971).

A polymerase from chloroplasts of Zea mays has also been isolated (Bottomley, Smith & Bogorad, 1971). The molecular weight appeared to exceed 500,000. The enzyme was more active with $Mg^{2+}$ than with $Mn^{2+}$, but no study of the effect of both together was reported. There was no inhibition by rifamycin SV.

All of the above polymerases did accept denatured DNA to some extent. Template specificity was otherwise found to be limited in those cases where template variation experiments were performed. Physically, the N. crassa enzyme most closely resembled the H. cutirubrum DNA-dependent RNA polymerase, but the greatest functional resemblance to the

latter is still found among the large bacterial polymerases.

4.2.4. Rate of RNA synthesis and the life of the cell

A rough calculation from the yield of units in the crude extract and the specific activity of the pure enzyme indicates the presence of about 6,700 αβ complexes per H. cutirubrum cell, assuming all of the α and β proteins to be associated. A second calculation yields a value of 0.56 nucleotides incorporated per second per active αβ complex, or 3,750 nucleotides per second per cell. A similar calculation for E. coli yields a rate of 15,900 nucleotides per second per cell. The mean generation time for H. cutirubrum, however, is at least 14 times longer than that for E.coli, so the activity of the H. cutirubrum polymerase is probably more than adequate for the work it has to do. (The implicit assumption that the total RNA-synthesizing capacity of the cell will be always employed is almost certainly false, but the comparison between total capacities may be none the less valid. It should be remembered, however, that the rate of synthesis in E. coli is inadequate to account for more than 10% of the total cell RNA (Davidson, 1969), a fact which has never been satisfactorily explained.)

## 4.3. The RNA-dependent enzyme as replicase

### 4.3.1. Possibility of viral origin

All of the discussion up to this point has tacitly assumed that the H. cutirubrum RNA-dependent RNA polymerase is endogenous to the bacterium and is not a virus-induced replicase. The assumption is made on the following grounds:

The similarity of amino acid composition between the RNA-dependent and DNA-dependent proteins has already been mentioned (s. 4.1.). Although this in itself lessens the probability that the former is wholly exogenous, it might still be argued that RNA-dependent activity arises from a virus-induced modification of an H. cutirubrum protein.

The H. cutirubrum strain used in these studies does not form plaques on agar, nor is its growth appreciably slower than that of other normal halophile strains. If a bacteriophage were involved, therefore, it would be an RNA phage which did not interfere with normal cell metabolism. In itself unlikely, this possibility is yet slighter in view of the fact that no report of phage of any kind capable of infecting Halobacterium species has appeared in the literature. Although the point must be regarded as uncertain until exhaustive studies have been completed, the present view

must be that the RNA-dependent RNA polymerase is a normal constituent of H. cutirubrum cells. It will be of interest to investigate whether the enzyme is present in other Halobacterium strains.

4.3.2. Comparison with virus-induced replicases

4.3.2.1. Coliphage

The RNA phage of E. coli have been classified according to the template specificities of their replicases (Miyaki, Haruna, Shiba, Itoh, Yamane & Watanabe, 1971). These accept only RNA templates similar to their related genomes. Thus, the three replicases from Group III phage (Qβ, VK and ST) all accept RNA from any of the three phages, without significant changes in their rate of RNA synthesis, whereas templates from Group IV (said to be somewhat similar to Group III) were copied at about 1/5 the normal rate and those from Groups I and II were not copied at all. This is in sharp contrast to H. cutirubrum RNA-dependent RNA polymerase, which apparently copies any high-molecular-weight RNA supplied.

Qβ replicase is the best-studied RNA-dependent RNA polymerase so far investigated. As described in s. 1.2., it consists of four subunits of which only one is translated from the phage genome. None of the other three appears to

correspond to an E. coli polymerase subunit. $Mg^{2+}$ only is required for activity. There would certainly seem to be little ground for imagining a relationship to the H. cutirubrum enzyme, which consists of one protein of molecular weight 17,000-18,000.

4.3.2.2. Other RNA viruses

The replicase of myxovirus strain WSN (influenza) is quite unlike that of Qβ, but is also unlike the H. cutirubrum enzyme. The influenza virus replicase (Chow & Simpson, 1971) required $Mn^{2+}$ only for activity, was inhibited by $Mg^{2+}$, and was strictly template-specific.

Cucumber mosaic virus replicase resembles that of Qβ in its size, $Mg^{2+}$ requirement and template specificity (May, Gilliland & Symons, 1970), accepting related RNA with facility and unrelated RNA less effectively as template.

Nearest to the H. cutirubrum enzyme among the viral replicases is that of tobacco mosaic virus (Brishammar, 1970). The molecular weight may be as low as 20,000, although the enzyme migrated on Sephadex as a 70,000-dalton aggregate for the most part. A 16:1 $Mg^{2+}/Mn^{2+}$ ratio gave the maximum activity. This enzyme, however, was also highly template-specific.

It may be concluded that the H. cutirubrum RNA-dependent

RNA polymerase is not functionally similar to any known virus-induced replicase.

4.3.3. Comparison with replicases from non-viral sources

Two non-viral RNA replicases have been the subject of preliminary reports. In Chinese cabbage leaves (Astier-Manifacier & Cornuet, 1971) is an $Mg^{2+}$-dependent, 60,000-100,000-dalton replicase which accepts a wide range of templates, though at different levels of activity. Poly (U,C) was the best template and poly C the worst, activity with natural RNA templates being about 0.2-0.4 of that obtained with poly (U,C). Functionally, this enzyme is clearly closer than the viral replicases to that of H. cutirubrum. Macrophage cells, on the other hand, contain an RNA-dependent polymerase specific for a class of quite heterogeneous RNA molecules, the 'informational' RNA synthesized after contact with an antigen which induces antibody synthesis in immunologically competent cells (Neuhoff, Schill & Jacherts, 1970). $Mg^{2+}$ only was sufficient for activity. The class restriction differentiates the macrophage replicase from the H. cutirubrum enzyme.

4.3.4. Comparison with DNA-dependent RNA polymerases from prokaryotes

The large bacterial RNA polymerases, as has been

pointed out earlier, accept RNA templates. Some specificity is displayed; for example, f2 RNA is a poor template for the E. coli enzyme (Gomatos, Krug & Tamm, 1964), while reovirus RNA is a relatively good one. However, detailed study of template specificity in the RNA-directed reaction has been carried out only with the M. lysodeikticus RNA polymerase (Fox, Robinson, Haselkorn & Weiss, 1964). A wide range of RNA templates both natural and synthetic were copied, at varying levels of activity. The poorest template was tRNA, as in the case of the H. cutirubrum enzyme; the best was turnip yellow mosaic virus RNA, which had about 5 times the template activity of tRNA. $Mn^{2+}$ was preferred, although $Mg^{2+}$ also permitted activity; unfortunately, the effect of both together was not reported. From a functional standpoint, however, it is clear that the H. cutirubrum RNA-dependent RNA polymerase most resembles the normal bacterial RNA polymerases (when the latter are RNA-directed), and is unlike the replicases induced by RNA viruses. The observation lends weight to the suggestion made in s. 3.5.2.5. that the role of RNA-directed synthesis in normal (i.e. uninfected) bacteria should be investigated.

## 4.4. The H. cutirubrum RNA polymerases as halophile enzymes

### 4.4.1. Effect of salt on activity and stability

The salt dependence of halophile enzymes was discussed in s. 1.3.2. Briefly summarizing, enzymes from extreme halophiles have uniformly required salt concentrations above 2 M for optimum activity as well as for stability, except for one enzyme believed to be vestigial. The RNA-dependent polymerase and the α protein from H. cutirubrum can be classed together as exceptions to the rule, since the effects of low ionic strength on their activity and stability are so much less dramatic than on the other enzymes so far studied. Both are indifferent to the salt concentration to a degree which argues highly stable conformations and limited interaction with the solvent. It is probable that other such proteins will be found as the halophiles are more thoroughly studied.

Protein β from H. cutirubrum, on the other hand, is like the enzymes so far described in requiring more than 2 M salt for optimum activity and in being rapidly inactivated in the absence of salt. The inactivation appears to be biphasic, in that there is an immediate alteration in template specificity when the salt is removed, followed by a gradual loss of activity which takes 24 h to complete. Once inactivated, the protein cannot be restored to its original state, like some of the enzymes mentioned in the introduction but unlike others such as isocitrate dehydrogenase,

which can be reactivated almost completely.

4.4.2. Divalent cation requirement

Many of the halophile enzymes studied are stabilized by $Mg^{2+}$. H. cutirubrum polynucleotide phosphorylase requires $Mn^{2+}$ for optimum activity but reaches a near-optimum level of activity at much lower $Mn^{2+}$ concentration if $Mg^{2+}$ is also present (Peterkin & Fitt, 1971). A phosphodiesterase from the same organism also displays similar requirements (P.S. Fitt & P.I. Peterkin, unpublished). It has been pointed out that some mitochondrial RNA polymerases have optimum $Mg^{2+}/Mn^{2+}$ ratios similar to that displayed by the H. cutirubrum polymerases. Nevertheless, the metal-ion-mediated association between the DNA-dependent polymerase proteins is without precedent. It is possible that such a mechanism will be found to be an adaptation to the high-salt environment and to be of frequent occurrence among the halophile enzymes, when the application of recently developed high-salt purification techniques allows the more extensive characterization of such enzymes on a broad and comprehensive basis.

## 4.5. Concluding remarks

Several interesting lines of investigation are suggested by the work presented here. Further studies on the

primed reaction are clearly necessary to determine its specificity of initiation, if any, and to characterize the elongation, termination and release functions apart from initiation. Concomitantly, investigation of the normal αβ-catalysed initiation alone should be possible with the aid of rifampicin. Structural studies, by sequencing and by X-ray diffraction, should receive a high priority, since the nucleic acid recognition enzymes isolated until recently have been too large to allow of such investigations (although a recent success in crystallization of the lac repressor protein gives reason to hope that diffraction studies will soon be performed in that case). A necessary pre-requisite to such work would be the scaling up of the preparation procedure for all three polymerase proteins, which would also be of interest in the methodology of high-salt enzyme purification. It will be of considerable interest to compare the H. cutirubrum polymerases with those of other halophiles, as well as with other H. cutirubrum enzymes of nucleic acid metabolism, to see whether other such small, stable proteins as α and the RNA-dependent enzyme will be found. The other halophiles will have to be searched in particular for RNA-dependent polymerases, in order to establish whether the separation of RNA-dependent and DNA-dependent activities is

common. The role of RNA-directed synthesis will require study, not only in halophiles but also in other bacteria. In the reaction catalysed by the complete DNA-dependent enzyme, termination and release remain to be investigated so as to explain the premature termination occurring in the standard assay conditions. Every answer, the saying goes, raises at least one more question; the present work has been no exception.

## 5. REFERENCES

Adler, J., Lehman, I.R., Bessman, M.J., Simms, E.S. & Kornberg, A. (1958) Proc. Nat. Acad. Sci. USA 44, 641-647

Anthony, D.D., Zeszotek, E. & Goldthwait, D.A. (1969) Biochim. Biophys. Acta 174, 458-475

Asano, S., Anraku, Y. & Mizuno, D. (1971) J. Biochem. (Tokyo) 70, 21-34

Asano, S., Kurashina, Y., Anraku, Y. & Mizuno, D. (1971) J. Biochem. (Tokyo) 70, 9-20

Astier-Manifacier, S. & Cornuet, P. (1971) Biochim. Biophys. Acta 232, 484-493

August, J.T. (1969) Nature 222, 121-123

Avila, J., Hermoso, J.M., Viñuela, E. & Salas, M. (1971) Eur. J. Biochem. 21, 526-535

Babinet, C. (1967) Biochem. Biophys. Res. Commun. 26, 639-644

Baillie, L.A. (1960) Intern. J. Appl. Radiation Isotopes 8, 1-7

Baltimore, D. (1970) Nature 226, 1209-1211

Bautz, E.K.F. & Bautz, F.A. (1970) Nature 226 1219-1222

Baxter, R.M. (1959) Can. J. Microbiol. 5, 47-57

Baxter, R.M. & Gibbons, N.E. (1956) Can. J. Microbiol. 2, 599-606

Bayley, S.T. (1966) J. Mol. Biol. 15, 420-427

Bayley, S.T. & Griffiths, E. (1968a) Biochemistry 1, 2249-2256

Bayley, S.T. & Griffiths, E. (1968b) Can. J. Biochem. 46, 937-944

Beckmann, J.S., Daniel, V., Tichauer, Y. & Littauer, U.Z. (1971) Biochem. Biophys. Res. Commun. 43, 806-813

Berg, D., Barrett, K., Hinkle, D., McGrath, J. & Chamberlin, M. (1969) Fed. Proc. 28, 659

Berg, D. & Chamberlin, M. (1970) Biochemistry 9, 5055-5064

Bessman, M.J., Lehman, I.R., Adler, J., Zimmerman, S.B., Simms, E.S. & Kornberg, A. (1958) Proc. Nat. Acad. Sci. USA 44, 633-640

Bessman, M.J., Lehman, I.R., Simms, E.S. & Kornberg, A. (1958) J. Biol. Chem. 233, 171-177

Bibilashvili, R.Sh. & Savochkina, L.P. (1971) Molekulyarnaya Biologiya 5, 252-258

Blatti, S.P., Ingles, C.J., Lindell, T.J., Morris, P.W., Weaver, R.F., Weinberg, F. & Rutter, W.J. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 649-658

Bollum, F.J. (1959) J. Biol. Chem. 234, 2733-2734

Bollum, F.J. (1966) Procedures in Nucleic Acid Research (Cantoni, G.L. & Davies, D.R., ed.; Harper and Row, New York) vol. 1, pp. 296-300

Bollum, F.J. (1968) Methods Enzymol. 12B, 169-173

Bolton, E.T. (1966) Procedures in Nucleic Acid Research (Cantoni, G.L. & Davies, D.R., ed.; Harper and Row, New York) vol. 1, pp. 437-443

Bottomley, W., Smith, H.J. & Bogorad, L. (1971) Proc. Nat. Acad. Sci. USA 68, 2412-2416

Bransome, E.D., Jr. & Grower, M.F. (1970) Anal. Biochem. 38, 401-408

Bremer, H. & Konrad, M.W. (1964) Proc. Nat. Acad. Sci. USA 51, 801-808

Bremer, H., Konrad, M. & Bruner, R. (1966) J. Mol. Biol. 16, 104-117

Bremer, H., Konrad, M., Gaines, K. & Stent, G.S. (1965) J. Mol. biol. 13, 540-553

Brewin, N. (1972) Nature New Biol. 235, 101

Brishammar, S. (1970) Biochem. Biophys. Res. Commun. 41, 506-511

Brogt, Th.M. & Planta, R.J. (1972) FEBS Lett. 20, 47-52

Brown, A.D. (1963) Biochim. Biophys. Acta 75, 425-435

Brown, A.D. (1964) Bacteriol. Rev. 28, 296-329

Burdon, R.H. (1960) Biochem. J. 77, 14p

Burdon, R.H. & Smellie, R.M.S. (1960a) Biochem. J. 76, 2p

Burdon, R.H. & Smellie, R.M.S. (1960b) Biochem. J. 76, 21p

Burdon, R.H. & Smellie, R.M.S. (1961a) Biochim. Biophys. Acta 47, 93-106

Burdon, R.H. & Smellie, R.M.S. (1961b) Biochim. Biophys. Acta 51, 153-162

Burgess, R.R. (1969a) J. Biol. Chem. 244, 6160-6167

Burgess, R.R. (1969b) J. Biol. Chem. 244, 6168-6176

Burgess, R.R. (1971) Ann. Rev. Biochem. 40, 711-740

Burgess, R.R. & Travers, A.A. (1970) Fed. Proc. 29, 1164-1169

Burgess, R.R. & Travers, A.A. (1971) Procedures in Nucleic Acid Research (Cantoni, G.L. & Davies, D.R., ed.; Harper & Row, New York) vol. 2, pp. 851-863

Burgess, R.R., Travers, A.A., Dunn, J.J. & Bautz, E.K.F. (1969) Nature 221, 43-46

Burma, D.P., Kröger, H., Ochoa, S., Warner, R.C. & Weill, J.D. (1961) Proc. Nat. Acad. Sci. USA 47, 749-752

Bush, E.T. (1963) Anal. Chem. 35, 1024-1029

Byfield, J.E., Lee, Y.C. & Bennett, L.R. (1970) Biochim. Biophys. Acta 204, 610-613

Canellakis, E.S. (1957) Biochim. Biophys. Acta 25, 217-218

Cassani, G., Burgess, R.R., Goodman, H.M. & Gold, L. (1971) Nature New Biol. 230, 197-200

Cato, A.E. & Jones, O.W. (1972) Biochim. Biophys. Acta 262, 154-159

Cazzulo, J.J. & Vidal, M.C. (1972) J. Bacteriol. 109, 437-439

Chamberlin, M. & Berg, P. (1962) Proc. Nat. Acad. Sci. USA 48, 81-94

Chamberlin, M. & Berg, P. (1964) J. Mol. Biol. 8, 708-726

Chamberlin, M., McGrath, J. & Waskell, L. (1970) Nature 228, 227-231

Chang, L.M.S. & Bollum, F.J. (1972) Biochem. Biophys. Res. Commun. 46, 1354-1360

Chazan, L. & Bayley, S.T. (1971) Proc. Canad. Fed. 14, 107

Chessin, H. & Summers, W.C. (1970) Biochem. Biophys. Res. Commun. 38, 40-45

Chow, N-L. & Simpson, R. (1971) Proc. Nat. Acad. Sci. USA 68, 752-756

Christian, J.H.B. & Waltho, J. (1962) Biochim. Biophys. Acta 65, 506-508

Clewell, D.B., Evenchik, B. & Cranston, J.W. (1972) Nature New Biol. 237, 29-31

Darlix, J.L., Fromageot, P. & Reich, E. (1971) Biochemistry 10, 1525-1531

Darlix, J.L., Sentenac, A. & Fromageot, P. (1971) FEBS Lett. 13, 165-168

Dausse, J.P., Sentenac, A. & Fromageot, P. (1972) Eur. J. Biochem. 26, 43-49

Davidson, J.N. (1969) The Biochemistry of the Nucleic Acids, 6th ed., p. 61, p. 158. Methuen, London

Davison, J., Brookman, K., Pilarski, L. & Echols, H. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 95-100

Davison, J., Pilarski, L.M. & Echols, H. (1969) Proc. Nat. Acad. Sci. USA 63, 168-174

Determann, H. (1967) Gelchromatographie (Springer-Verlag, Berlin)

Dezelee, S., Sentenac, A. & Fromageot, P. (1972) FEBS Lett. 21, 1-6

Dische, Z. (1955) The Nucleic Acids (Chargaff, E. & Davidson, E.N., ed.; Academic Press, New York) vol. 1, pp. 285-305

Dunn, J.J., Bautz, E.K.F. & Bautz, F.A. (1971) Nature New Biol. 230, 94-96

Eccleshall, R. & Criddle, R.S. (1972) Fed. Proc. 31, A472

Edmonds, M. & Abrams, R. (1957) Biochim. Biophys. Acta 26, 226-227

Fischer, L. (1969) Laboratory Techniques in Biochemistry and Molecular Biology (Work, T.S. & Work, E., ed.; John Wiley and Sons, Inc., New York) vol. 1, pp. 157-396

Fish, W.W., Mann, K.G. & Tanford, C. (1969) J. Biol. Chem. 244, 4989-4994

Fleishman, D.G. & Glazunov, V.V. (1962) Pribory i Tekhnika Eksperimenta 3, 55-63

Fox, C.F., Robinson, W.S., Haselborn, R. & Weiss, S.B. (1964) J. Biol. Chem. 239, 186-195

Fox, C.F. & Weiss, S.B. (1964) J. Biol. Chem. 239, 175-185

Frederick, E.W., Maitra, U. & Hurwitz, J. (1969) J. Biol. Chem. 244, 413-424

Fromageot, P., Darlix, J.L. & Sentenac, A. (1970) RNA Polymerase and Transcription (Silvestri, L., ed.; North-Holland, Amsterdam) pp. 46-54

Fuchs, E., Millette, R.L., Zillig, W. & Walter, G. (1967) Eur. J. Biochem. 3, 183-193

Fukuda, R. & Ishihama, A. (1971) Biochem. Biophys. Res. Commun. 45, 1255-1261

Furth, J.J., Hurwitz, J. & Goldmann, M. (1961a) Biochem. Biophys. Res. Commun. 4, 362-367

Furth, J.J., Hurwitz, J. & Goldmann, M. (1961b) Biochem. Biophys. Res. Commun. 4, 431-435

Gabbay, E., DeStefano, R. & Sanford, K. (1972) Biochem. Biophys. Res. Commun. 46, 155-161

Gallerani, R., Saccone, C., Cantatore, P. & Gadaleta, M.N. (1972) FEBS Lett. 22, 37-40

Geiduschek, E.P., Nakamoto, T. & Weiss, S.B. (1961) Proc. Nat. Acad. Sci. USA 47, 1405-1415

Gerard, G.F., Johnson, J.C. & Boezi, J.A. (1972) Biochemistry 11, 989-997

Ginzburg, M., Ginzburg, B.Z. & Tosteson, D.C. (1971) J. Membrane Biol. 6, 259-268

Ginzburg, M., Sachs, L. & Ginzburg, B.Z. (1970) J. Membrane Biol. 5, 78-101

Gochnauer, M.B. & Kushner, D.J. (1969) Can. J. Microbiol. 15, 1157-1165

Goldthwait, D.A., Anthony, D.A. & Wu, C-W. (1970) RNA Polymerase and Transcription (Silvestri, L., ed.; North-Holland, Amsterdam) pp. 10-27

Gomatos, P.J., Krug, R.M. & Tamm, I. (1964) J. Mol. Biol. 9, 193-207

Green, A.A. & Hughes, W.L. (1955) Methods Enzymol. 1, 67-90

Griffiths, E. & Bayley, S.T. (1969) Biochemistry 8, 541-551

Grunberg-Manago, M. & Ochoa, S. (1955) J. Am. Chem. Soc. 77, 3165-3166

Gumport, R.F. & Weiss, S.B. (1969) Biochemistry 8, 3618-3628

Hecht, L.I., Zamecnik, P.C., Stephenson, M.L. & Scott, J.F. (1958) J. Biol. Chem. 233, 954-963

Heidelberger, C., Harbers, E., Leibman, K.C., Takagi, Y. & Potter, V.R. (1956) Biochim. Biophys. Acta 20, 445-446

Heil, A. & Zillig, W. (1970) FEBS Lett. 11, 165-168

Herbert, E. (1958) J. Biol. Chem. 231, 975-986

Herzfeld, F. & Zillig, W. (1971) Eur. J. Biochem. 24, 242-248

Holmes, P.K. & Halvorson, H.O. (1965) J. Bacteriol. 90, 316-326

Huang, R.-C.C., Maheshwari, N. & Bonner, J. (1960) Biochem. Biophys. Res. Commun. 3, 689-694

Hubbard, J.S. & Miller, A.B. (1969) J. Bacteriol. 99, 161-168

Hurwitz, J. & August, J.T. (1963) Progr. Nucl. Acid Res. 1, 59-92

Hurwitz, J., Bresler, A. & Diringer, R. (1960) Biochem. Biophys. Res. Commun. 3, 15-19

Hurwitz, J., Furth, J.J., Anders, M., Ortiz, P. & August, J.T. (1961) Cold Spring Harbor Symp. Quant. Biol. 26, 91-100

Hussey, C., Pero, J., Shorenstein, R.G. & Losick, R. (1972) Proc. Nat. Acad. Sci. USA 69, 407-411

Ingram, M. (1947) Proc. Roy. Soc. B 134, 181-201

Ishihama, A. (1972) Biochemistry 11, 1250-1258

Ishihama, A., Murakami, S., Fukuda, R., Matsukage, A. & Kameyama, T. (1971) Molec. Gen. Genet. 111, 66-76

Jacquet, M., Cukier-Kahn, R., Pla, J. & Gros, F. (1971) Biochim. Biophys. Res. Commun. 45, 1597-1607

Jenkins, J.H., Crist, S.B. & Jones, O.W. (1971) Biochim. Biophys. Acta 246, 442-449

Johnson, J.C., De Backer, H. & Boezi, J.A. (1971) J. Biol. Chem. 246, 1222-1232

Jones, O.W. & Berg, P. (1966) J. Mol. Biol. 22, 199-209

Joshi, J., Guild, W.R. & Handler, P. (1963) J. Mol. Biol. 6, 34-38

Kamen, R. (1970) Nature 228, 527-533

Kapuler, A.M. & Reich, E. (1971) Biochemistry 10, 4050-4061

Karkas, J.D., Stavrianopoulos, J.G. & Chargaff, E. (1972) Proc. Nat. Acad. Sci. USA 69, 398-402

Karstadt, M. & Krakow, J.S. (1970) J. Biol. Chem. 245, 746-751

King, A.M.Q. & Nicholson, B.H. (1971) J. Mol. Biol. 62, 303-319

Kondo, M., Gallerani, R. & Weissman, C. (1970) Nature 228, 525-527

Krakow, J.S. (1963) Biochim. Biophys. Acta 72, 566-571

Krakow, J. & Horsley, W.J. (1967) J. Biol. Chem. 242, 4796-4800

Krakow, J.S. & Karstadt, M. (1967) Proc. Nat. Acad. Sci. USA 58, 2094-2101

Krakow, J.S. & Ochoa, S. (1963) Proc. Nat. Acad. Sci. USA 49, 88-94

Krakow, J.S. & von der Helm, K. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 73-83

Küntzel, H. & Schäfer, K.P. (1971) Nature New Biol. 231, 265-269

Kurtz, S. & Pearlman, R.E. (1972) Can. J. Biochem. 50, 154-157

Kushner, D.J. & Bayley, S.T. (1963) Can. J. Microbiol. 9, 53-63

Kushner, D.J. & Onishi, H. (1966) J. Bacteriol. 91, 653-660

Langridge, R. (1967) Seventh Int. Congr. Biochem. Abstracts 1, 57

Lanyi, J.K. (1971) J. Biol. Chem. 246, 4552-4559

Lanyi, J.K. & Stevenson, J. (1969) J. Bacteriol. 98, 611-616

Lanyi, J.K. & Stevenson, J. (1970) J. Biol. Chem. 245, 4074-4080

Lark, K.G. (1972) J. Mol. Biol. 64, 47-60

Larson, H. (1967) Advances in Microbiol. Physiology (Rose, A.H. & Wilkinson, J.F., ed.; Academic Press, New York) pp. 97-132

Lecocq, J.P. (1971) FEBS Lett. 16, 213-215

Lee-Huang, S. & Warner, R. (1969) J. Biol. Chem. 244, 3795-3802

Lehman, I.R., Bessman, M.J., Simms, E.S. & Kornberg, A. (1958) J. Biol. Chem. 233, 163-170

Lill, U.T. & Hartman, G.R. (1970) Biochem. Biophys. Res. Commun. 39, 930-934

Litvak, S., Carré, D.S. & Chapeville, F. (1970) FEBS Lett. 11, 316-319

Lochhead, A.G. (1934) Can. J. Research 10, 275-286

Loeb, J. & Chauveau, J. (1969) Biochim. Biophys. Acta 182, 225-234

Losick, R., Sonenshein, A.L., Shorenstein, R.G. & Hussey, C. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 443-450

Louis, B.G. & Fitt, P.S. (1971a) Biochem. J. 121, 621-627

Louis, B.G. & Fitt, P.S. (1971b) Biochem. J. 121, 629-633

Louis, B.G. & Fitt, P.S. (1971c) FEBS Lett. 14, 143-145

Louis, B.G. & Fitt, P.S. (1972a) Biochem. J. 127, 69-80

Louis, B.G. & Fitt, P.S. (1972b) Biochem. J. 127, 81-86

Louis, B.G. & Fitt, P.S. (1972c) Biochem. J. 129, in press

Louis, B.G., Peterkin, P.I. & Fitt, P.S. (1971) Biochem. J. 121, 635-641

Mahler, H.R. (1955) Methods Enzymol. 1, 553-559

Maitra, U. (1970) Biochem. Biophys. Res. Commun. 41, 1255-1260

Maitra, U. (1971) Biochem. Biophys. Res. Commun. 43, 443-450

Maitra, U. & Barash, F. (1969) Proc. Nat. Acad. Sci. USA 64, 779-786

Maitra, U. & Hurwitz, J. (1965) Proc. Nat. Acad. Sci. USA 54, 815-822

Maitra, U. & Hurwitz, J. (1967) J. Biol. Chem. 242, 4897-4907

Maitra, U., Lockwood, A.H., Dubnoff, J.S. & Guha, A. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 143-156

Maitra, U., Novogrodsky, A., Baltimore, D. & Hurwitz, J. (1965) Biochem. Biophys. Res. Commun. 18, 801-811

Marmur, J. (1961) J. Mol. Biol. 3, 208-218

Marshall, C.L., Wicken, A.J. & Brown, A.D. (1969) Can. J. Biochem. 47, 71-74

Martin, R.G. & Ames, B.N. (1961) J. Biol. Chem. 236, 1372-1379

May, J.T., Gilliland, J.M. & Symons, R.H. (1970) Virology 41, 653-664

McClare, C.W.F. (1967) Nature 216, 766-771

Millette, R.L., Trotter, C.D., Herrlich, P. & Schweiger, M. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 135-142

Miyake, T., Haruna, I., Shiba, T., Itoh, Y.H., Yamane, K. & Watanabe, I. (1971) Proc. Nat. Acad. Sci. USA 68, 2022-2024

Mondal, H., Mandal, R.K. & Biswas, B.B. (1972) Eur. J. Biochem. 25, 463-470

Moore, R.L. & McCarthy, B.J. (1969a) J. Bacteriol. 99, 248-254

Moore, R.L. & McCarthy, B.J. (1969b) J. Bacteriol. 99, 255-262

Müller, K. (1971) Molec. Gen. Genet. 111, 273-296

Müller, K. & Bremer, H. (1969) J. Mol. Biol. 43, 89-107

Nakamoto, T., Fox, C.F. & Weiss, S.B. (1964) J. Biol. Chem. 239, 167-174

Neuhoff, V. (1968) Arzneimittelforschung 18, 35-39

Neuhoff, V., Schill, W.-B. & Sternbach, H. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 1126-1136

Neuhoff, V. (1970) Mitteil. Deutschen Pharm. Ges. 40, 289-314

Neuhoff, V., Schill, W.-B. & Jacherts, D. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 157-162

Neuhoff, V., Schill, W.-B. & Sternbach, H. (1970) Biochem. J. 117, 623-631

Neuhoff, V., Weise, M. & Sternbach, H. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 1395-1401

Nicholson, B.H. (1971) Biochem. J. 123, 117-122

Niyogi, S.K. (1972) J. Mol. Biol. 64, 609-618

Niyogi, S.K. & Stevens, A. (1964) Biochem. Biophys. Res. Commun. 16, 272-277

Niyogi, S.K. & Stevens, A. (1965a) J. Biol. Chem. 240, 2587-2592

Niyogi, S.K. & Stevens, A. (1965b) J. Biol. Chem. 240, 2593-2598

Niyogi, S.K. & Wilton, P.E. (1969) J. Mol. Biol. 41, 149-153

Ochoa, S., Burma, D.P., Kröger, H. & Weill, J.D. (1961) Proc. Nat. Acad. Sci. USA 47, 670-679

Paterson, A.R.P. & LePage, G.A. (1957) Cancer Res. 17, 409-417

Peng, C.T. (1970) Modern Aspects of Liquid Scintillation Counting (Neary, M.P., ed.; Beckman Instruments, Inc., Fullerton, Calif.) ch. 10

Peterkin, P.I. (L970) Thesis submitted to the Dept. of Biochemistry, Univ. of Ottawa

Peterkin, P.I. & Fitt, P.S. (1971) Biochem. J. 121, 613-620

Pinck, L. (1970) Bull. Soc. Chim. Biol. 52, 843-855

Preiss, H. & Zillig, W. (1967) Biochim. Biophys. Acta 140, 540-542

Preiss, J., Dieckmann, M. & Berg, P. (1961) J. Biol. Chem. 236, 1748-1757

Pugh, E.L., Wassef, M.K. & Kates, M. (1971) Can. J. Biochem. 49, 953-958

Qasba, P.K. & Zillig, W. (1969) Eur. J. Biochem. 7, 315-317

Randerath, K. (1964) Thin-Layer Chromatography (Academic Press, New York) ch. 10

Randerath, K. & Randerath, E. (1967) Methods Enzymol. 12A, 323-347

Reid, B.D. & Parsons, P. (1971) Proc. Nat. Acad. Sci. USA 68, 2830-2834

Reistad, R. (1970) Arch. Mikrobiol. 71, 353-360

Remold-O'Donnell, E. & Zillig, W. (1969) Eur. J. Biochem. 7, 318-323

Richardson, J.P. (1966a) Proc. Nat. Acad. Sci. USA 55, 1616-1623

Richardson, J.P. (1966b) J. Mol. Biol. 21, 83-114

Richardson, J.P. (1969) Progr. Nucl. Acid Res. 9, 75-116

Richardson, J.P. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 127-134

Roberts, J.W. (1969) Nature 224, 1168-1174

Roeder, R.G. & Rutter, W.J. (1969) Nature 224, 234-237

Schachman, H.K., Adler, J., Radding, C.M., Lehman, I.R. & Kornberg, A. (1960) J. Biol. Chem. 235, 3242-3249

Schäfer, K.P., Bugge, G., Grandi, M. & Küntzel, H. (1971) Eur. J. Biochem. 21, 478-488

Schales, O. & Schales, S.S. (1941) J. Biol. Chem. 140, 879-884

Schröder, E. & Lübke, K. (1966) The Peptides (Academic Press, New York) vol. 2, p. 448

Scrutton, M.C., Wu, C.-W. & Goldthwait, D.A. (1971) Proc. Nat. Acad. Sci. USA 68, 2497-2501

Sehgal, S.N. & Gibbons, N.E. (1960) Can. J. Microbiol. 6, 165-169

Seifart, K.H. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 719-726

Seifert, W. & Zillig, W. (1969) Coll. Gesell. Biol. Chem. 20, 32-47

Siegel, R.B. & Summers, W.C. (1970) J. Mol. Biol. 49, 115-123

Singh, V.K. & Sung, S.C. (1972) Can. J. Biochem. 50, 299-304

Smith, D.A., Ratliff, R.L., Williams, D.L. & Martinez, A.M. (1967) J. Biol. Chem. 242, 590-595

Snedecor, G.W. (1946) Statistical Methods, 4th Ed. (Iowa State College Press, Ames, Iowa)

Spiegelman, S., Pace, N.R., Mills, D.R., Levisohn, R., Eikhom, T.S., Taylor, M.M., Peterson, R.L. & Bishop, D.H.L. (1968) Cold Spring Harbor Symp. Quant. Biol. 33, 101-124

Stead, N.W. & Jones, O.W. (1967) J. Mol. Biol. 26, 131-135

Steensland, H. & Larsen, H. (1969) J. Gen. Microbiol. 55, 325-336

Sternbach, H. & Eckstein, F. (1970) FEBS Lett. 8, 205-206

Stevens, A. (1960) Biochem. Biophys. Res. Commun. 3, 92-96

Stevens, A. (1961) J. Biol. Chem. 236, PC43-PC44

Stoeckenius, W. & Rowen, R. (1967) J. Cell. Biol. 34, 365-393

Straat, P.A., Pongs, O. & Ts'o, P.O.P. (1971) Biochem. Biophys. Res. Commun. 44, 905-911

Strain, G.C., Mullinix, K.P. & Bogorad, L. (1971) Proc. Nat. Acad. Sci. USA 68, 2647-2651

Stumpf, P. (1947) J. Biol. Chem. 169, 367-371

Sugiura, M., Okamoto, T., Takanami, M. (1970) Nature 225, 598-600

Sümegi, J., Sanner, T. & Pihl, A. (1971) FEBS Lett. 16, 125-127

Sümegi, J., Sanner, T. & Pihl, A. (1972) Biochim. Biophys. Acta 262, 145-153

Summers, W.C. & Siegel, R.B. (1969) Nature 223, 1111-1113

Summers, W.C. & Siegel, R.B. (1970) Nature 228, 1160-1162

Summers, W.C. & Szybalski, W. (1968) Virology 34, 9-16

Swank, R.T. & Munkres, K.D. (1971) Anal. Biochem. 39, 462-477

Szybalski, W., Kubinski, H. & Sheldrick, P. (1966) Cold Spring Harbor Symp. Quant. Biol. 31, 123-127

Takanami, M., Okamoto, T. & Sugiura, M. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 179-188

Temin, H. & Mizutani, S. (1970) Nature 226, 1211-1218

Travers, A.A. & Burgess, R.R. (1969) Nature 222, 537-540

Travers, A.A., Kamen, R.I. & Cashel, M. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 415-418

Travers, A.A., Kamen, R.I. & Schleif, R.F. (1970) Nature 228, 748-751

Voigt, H.-P., Kaufmann, R. & Matthaei, H. (1970) FEBS Lett. 10, 257-260

Volken, E. & Cohn, W.E. (1954) Methods Biochem. Anal. 1, 287-305

von der Helm, K. & Krakow, J. (1972) Nature New Biol. 235, 82-83

von der Helm, K. & Zillig, W. (1967) Hoppe-Seyler's Z. Physiol. Chem. 348, 902-912

Warburg, O. & Christian, W. (1942) Biochem. Z. 310, 384-421

Weaver, R.F., Blatti, S.P. & Rutter, N.J. (1971) Proc. Nat. Acad. Sci. USA 68, 2994-2999

Weiss, S.B. (1960) Proc. Nat. Acad. Sci. USA 46, 1020-1030

Weiss, S.B. & Gladstone, L. (1959) J. Am. Chem. Soc. 81, 4118

Weiss, S.B. & Nakamoto, T. (1961a) J. Biol. Chem. 236, PC18-PC20

Weiss, S.B. & Nakamoto, T. (1961b) Proc. Nat. Acad. Sci. USA 47, 1400-1405

Whiteley, H.R. & Hemphill, E. (1970) Biochem. Biophys. Res. Commun. 41, 647-654

Wickner, W., Brutlag, D., Schekman, R. & Kornberg, A. (1972) Proc. Nat. Acad. Sci. USA 69, 965-969

Wilzbach, K.E. (1957) J. Am. Chem. Soc. 79, 1013

Wilzbach, K.E. (1963) Adv. Tracer Methodol. 1, 4-11

Yoshida, H. & Shibata, M. (1969) J. Biochem. (Tokyo) 66, 737-738

Zamenhof, S. (1957) Methods Enzymol. 3, 696-704

Zillig, W., Fuchs, E. & Millette, R. (1966) Procedures in Nucleic Acid Research (Cantoni, G.L. & Davies, D.R., ed.; Harper and Row, New York) vol. 1, pp. 323-339

Zillig, W., Fuchs, E., Palm, P., Rabussay, D. & Zechel, K. (1970) RNA Polymerase and Transcription (Silvestri, L., ed.; North-Holland Amsterdam) pp. 151-157

Zillig, W., Zechel, K., Rabussay, D., Schachner, M., Sethi, V.S., Palm, P.,Heil, A. & Seifert, W. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 47-58

# APPENDICES

# 6. APPENDICES

## 6.1. Programmes for the Olivetti Programma 101

### 6.1.1. Quench correction by least squares fit

The programme appears in Fig. 6.1., and the equations it is designed to solve are stated in s. 2.2.6.2.3. The symbol S in Fig. 6.1. replaces R in s. 2.2.6.2.3.

### 6.1.2. dpm from cpm

The programme (Fig. 6.2.) solves the equation

$$dpm = \frac{cpm}{f(aS^2 + bS + c)}$$

(see s. 2.2.6.2.3. for derivation).

### 6.1.3. $dpm_\ell$ from double-labelling data

The programme (Fig. 6.3.) solves the equation

$$dpm_\ell = \frac{dpm_\ell - cpm_h(xS^2 + yS + z)}{f(aS^2 + bS + c)}$$

(see s. 2.2.6.2.3. for derivation).

## 6.2. Buffer changes in the standard assay

During these studies, an unexplained difficulty arose because of rapid oxidation of $Mn^{2+}$, and consequent high blanks, when using tris-HCl buffers in the assays for the two

FIGURE 6.1.

olivetti programma 101

Title QUENCH CORRECTION $E=aS^2+bS+c$ BY LEAST SQUARES, FOR ANY LSC TWO CARD SIDES

| Date | | Code | | | | No. of cards | No. of instructions |
|---|---|---|---|---|---|---|---|
| m | yr. | class | | | | | |
| | | | | | | | |

PROGRAM INSTRUCTIONS **SIDE A** CARD NO._____

| REG. 1 | | | | REG. 2 | | | | REG. F | | | | REG. E | | | | REG. D | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | B | W | | 25 | / | ↕ | | 49 | | S | * | 73 | | S | * | 97 | | S | * |
| 2 | | S | * | 26 | | − | | 50 | | S | | 74 | | S | | 98 | | S | |
| 3 | E | ↑ | | 27 | | S | * | 51 | | S | | 75 | | S | | 99 | | S | |
| 4 | B | V | | 28 | | ↕ | | 52 | | S | | 76 | | S | | 100 | | S | |
| 5 | | S | * | 29 | | ÷ | | 53 | | S | | 77 | | S | | 101 | | S | |
| 6 | f | ↑ | | 30 | f | ↕ | | 54 | A | Σ | | 78 | | S | | 102 | a | V | |
| 7 | f | ↓ | | 31 | f | x | | 55 | E | ↓ | * | 79 | | S | | 103 | c/ | − | |
| 8 | b | + | | 32 | d | + | | 56 | / | ↕ | | 80 | A | W | | 104 | c/ | ↕ | |
| 9 | b | ↕ | | 33 | d | ↕ | | 57 | E | ↑ | | 81 | E | x | | 105 | E | ↓ | * |
| 10 | f | ↓ | | 34 | F | ↓ | | 58 | | V | | 82 | E | ↕ | | 106 | / | ◇ | |
| 11 | A | x | | 35 | f | x | | 59 | | S | * | 83 | | Y | | 107 | / | W | |
| 12 | F | ↕ | | 36 | D | + | | 60 | | S | * | 84 | | S | * | 108 | | S | * |
| 13 | F | ↓ | | 37 | D | ↕ | | 61 | | S | * | 85 | | S | * | 109 | | S | * |
| 14 | B | + | | 38 | f | ↓ | | 62 | | S | | 86 | | S | | 110 | | S | |
| 15 | B | ↕ | | 39 | e | + | | 63 | | S | | 87 | | S | | 111 | | S | |
| 16 | F | ↓ | | 40 | e | ↕ | | 64 | | S | | 88 | | S | | 112 | | S | |
| 17 | f | x | | 41 | a | ↑ | * | 65 | | S | | 89 | | S | | 113 | | S | |
| 18 | c/ | + | | 42 | R | ↓ | | 66 | A | V | | 90 | A | Y | | 114 | a | W | |
| 19 | c/ | ↕ | | 43 | d | S | | 67 | a | ↑ | * | 91 | b | ↓ | | 115 | | ↕ | |
| 20 | F | ↓ | | 44 | | ↓ | | 68 | r | S | | 92 | B | x | | 116 | e | ↓ | |
| 21 | A | x | | 45 | E | + | | 69 | D | ↓ | | 93 | E | ÷ | | 117 | / | ◇ | |
| 22 | C | + | | 46 | E | ↕ | | 70 | | ↓ | | 94 | c/ | ↕ | | 118 | R | S | * |
| 23 | C | ↕ | | 47 | C | V | | 71 | | W | | 95 | / | V | * | 119 | | S | |
| 24 | E | ↓ | * | 48 | | Σ | * | 72 | | S | * | 96 | | S | * | 120 | | S | |
| REG. 1 | | | | REG. 2 | | | | REG. 1/ | | | | REG. e/ | | | | REG. d/ | | | |

CONTENTS OF REGISTER

| | AT #47 | AT #118 |
|---|---|---|
| M | | n |
| A | | $\Sigma E$ |
| R | | $\Sigma s^2E$ |
| | | $\Sigma SE$ |
| b/ | $\Sigma S$ | $\Sigma S$ |
| B | $\Sigma s^2$ | $\Sigma s^2$ |
| c/ | $\Sigma s^3$ | $\Sigma s^3-\left(\frac{\Sigma s \Sigma s^2}{n}\right)$ |
| C | $\Sigma s^4$ | $\Sigma s^4$ |
| d/ | $\Sigma SE$ | |
| D | $\Sigma s^2E$ | |
| e/ | $\Sigma E$ | $\Sigma E$ |
| E | Q+0.1n | n |
| 1/ | $E_n$ | $E_n$ |
| F | $S_n^2$ | $S_n^2$ |

* SEE TEXT

| CONSTANTS ON CARD | | CONSTANTS ON CARD | |
|---|---|---|---|
| | | | |
| | | | |
| | | | |

Decimal Wheel = 4



M. P. 42B REV. 6/70

Figure 6.1. continued

| | INSTRUCTION | | | |
|---|---|---|---|---|
| | # | | | |
| * | 2 | | S | Entry of dpm for quenched standards |
| * | 5 | | S | Entry of ratio (a/b or int. std.) |
| * | 24 | E | ↓ | Unpacking routine for figure entered at 2 |
| | 25 | / | ↕ | |
| | 26 | | - | |
| * | 27 | | S | Entry of cpm |
| * | 41 | a | ↑ | Generate constant 0.1 |
| | 42 | R | ↓ | |
| | 43 | d | S | |
| | 44 | | ↓ | Packing n into decimal section of register E |
| | 45 | E | + | |
| | 46 | E | ↕ | |
| * | 48 | | Z | This instruction is never executed |
| * | 49 | | S | Begins F storage region |
| * | 55 | E | ↓ | Unpacking routine for 0.1 x n |
| | 56 | / | ↕ | |
| | 57 | E | ↑ | |
| * | 59 | | S | This instruction is never executed |
| * | 60 | | S | Signal stop |
| * | 61 | | S | Begins f storage region |

Figure 6.1. continued

| INSTRUCTION # | | | |
|---|---|---|---|
| * 67 | a ↑ | } Generate constant 10 | } Replace 0.1 x n by n |
| 68 | r S | | |
| 69 | D ↓ | | |
| 70 | ↓ | | |
| 81 | E x | | |
| 82 | E ↕ | | |
| * 72 | S | Signal stop | |
| * 73 | S | Begins E storage region (6-fig. dpm + decimal) | |
| * 84 | S | Signal stop | |
| * 85 | S | Begins e storage region | |
| * 95 | / V | $\Sigma S^3$ is >0 so jump always occurs (as is n for # 107) | |
| * 96 | S | Signal stop | |
| * 97 | S | Begins D storage region | |
| * 105 | E ↓ | n moved to register M for card change | |
| 115 | ↕ | | |
| 116 | e ↓ | $\Sigma y$ moved to register A for card change | |
| * 108 | S | Signal stop | |
| * 109 | S | Begins d storage region | |
| * 118 | R S | d & D to R for card change: stops execution | |

FIGURE 6.1. CONTINUED

olivetti programma 101

Title QUENCH CORRECTION $E = aS^2 + bS + c$ BY LEAST SQUARES, FOR ANY LSC TWO CARD SIDES

| Date | | Code | | No. of cards | No. of instructions |
|---|---|---|---|---|---|
| m | yr. | class | | | |
| | | | | | |

PROGRAM INSTRUCTIONS SIDE B CARD NO. _____

| REG. 1 | | | | REG. 2 | | | | REG. F | | | | REG. E | | | | REG. D | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | B | Y | | 25 | e | x | | 49 | | S | * | 73 | | S | * | 97 | | | |
| 2 | R | S | * | 26 | E | ÷ | | 50 | | S | | 74 | | S | | 98 | | | |
| 3 | E | ↑ | | 27 | D | ↕ | | 51 | | S | | 75 | | S | | 99 | | | |
| 4 | e | ↕ | | 28 | D | - | | 52 | | S | | 76 | | S | | 100 | | | |
| 5 | B | ↓ | | 29 | D | ↕ | | 53 | | S | | 77 | | S | | 101 | | | |
| 6 | A | x | | 30 | f | ↓ | | 54 | | S | | 78 | A | Y | | 102 | | | |
| 7 | E | ÷ | | 31 | C | x | | 55 | | S | | 79 | E | ÷ | | 103 | | | |
| 8 | C | ↕ | | 32 | c/ | ÷ | | 56 | A | V | | 80 | c/ | ↕ | * | 104 | | | |
| 9 | C | - | | 33 | c/ | - | | 57 | B | ↓ | | 81 | C | ↓ | | 105 | | | |
| 10 | C | ↕ | | 34 | F | ↕ | | 58 | C | x | | 82 | | S | * | 106 | | | |
| 11 | b | ↓ | | 35 | f | ↓ | | 59 | | W | | 83 | | S | | 107 | | | |
| 12 | e | x | | 36 | D | x | | 60 | | S | * | 84 | | S | * | 108 | | | |
| 13 | E | ÷ | | 37 | c/ | ÷ | | 61 | | S | * | 85 | | S | * | 109 | | | |
| 14 | d | ↕ | | 38 | d | - | | 62 | | S | | 86 | | S | | 110 | | | |
| 15 | d | - | | 39 | F | ÷ | | 63 | | S | | 87 | | S | | 111 | | | |
| 16 | d | ↕ | | 40 | C | ↕ | * | 64 | | S | | 88 | | S | | 112 | | | |
| 17 | b | ↓ | | 41 | d | x | | 65 | | S | | 89 | | S | | 113 | | | |
| 18 | A | x | | 42 | c/ | ÷ | | 66 | A | W | | 90 | | S | | 114 | | | |
| 19 | E | ÷ | | 43 | D | - | | 67 | B | ↕ | | 91 | A | ≠ | | 115 | | | |
| 20 | f | ↕ | | 44 | F | ÷ | | 68 | e | ↓ | | 92 | a | ◇ | | 116 | | | |
| 21 | B | ↓ | | 45 | b | ↕ | * | 69 | D | - | | 93 | b | ◇ | | 117 | | | |
| 22 | f | - | | 46 | b | x | | 70 | B | - | | 94 | c | ◇ | | 118 | | | |
| 23 | f | ↕ | | 47 | D | ↕ | | 71 | | Y | | 95 | / | ◇ | | 119 | | | |
| 24 | B | ↓ | | 48 | | V | | 72 | | S | * | 96 | | S | * | 120 | | | |
| REG. 1 | | | | REG. 2 | | | | REG. I/ | | | | REG. e/ | | | | REG. d/ | | | |

CONTENTS OF REGISTER

| | | |
|---|---|---|
| M | | |
| A | | |
| R | | |
| b/ | TILL # 45 $\Sigma S$ | AT #60 b |
| B | TILL 58 $\Sigma S^2$ ; AT 67 $a\Sigma S^2$ | |
| c/ | TILL #80 $\Sigma S^3 - \frac{\Sigma S \Sigma S^2}{n}$ | AT #20 c |
| C | AT # 10 $\Sigma S^4 - \frac{(\Sigma S^2)^2}{n}$ | AT #80 a |
| d/ | AT #16 $\Sigma SE - \frac{\Sigma S \Sigma E}{n}$ | |
| D | AT #29 $\Sigma S^2 E - \frac{\Sigma S^2 \Sigma E}{n}$ ; AT #47 $b\Sigma S$ | |
| e/ | $\Sigma E$ | |
| E | $n$ | |
| I/ | AT # 23 $\Sigma S^2 - \frac{(\Sigma S)^2}{n}$ | |
| F | $\frac{f \times C}{c/}$ AT #34 $- c/$ | |

* SEE TEXT

| CONSTANTS ON CARD | | CONSTANTS ON CARD | |
|---|---|---|---|
| | | | |
| | | | |
| | | | |

Decimal Wheel = 4



M. P. 42B REV. 6/70

Figure 6.1. continued

| INSTRUCTION # | | | |
|---|---|---|---|
| * | 2 | R S | Routine for restoring d, D, e, E contents |
| | 3 | E ↑ | |
| | 4 | e ↕ | |
| * | 40 | C ↕ | Storage of a |
| * | 45 | b ↕ | Storage of b |
| * | 49 | S | Begins F storage region |
| * | 60 | S | Signal stop |
| * | 61 | S | Begins f storage region |
| * | 72 | S | Signal stop |
| * | 73 | S | Begins E storage region |
| * | 80 | c/↕ | Storage of c |
| * | 82 | S | Execution ceases to allow choice of printout option (Z) or direct continuation |
| * | 84 | S | Signal stop |
| * | 85 | S | Begins e storage region |
| * | 96 | S | Signal stop and end of execution |

**FIGURE 6.2.**

**olivetti programma 101**

Title QUENCH PLUS SPILLOVER

$$dpm_x = \frac{cpm_x - cpm_h(zS^2+yS+z)}{f(aS^2+bS+c)}$$

| Date | | Code | | No. of cards | No. of instructions |
|---|---|---|---|---|---|
| m | yr. | class | | | |

PROGRAM INSTRUCTIONS CARD NO._____

| REG. 1 | REG. 2 | REG. F | REG. E | REG. D |
|---|---|---|---|---|
| 1 A V | 25 B ↓ | 49 S * | 73 | 97 |
| 2 S * | 26 A X | 50 S | 74 | 98 |
| 3 D ↑ | 27 d X | 51 S | 75 | 99 |
| 4 S * | 28 b + | 52 S | 76 | 100 |
| 5 E ↑ | 29 f + | 53 S | 77 | 101 |
| 6 S * | 30 b ↕ | 54 S | 78 | 102 |
| 7 F ↑ | 31 B ↓ | 55 S | 79 | 103 |
| 8 S * | 32 E X | 56 A W | 80 | 104 |
| 9 d ↑ | 33 B ↕ | 57 c/ ÷ | 81 | 105 |
| 10 S * | 34 A X | 58 A ◇ | 82 | 106 |
| 11 e ↑ | 35 D X | 59 C V * | 83 | 107 |
| 12 S * | 36 B + | 60 S * | 84 | 108 |
| 13 f ↑ | 37 F + | 61 | 85 | 109 |
| 14 S * | 38 B ↕ | 62 | 86 | 110 |
| 15 C ↑ | 39 S * | 63 | 87 | 111 |
| 16 S * | 40 ↓ | 64 | 88 | 112 |
| 17 c/ ↑ | 41 b X | 65 | 89 | 113 |
| 18 B V * | 42 S | 66 | 90 | 114 |
| 19 / ◇ | 43 ↕ | 67 | 91 | 115 |
| 20 S * | 44 - | 68 | 92 | 116 |
| 21 B ↑ | 45 B ÷ | 69 | 93 | 117 |
| 22 ↓ | 46 C ÷ | 70 | 94 | 118 |
| 23 e X | 47 a ◇ * | 71 | 95 | 119 |
| 24 b ↕ | 48 W | 72 | 96 | 120 |
| REG. 1 | REG. 2 | REG. 11 | REG. w | REG. d/ |

| CONTENTS OF REGISTER | |
|---|---|
| M | |
| A | |
| R | |
| b/ | GENERAL STORAGE |
| B | GENERAL STORAGE |
| c/ | dpm/nmol |
| C | f |
| d/ | x |
| D | a |
| e/ | y |
| E | b |
| f/ | z |
| F | c |

| CONSTANTS ON CARD | | CONSTANTS ON CARD | |
|---|---|---|---|
| | | | |
| | | | |
| | | | |

Decimal Wheel = _____

Page No.____ of ____

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Figure 6.2. continued

| | INSTRUCTION # | | |
|---|---|---|---|
| * | 1 | A V | Routine for entering a, b and c manually |
| | 2 | S | |
| | 3 | C ↑ | |
| | 4 | S | |
| | 5 | b ↑ | |
| | 6 | S | |
| | 7 | c/↑ | |
| * | 8 | B Z | Entry point for automatic a, b, c transfer |
| * | 9 | S | Entry of fraction of assay counted |
| * | 11 | S | Entry of background |
| * | 13 | S | Entry of dpm/nmol |
| * | 15 | B V | Start of calculation loop |
| * | 17 | S | Entry of S |
| * | 26 | S | Entry of cpm |
| * | 32 | a ◊ | dpm |
| * | 34 | A ◊ | nmol |
| * | 35 | C V | End of calculation loop |

FIGURE 6.3.

olivetti programma 101

Title dpm FROM cpm
$E = aS^2 + bS + c$
$dpm = cpm / f(aS^2 + bS + c)$

| Date | | Code | | No. of cards | No. of instructions |
|---|---|---|---|---|---|
| m | yr. | class | | | |
| | | | | | |

PROGRAM INSTRUCTIONS CARD NO.

| | REG. 1 | REG. 2 | REG. F | REG. E | REG. D |
|---|---|---|---|---|---|
| 1 | A V * | 25 c/ + | 49 | 73 | 97 |
| 2 | S | 26 S * | 50 | 74 | 98 |
| 3 | C ↑ | 27 B ↑ | 51 | 75 | 99 |
| 4 | S | 28 B ↕ | 52 | 76 | 100 |
| 5 | b ↑ | 29 D − | 53 | 77 | 101 |
| 6 | S | 30 d ÷ | 54 | 78 | 102 |
| 7 | c/ ↑ | 31 B ÷ | 55 | 79 | 103 |
| 8 | B Z * | 32 a ◇ * | 56 | 80 | 104 |
| 9 | S * | 33 E ÷ | 57 | 81 | 105 |
| 10 | d ↑ | 34 A ◇ * | 58 | 82 | 106 |
| 11 | S * | 35 C V * | 59 | 83 | 107 |
| 12 | D ↑ | 36 | 60 | 84 | 108 |
| 13 | S * | 37 | 61 | 85 | 109 |
| 14 | E ↑ | 38 | 62 | 86 | 110 |
| 15 | B V * | 39 | 63 | 87 | 111 |
| 16 | / ◇ | 40 | 64 | 88 | 112 |
| 17 | S * | 41 | 65 | 89 | 113 |
| 18 | ↓ | 42 | 66 | 90 | 114 |
| 19 | B ↑ | 43 | 67 | 91 | 115 |
| 20 | b x | 44 | 68 | 92 | 116 |
| 21 | B ↕ | 45 | 69 | 93 | 117 |
| 22 | A X | 46 | 70 | 94 | 118 |
| 23 | C X | 47 | 71 | 95 | 119 |
| 24 | B + | 48 | 72 | 96 | 120 |
| | REG. 1 | REG. 2 | REG. f/ | REG. e/ | REG. d/ |

| CONTENTS OF REGISTER | |
|---|---|
| M | |
| A | |
| R | |
| b/ | b |
| B | GENERAL STORAGE |
| c/ | c |
| C | a |
| d/ | f |
| D | cpm BACKGROUND |
| e/ | |
| E | dpm/nmol |
| f/ | |
| F | |

| CONSTANTS ON CARD | | CONSTANTS ON CARD | |
|---|---|---|---|
| | ↑ | | ↑ |
| | ↑ | | ↑ |
| | ↑ | | ↑ |

Decimal Wheel = _____

M. P. 42B REV. 6/70

Page No. ____ of ____

Figure 6.3. continued

| INSTRUCTION # | | | |
|---|---|---|---|
| * | 2 | S | Entry of a |
| * | 4 | S | Entry of b |
| * | 6 | S | Entry of c |
| * | 8 | S | Entry of x |
| * | 10 | S | Entry of y |
| * | 12 | S | Entry of z |
| * | 14 | S | Entry of f |
| * | 16 | S | Entry of dpm/nmol |
| * | 18 | B V | Start of calculation loop |
| * | 20 | S | Entry of S |
| * | 39 | S | Entry of $cpm_h$ |
| * | 42 | S | Entry of $cpm_\ell$ |
| * | 47 | a ◊ | $dpm_\ell$ |
| * | 49 | S | Start of F storage region |
| * | 58 | A ◊ | $nmol_\ell$ |
| * | 59 | C V | End of loop |
| * | 60 | S | Signal stop |

polymerases as previously described (s. 2.2.7.). In the case of the DNA-dependent RNA polymerase, this problem was eliminated by any of the following three methods: (i) use of glycine-NaOH buffer, pH 9.5; (ii) use of tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid-NaOH buffer, pH 8.6; (iii) addition of 2-mercaptoethanol (50 mM) to the assay medium containing tris. In all three cases, the results of detailed studies were the same as those previously obtained with tris buffer apart from the higher pH optimum with glycine-NaOH buffer. Similarly, the RNA-dependent enzyme may be assayed satisfactorily with either tris(hydroxymethyl)methylaminopropanesulphonic acid- or cyclohexylaminopropanesulphonic acid-NaOH buffers, pH 9.5, instead of tris-HCl buffer, pH 9.5. The problem has now disappeared and it has been confirmed that use of these buffers instead of tris affects neither the activity nor the requirements of the two enzymes. It is believed that the oxidation of $Mn^{2+}$ was due to the presence of a steam-distillable oxidant in the public water supply.