RNA Metabolism in Plants

during the Initiation of Cell Division

by

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The Lady is withdrawn
In a white gown, to contemplation, in a white gown.
Let the whiteness of bones atone to forgetfulness.
There is no life in them. As I am forgotten
And would be forgotten, so I would forget
Thus devoted, concentrated in purpose.

Ash-Wednesday, 1930
T.S. Eliot
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In the present study, changes of total RNA, various RNA fractions and RNA polymerase activities have been examined during the first cell cycle and subsequent growth, in the presence and absence of 2,4-D, of artichoke explants. The initial increases of RNA synthesis and accumulation and RNA polymerase activities are independent of the presence of 2,4-D in the culture medium. In the absence of 2,4-D, after longer periods of growth, incorporation of label \( (P^{32}\text{-orthophosphate}) \) into rRNA is reduced; however, the rate of synthesis of the precursor molecule is only slightly inhibited so that a post-transcriptional control mechanism appears to be operative. The increase in percentage of polydisperse RNA can be mainly attributed to the decreased labelling of the rRNA. In the presence of 2,4-D there is no change in the relative rates of synthesis of the various classes of RNA during the increase of total RNA per explant.

RNA polymerase activities have been demonstrated in both the chromatin-bound and the 10,000 x g fractions. They differ in their response to monovalent and divalent salts, and to added DNA. Chromatin-bound activity is stimulated by increasing concentrations of ammonium sulphate and potassium chloride (up to \( 0.1M(NH_4)_2SO_4 \) and \( 0.3M \text{KCl} \)), whilst the soluble activity is inhibited by monovalent salt. The chromatin-bound fraction requires only the presence of manganese for optimal activity, whereas the soluble polymerase requires the presence of both manganese and magnesium ions. Exogenous DNA may stimulate (denatured) or inhibit (native) the chromatin activity but it is not essential for its activity, whereas the soluble activity is completely dependent on the presence of denatured DNA. It has not been possible to solubilise the chromatin-bound activity and
render it DNA dependent, although, by sonication and high salt treatment, the activity can be transferred to the 10,000 x g supernatant. Despite these differences it is possible that the activity found in the chromatin fraction is a bound form of the soluble activity.

During culture of explants the percentage of activity found bound to the chromatin increases and this could be correlated with total RNA synthesis, but not with any changes of the classes of RNA synthesised. The presence of 2,4-D in the growth medium maintains and subsequently increases the stimulation of RNA polymerase activities and RNA synthesis observed in the first 15hr. growth. In the absence of 2,4-D after 15hr. growth, total RNA synthesis decreases, accumulation ceases, and the percentage of activity bound to the chromatin is found to decrease.

The soluble activity has been further fractionated by DEAE cellulose chromatography into two activities, I and II. They have been shown to differ in their divalent ion requirements and monovalent salt response. Whilst both activities utilise denatured DNA as template, polymerase II can also utilise native DNA with about 25% of the efficiency; however, polymerase II activity cannot be further fractionated on this basis. The fungal toxin, α amanitin completely inhibits the activity of polymerase II but does not affect polymerase I at all. Some evidence was found for the existence of a 2,4-D binding factor which stimulates chromatin-bound RNA polymerase activity.
INTRODUCTION

Our present knowledge of the genome of eucaryotes suggests that the cell contains much information that is not expressed at any one time and, during the course of development and specialisation of any particular cell line, has the ability to sequentially express specific parts of the genome. Mechanisms for the control of this process were for some time classed into two types - transcriptional and translational, but there is evidence, now, that many other points for control exist, e.g. processing of the transcription product, controlled transport out of the nucleus of the mRNA and regulation by turnover of the protein products.

Control of transcription may be reversible (i.e. genes may be turned on or off), whilst irreversible inhibition of gene expression may also occur as the result of loss of whole chromosomes, e.g. *Parascaris equorum*, *Cyclops* (Lewis and John, 1963) where many of the chromosomes are lost in the soma cell line. Heterochromatisation of regions of the chromosome may result in an apparent irreversible loss of gene activity, e.g. *Zea* (McClintock, 1950). The gene for leaf colour can be located close to the heterochromatic region of the chromosome, resulting in a variegated phenotype. By an inversion mutation this gene can be separated from the heterochromatic region and become fully active once more to give a green phenotype.

The classical model for gene action (Jacob and Monod, 1961) involves the synthesis of a messenger RNA molecule as the intermediate between the information of the DNA and the final protein. Three requirements must be fulfilled to procure such a molecule; specific initiation, faithful transcription from the DNA template, presumably by Watson-Crick base
pairing and, finally, accurate termination of transcription.

The product of this reaction may be termed the transcription unit and in bacteria is often a polycistronic messenger, including the regulator genes and the initiation and termination sequences for protein synthesis. In eucaryotes the product of transcription in nuclei appears to be heterodisperse RNA, a rapidly labelled class of RNA of size 10S to 100S, most of which is far larger than that necessary to code for a protein of average molecular weight. Post-transcriptional modification may cleave out smaller molecules which may still contain sequences of RNA that will not be translated into a polypeptide chain. Such sequences have recently been identified as poly-adenylic acid in the HeLa messenger RNA (Darnell, Wall and Tushinski, 1971), and haemoglobin messenger (Lim and Canellakis, 1970; Burr and Lingrel, 1971). This poly A sequence appears as 100-200 nucleotides at the 5' end of the messenger (Ryskov, Farashyan and Georgiev, 1972); some poly A (10-20 nucleotides) is also present at the 3' end of the messenger RNA.

Ryskov et al (1972) propose that the heterodisperse RNA (Hn RNA) is reduced in size by a 5'-acting nuclease which digests nucleotides from the HnRNA until it reaches the poly A sequence which was originally an internal part of the transcription product. Post-transcriptional modification has been conclusively demonstrated in the maturation of ribosomal RNA, where a larger precursor molecule, 45S in HeLa cells, is transcribed from the DNA, and is subsequently cleaved to yield the ribosomal RNAs, (e.g. Maden, 1970). Whilst it is of obvious importance to study post-transcriptional control in the expression of information, it is the initial step of trancriptive control that will be considered here in some detail.
Selective gene transcription in eucaryotes has been ascribed variously as a function of the template or a function of the enzyme concerned in transcription.

The first view holds that the availability of a particular gene is regulated by the macromolecules associated with the DNA in the chromatin matrix (Stedman and Stedman, 1950; Bonner, Dahmus, Fambrough, Huang, Marushige and Tuan, 1968; Davidson, 1969). It is assumed here that there is only one specific nucleotide sequence for initiation on the DNA and transcription will occur on those cistrons which are not repressed. Persuasive evidence supporting this view is the demonstration that bacterial RNA polymerase exhibits apparent transcriptive specificity on different sources of chromatin. Thus, chromatin isolated from different organs of mouse will direct the synthesis of specific RNA molecules as detected by competitive hybridisation techniques (Paul and Gilmour, 1968; Smith, Church and McCarthy, 1969). The alternative possibility, and the subject of this thesis, requires the transcriptive specificity to reside in the polymerase or in multiple polymerases.

Of the three stages in the production of a defined transcription product, specific initiation, faithful copying of the template during chain elongation and specific termination, the most obvious point at which efficient control can be exerted is at the point of initiation. In order to consider the mechanisms by which control of initiation can be mediated, it is necessary to consider in some detail the machinery involved in transcription. Since considerable progress has been achieved with the bacterial systems and the mechanisms that have been elucidated have been held as examples to direct eucaryotic research, I shall consider in some detail the transcription control mechanisms of procaryotes.
All bacterial RNA is thought to be synthesised by a single RNA polymerase which, after extensive purification, was shown to consist of four main sub-units, $\alpha$ (MW-41,000), $\beta$ (155,000), $\beta'$ (165,000), $\sigma$ (95,000) so that the complete (holoenzyme) structure is $\alpha_2\beta\beta'\sigma$ (Travers and Burgess, 1969). In addition, a small subunit $\omega$ (12,000) is found associated with the enzyme (Burgess, 1969), which may or may not be a true component of the holoenzyme.

Functions have been ascribed to some of the subunits; for instance $\beta'$ is required for the binding of RNA polymerase to the DNA (Zillig, Fuchs, Palm, Rabassay and Zechel, 1970), whilst $\beta$ interacts with rifampicin, an antibiotic that inhibits initiation by the enzyme in vivo and in vitro (Lancini, Pallanza and Silvestri, 1969; Umezawa, Mizuno, Yamazaki and Nitta, 1968). The $\sigma$ factor is required for accurate initiation of RNA synthesis, and can be dissociated from the holoenzyme ($\alpha_2\beta\beta'\sigma$) to give the core enzyme ($\alpha_2\beta\beta'$). Both the core and the holoenzyme transcribe calf thymus DNA and poly d AT efficiently but differ in their ability to transcribe T4 DNA - which is transcribed efficiently only by the holoenzyme. (T4 DNA is used as an example of the endogenous template which the RNA polymerase would normally transcribe). The holoenzyme can be re-constituted by mixing core polymerase and purified $\sigma$-factor, and the template specificity for T4 DNA is then restored. (Burgess and Travers, 1971). A measure of the accuracy of initiation is the initiation of transcription on the sense strand of the DNA helix as measured by asymmetry of the RNA product. An RNA product is said to be symmetrical if it binds with equal facility to both strands of the DNA helix, and asymmetrical if it binds only to one strand. In vivo transcription is presumably from only one strand of the helix, the sense strand, and is asymmetric.
Holoenzyme transcribes any phage DNA asymmetrically (e.g. Geiduschek, Tocchini-Valentini and Sarnat, 1964) whilst core enzyme on the same template transcribes a product that is more nearly symmetrical (transcribed from both strands of DNA of the helix, e.g. Sugiura, Okamoto and Takanami, 1970). Moreover, the in vitro transcription product of holoenzyme on phage DNA corresponds closely to that RNA synthesised during the initial stages of infection, whereas that transcribed by the core polymerase is complementary to the RNA transcribed during the total infection process (Bautz, Bautz and Dunn, 1969).

This suggests that the $\sigma$ factor is necessary for accurate initiation and, therefore, might be involved directly in recognising specific sequences of DNA. This conclusion is confirmed by the isolation of promoter mutants (with mutations in the region of DNA recognised by the polymerase during initiation) (Roberts, 1969), which cause a reduced amount of RNA synthesis both in vivo and in vitro by the holoenzyme while having little effect on transcription by the core enzyme.

Escherichia coli holoenzyme ($\alpha_2\beta\beta'\sigma^-$) transcribes phage DNA efficiently but not bacterial DNA (Travers, Kamen and Schleif, 1970). The existence of a positive control factor, termed psi factor ($\psi$), was postulated on theoretical grounds to alter the initiation specificity of the holoenzyme to allow efficient transcription of bacterial DNA. Several types of psi factor could exist in a bacterial cell, each regulating the initiation of a class of transcription units. Such a factor has been isolated by Travers, Kamen and Schleif (1970) which exerts a positive control over the transcription of rRNA and tRNA cistrons, termed $\psi_r$. Sigma factor controls the specificity of the polymerase for the template, whilst psi factor, working only in the presence of a sigma factor, specifies which part of that template is to be transcribed.
The process of initiation has been to some extent elucidated for bacteria. The first step is a rapid and completely reversible binding of polymerase to DNA template (Richardson, 1966; Jones and Berg, 1966), which is non-specific and can be carried out with equal facility by both holoenzyme and core enzyme (di Mauro, Snyder, Marino, Lamberti, Copp and Tocchini-Valentini, 1969) so that presumably the sigma factor is not required. When the holoenzyme binds to a promoter site the binding is stable and at 37°C has a half life of 60 hr. (Hinkle and Chamberlin, 1972; Zillig, Zechel, Rabussay, Schachner, Sethi, Palm, Heil and Seifert, 1970). Sigma factor is necessary for the production of this stable complex and the appearance of rifampicin resistance of transcription is associated with this reaction. Hinkle, Mangel and Chamberlin (1972), suggest that the relative resistance of the RNA polymerase holoenzyme-DNA complex is due to the rapid rate of RNA chain initiation by this complex. The process of initiation is summarised in Figure 1.1.

The sigma subunit acts catalytically (Travers and Burgess, 1969) and is not required for chain elongation. Krakow, Daley and Karstadt (1969) and Travers and Burgess (1969), observed that σ is released from the polymerase-DNA complex shortly after initiation in vitro and can be recycled, and Pettijohn, Stonington and Kosman (1970), demonstrated this might occur in vivo, since σ was not present in a gently isolated RNA polymerase-DNA complex actively engaged in RNA synthesis. The step at which this release occurs is not clearly identified but is possibly when a single-stranded polynucleotide structure is generated, (Krakow, Daley and Karstadt, 1969), either a nascent RNA chain or a single-stranded DNA chain. The transition of state I to state II of the promoter complex is another likely candidate for the step at which σ is released from the initiation complex.
Fig. 1.1  Model for the Initiation of RNA synthesis by the 
Escherichia coli RNA polymerase

\[
\begin{align*}
E\sigma + DNA & \xrightarrow{\text{RIFAMPICIN}} E\sigma' pDNA \\
E\sigma - DNA & \xrightarrow{\text{(non-specific binding)}} E\sigma pDNA \\
& \xrightarrow{\text{(specific binding)}} E\sigma' pDNA \\
\text{sigma factor} & \xrightarrow{\text{purine triphosphate}} pDNA \\
\text{PuT.p.} & \xrightarrow{\text{chain elongation}} 
\end{align*}
\]
It is possible that as well as containing information for promoter recognition, \( \sigma \) may function in generating a suitable single-stranded DNA structure on which transcription may proceed (Travers, 1971).

The types of transcriptional transitions that bacteria can undergo have been divided by Travers (1971), into two classes; those that are normally unidirectional (Class I), such as bacterial sporulation and lytic phage development, and those that are easily reversible (Class II), such as growth transitions. These classes of transitions employ two rather different mechanisms for altering the specificity of the enzyme so that different genes can be transcribed.

Class II transcription changes are easily reversible and are mediated by the substitution of positive control (psi) factors. Fine control of Class II transitions is thought to be mediated by a category of regulatory proteins (such as the lac and \( \lambda \) repressors), which interact directly with the DNA at or near the promoter site to prevent transcription. Class I transitions represent a more fundamental change in transcription product, involving the turning off of a large number of genes followed by the transcription of another set of previously unused genes. The mechanism for changing the specificity of initiation in this case involves not only a substitution of psi factor, but the modification of various subunits of the core polymerase and the production of a new sigma factor, so that the new set of genes can be transcribed. A two-tiered system of initiation factors has therefore been postulated for the control of transcriptional transitions. The basic enzyme for transcribing bacterial DNA is the complete holoenzyme (including sigma factor). Course control over which class of transcription units are
to be read is mediated by the psi factor, which binds to the enzyme altering its initiation specificity on bacterial DNA. The finer control of a small number of transcription units, for example, genes for three proteins in the case of the lac repressor, is mediated by a class of proteins, the repressors, which interact directly with the DNA.

Major changes in the pattern of transcription, Class I transitions, involve both positive and negative aspects of control. Not only must a large class of transcription units be turned off, but a second class must also be turned on. This may be achieved by the de novo synthesis of a new RNA polymerase, whilst the original polymerase is functionally inactivated, or by the modification of the polymerase initiation specificity by core modification or substitution of sigma factor, or by a combination of these mechanisms. During T₇ infection two classes of RNA are synthesised; early and late. The early RNA is synthesised in vitro by the E. coli holoenzyme (Geiduschek, Tocchini-Valentini and Sarnat, 1964; Summers and Siegel, 1969) while late RNA synthesis depends on the function of T₇ gene 1. (Siegel and Summers, 1970) which codes for a new RNA polymerase (Mol. weight of 107,000), which then transcribes the late T₇ RNA (Chamberlin, McGrath and Waskell, 1970). This new polymerase is quite different from the E. coli polymerase on the basis of size, drug-resistance, and antibody data.

The second mechanism for change of initiation specificity which conserves at least part of the pre-existing enzyme, has been detected in several cases of differing complexity. The simplest example is sporulation of Bacillus subtilis (Losick and Sonenshein, 1969).
Examination of the subunit structure of polymerase from vegetative and sporulating cells showed the presence of a much smaller $\beta$ subunit of the core enzyme from sporulating cells (Losick, Shorenstein and Sonenshein, 1970), whilst the other components of the core enzyme were unmodified. The change of $\beta$ subunit may be a result of de novo synthesis and substitution of a new subunit or modification of the pre-existing subunit. Since rifampicin sensitivity is maintained throughout sporulation (a function of the $\beta$ subunit), the latter explanation is favoured. The $\beta$ subunit is important for sporulation, since a mutation to rifampicin-resistance renders the organism unable to sporulate (Sonenshein and Losick, 1970). Modification of the $\beta$ subunit during sporulation may be responsible for turning off vegetative RNA synthesis, since re-constitution of vegetative sigma factor with sporulative core enzyme does not re-activate the transcriptive pattern of vegetative holoenzyme. A new sigma factor has been identified (Losick, 1971) in sporulating cells, which alters the specificity of initiation so that only sporulation RNA is synthesised. In this example of sporulation, the necessary negative and positive control changes are achieved by modification of the $\beta$ subunit to reduce its affinity for vegetative sigma factor so that vegetative genes are turned off, and the synthesis of a new sporulative sigma factor to turn on transcription of the genes for sporulation.

The development of coliphage T4 and Bacillus subtilis phage SP01 presents a more complex case of subunit modification, involving first, repression of transcription of the bacterial genome, followed by the sequential expression of immediate early, delayed early and late phage genes.
Pre-labelling of RNA polymerase before infection by these phages has shown that the $\alpha$, $\beta$ and $\beta'$ subunits are partially conserved (Goff and Weber, 1970), although the $\alpha$ chain is modified very rapidly on infection by the covalent addition of 5' adenylate, followed by a modification of the $\beta'$ subunit after 10-15 min. of infection. A newly-synthesised 10,000 Mol. weight subunit $\omega$ is found associated with the enzyme early in infection, replacing the host $\omega$ subunit.

The changes of core polymerase on infection may be represented as $\alpha_2 \beta \beta' \omega_2$ host (host core polymerase) being changed to $\alpha_{T4}^T \beta \beta' \omega_{T4}$ in early T4 infection, and to $\alpha_2 \beta T_4 \beta' \omega_2 \omega_{T4}$ in the late core polymerase. The net result of these core polymerase modifications is a loss of affinity for host $\sigma$ factor and, therefore, no transcription of the bacterial genome (Travers, 1971). However, all three core polymerases lack initiation specificity when transcribing T4DNA and so further specificity factors must be present. A $\sigma$-T4 early factor has been isolated from T4-infected cells that directs the host and modified (early T4) core polymerase to transcribe delayed early RNA (Travers, 1970). Late RNA synthesis is dependent on the product of T4 gene 55 (delayed early) and from the evidence of Snyder and Geidusheck (1968) and Travers (1971) this could well be a further T4 specified sigma factor (denoted $\sigma$-T4 late). The situation in T4 infection is to some extent further complicated by the presence of quasialate genes which may be transcribed by either the delayed early or late polymerase.
This more complex example of a class I transition seen in T4 and SP01 infections follows the same basic pattern as the simpler case of bacterial sporulation, in which there is a modification of the subunits of the core enzyme, possibly acting as a negative control of bacterial DNA transcription either directly or by reducing the affinity for the bacterial sigma factor, and an associated change of sigma factor necessary for change of specificity of initiation.

Sequential modification of the subunits together with changes of sigma factors could explain the changing pattern of RNA synthesis during phage infection. It is pertinent at this point to consider what is known of the structure and functioning of the eucaryotic RNA polymerase, particularly to determine whether these control mechanisms are applicable.

Although RNA polymerase was first detected in rat liver by Weiss and Gladstone (1959), progress with eucaryotic systems proceeded slowly when compared with bacterial, due to the low activities of the extracted enzyme, its instability and the difficulty of solubilising it free from the endogenous DNA. Recently these problems have been largely overcome by the use of alkaline extraction media (pH 8.0) to solubilise the activity, and glycerol to stabilise the enzyme (Seifert and Sekeris, 1969; Cunningham, Cho and Steiner, 1969; Goldberg, Moon and Rosenau, 1969; Roeder and Rutter, 1969). In some plants, RNA polymerase has been extracted as a soluble enzyme, free of DNA, e.g. maize (Strain, Mullinix and Bogorad, 1971), and coconut endosperm (Mondal, Mandal and Biswas, 1970), while in others it has been found bound to the chromatin from which it has not been successfully isolated in a stable form, e.g. soya bean (O'Brien, Jarvis, Cherry and Hanson, 1968) and sugar beet (Duda and Cherry, 1971).
One of the first differences noted is that eucaryotic RNA polymerase exists in the cell in a number of distinct molecular forms, whereas bacterial polymerase exists only as a single species. These forms of polymerase are resolved by DEAE chromatography and have been designated I II III and IV (Blatti, Ingles, Lindell, Morris, Weaver, Weinberg and Rutter, 1970). These multiple forms of the enzyme have each been ascribed a different location in the cell, (Jacob, Sajdel and Munro, 1970; Jacob, Sajdel, Muecke and Munro, 1970; Blatti et al, 1970; Roeder and Rutter, 1970a). RNA polymerase I is found in the nucleolus, II and III in the nucleoplasm, and IV has tentatively been described as a mitochondrial polymerase (Blatti et al, 1970). The multiplicity of polymerases probably reflects the complexity of the larger genome found in eucaryotic cells, and the degree of compartmentalisation of the genetic information. It may be analogous to the situation found in T7 infected bacteria where a separate polymerase exists to transcribe part of the DNA (the late RNA genes in this case).

RNA polymerase I and II have been purified and characterised from many organisms; for example, rat liver (Jacob, Sajdel, Muecke and Munro, 1970; Roeder and Rutter, 1969), calf thymus (Chambon, Gissinger, Mandel, Kedinger, Gniazdowski and Meihlac, 1970; Stein and Hausen, 1970a), yeast (Roeder, 1969), Blastocladia (Horgen and Griffen, 1971), maize (Strain, Mullinix and Bogorad, 1971). RNA polymerase I (nucleolar) is active with either Mn$^{2+}$ and Mg$^{2+}$ as the divalent metal ion and shows a greater activity with manganese. The enzyme uses native DNA in preference to denatured DNA and its activity is not very sensitive to ionic strength. The molecular weight of the enzyme is estimated to be between 500,000 and 700,000 (Chambon et al, 1970) and by analysis by SDS-acrylamide gel
electrophoresis has been fractionated into a limited number of protein components of 135,000-210,000 mol. weight.

The nucleoplasmic RNA polymerase II differs in its ionic requirements; it is much more active with \( \text{Mn}^{2+} \) than \( \text{Mg}^{2+} \) and is stimulated by high ionic strength (0.2M). Native template DNA is most efficiently used by polymerase II, although denatured template can sustain 75% of the activity measured on the native template. Polymerase II is specifically inhibited by the toxin \( \alpha \)-amanitin which stoichiometrically inhibits chain growth (Chambon et al, 1970; Jacob, Sajdel, Muecke and Munro, 1970). From the sedimentation coefficient the molecular weight has been estimated as 700,000 and fractionation on SDS-acrylamide gels shows a small number of discrete subunits of mol. weight 150,000, 185,000 and 215,000 (Chambon et al, 1970).

Polymerase III is located in the nucleoplasmic fraction but is only found in some organisms, e.g. sea urchin (Roeder and Rutter, 1969) and polymerase IV has been ascribed to the mitochondria. Chloroplasts contain a unique membrane-bound polymerase which has been solubilised and characterised by Bottomley, Smith and Bogorad (1971).

The assay of RNA polymerase in eucaryotic cells is complicated by the choice of an appropriate template for the enzyme, since in vivo the DNA is complexed with chromosomal proteins. The complexity of chromatin structure and the varying extent to which this structure may be expanded or condensed appears to control at least in part the availability of the template for transcription. The structure of the DNA itself, whether single or double-stranded, has a marked effect on the activity of the polymerases. It is therefore with these difficulties in mind that the template specificity of eucaryotic polymerases has been examined.
Blatti et al. (1970) and Chambon et al. (1970) reported that calf thymus polymerase II is active on native calf thymus DNA, dGdC and dIdC homopolymer pairs as template but not on phage DNAs. However, other eucaryotic polymerases (Xenopus) appear to transcribe both phage and viral DNA (Sugden and Sambrook, 1970; Keller and Goor, 1970). It seems that at least some polymerase preparations do exhibit a template specificity as assayed on purified DNAs from a variety of sources, which implies the recognition of some feature in the template DNA. The absence of template specificities in preparations may reflect the loss of some specificity factor during the preparation of that enzyme. Polymerase I and II isolated from Xenopus laevis exhibit different specificities for different regions of the DNA (Tocchini-Valentini and Crippa, 1970). The DNA containing the information for rRNA (rDNA) can reasonably easily be isolated from Xenopus, and it was found that only polymerase I could preferentially bind to it. This is the first example of differential specificity for template binding exhibited by the multiple polymerase activities of eucaryotic cells.

It is therefore of considerable interest to determine whether correlations exist between in vivo changes of transcription and in vitro changes of the multiple polymerase activities. During the development of the embryo of Xenopus laevis there is intensive rRNA synthesis between stage 4 (lampbrush) and stage 6 (mature oocytes) and outwith of this period very little rRNA is synthesised. This provides an ideal system to study the control of transcription. By micro-injection of α-amanitin (specific inhibitor of polymerase II) the rRNA synthesis was shown to be insensitive to the toxin (therefore presumably synthesised by polymerase I) and the early RNA synthesis (in early cleavage embryos) completely inhibited by it; therefore, synthesised by polymerase II (Tocchini-Valentini and Crippa, 1970).
The relative activities in vivo of the two polymerases therefore differ widely during the development of *Xenopus* embryos. Injection of bacterial sigma factor into early embryos or stage 4-6 embryos - results in a stimulation of RNA synthesis (Crippa and Tocchini-Valentini, 1970). About half of this stimulation is αamanitin sensitive and half is αamanitin insensitive, so it seems that both polymerases are present at both stages. Thus, while both polymerases are always present, only one may be active; the control of this may be by endogenous initiation factors. There is, moreover, very little change in relative activities of polymerase I and II in vitro during the development of the embryo (Roeder, Reeder and Brown, 1970).

Changes in polymerase activity ratios during functional transitions induced in rat tissues by hormonal treatments (Blatti et al, 1970) have been followed by assaying isolated nuclei in the presence and absence of αamanitin. These experiments are subject to the criticism that they may not represent the in vivo situation since any cytoplasmic factors that regulate the polymerase activities would be lost. In vitro changes in amounts of polymerase I and II fractionated on DEAE cellulose confirm the results, however, that significant changes in polymerase I and II activities in the target tissues occur after the hormonal treatments, e.g. glucocorticoid treatments stimulated relatively polymerase I activity of liver nuclei and oestrogen treatment likewise stimulated polymerase I activity of uterus nuclei. In each case, the hormonal treatments induced a synthesis of rRNA in the target tissues. A similar correlation of changes in the ratio of RNA polymerase activities with changes in RNA synthesis occur in developing sea urchin embryos (Roeder and Rutter, 1970b).
These two results, in rat tissue treated with hormones, and developing sea urchin embryos contrast with the results from *Xenopus* embryos where no correlation is observed between changes of the ratio of RNA polymerase activities and RNA synthesis.

The multiplicity of eucaryotic RNA polymerases does not correspond to the complexity required to control RNA synthesis. Multiple regulatory units, similar to the bacterial specificity factors, could, in principle provide the appropriate selectivity, as could alteration in the chromosomal template.

Several attempts have been made to demonstrate specificity factors in eucaryotic cells analogous to the sigma and psi factors of bacteria. Two types of approach have been used; the first involves the injection of bacterial sigma factor into *Xenopus* oocytes, to try to mimic the effect of an endogenous positive control factor, while the second approach depends on the stimulation of purified RNA polymerase (presumably minus factor) by factors isolated from the homogenate.

The injection of bacterial sigma factor into stage 4 *Xenopus* oocytes, resulted in an increase of RNA synthesis which was rifampicin-resistant (rifampicin inhibits bacterial core enzyme activity but not eucaryotic polymerase activity), whereas injection of the bacterial holoenzyme gave an increase of RNA synthesis which was rifampicin-sensitive (Crippa and Tocchini-Valentini, 1970). Since the sigma factor alone is unable to catalyse RNA synthesis in vitro the stimulation of RNA synthesis, resulting from injection of bacterial sigma factor, was due to the interaction of the sigma factor with an eucaryotic "core
enzyme". Based on this observation, the existence of positive control factors has been postulated. However, no proof has been put forward that the stimulation of RNA synthesis constitutes an increased rate of initiation rather than one of chain elongation, nor that this initiation is specific for any part of the genome.

Factors reported to stimulate the activity of the purified RNA polymerase have been isolated from calf thymus (Stein and Hausen, 1970a, 1970b), and rat liver (Seifart, 1970). Stein and Hausen (1970a) isolated and partially purified a protein of sedimentation constant 35 which specifically stimulated the activity of polymerase II but not polymerase I. The factor, termed S, was bound to the polymerase under low salt conditions but not under high salt conditions. The addition of S factor to the polymerase preparation changed a number of the characteristics of the reaction. The ability to transcribe native DNA was increased by nearly tenfold but with no effect on transcription of denatured DNA. The salt response of the polymerase plus and minus S factor revealed two quite different patterns. In the presence of S factor the polymerase was progressively inhibited (activity as measured on native DNA) with higher salt, as the S factor became dissociated, whilst on the absence of S factor the polymerase was stimulated by about 50% at 0.25M KCl. There is evidence that the S factor can act catalytically and displays a binding specificity for polymerase II. Bacterial sigma factor cannot substitute for the effect of S factor on polymerase II, nor can S factor substitute for the sigma factor on bacterial core enzyme.
The results compare well with the effects of the sigma factor on bacterial RNA polymerase. The factor changed the template specificity of the enzyme, bound specifically to the enzyme and stimulated RNA synthesis in a catalytic fashion. However, it has not yet been shown to act at the level of initiation rather than chain elongation, nor has it been shown to promote initiation at specific sites on the DNA, so that comparisons with the bacterial sigma factor must still be circumstantial.

Seifart (1970) has suggested that since most of the purified eucaryotic polymerases are more active on denatured rather than native DNA they have most probably lost a factor during their purification which allows them to utilise native DNA, which he assumes is the in vivo state of the DNA. He points out that in cruder preparation from some tissues (Ballard and Williams-Ashmann, 1966; Furth and Loh, 1964), the enzyme could utilise native DNA more efficiently. Seifart (1970) isolated a factor from rat liver cytoplasm that was able to stimulate eightfold RNA polymerase activity on native DNA from a variety of sources but not denatured DNA. However, contamination of this factor with nucleases which could stimulate initiation by the introduction of nicks in the DNA, complicated the analysis of the results. Again, these experiments give no indication that the effect of the factor is at the level of initiation rather than chain elongation, nor that the increased levels of synthesis represent a specific rather than non-specific transcription.

Other reports of factors stimulating RNA polymerase activity (Mondal, Mandal and Biswas, 1970; Matthyse and Phillips, 1969; Matthyse, 1969) are less well documented and no purification of such factors has been attempted.
A factor has been isolated from crude extracts of pea and corn by 2,4-D sepharose affinity chromatography which stimulates *E. coli* RNA polymerase activity on pea DNA (Venis, 1971) and this stimulation appears to be at the level of initiation since it is rifampicin-sensitive. It is not clear to what extent this situation is similar to the in vivo eucaryotic polymerase although the bacterial sigma factor stimulation of *Xenopus* RNA polymerase (Crippa and Tocchini-Valentini, 1970) argues in favour of a close similarity.

Hardin, O'Brien and Cherry (1970) have isolated a 2,4-D binding protein from soyabean that stimulates the endogenous polymerase activity and have postulated a role for it in the 2,4-D stimulation of RNA synthesis, which will be discussed later.

There is, therefore, some experimental evidence that factors which can stimulate RNA synthesis exist in eucaryotic systems. In some cases these factors have been shown to interact with the RNA polymerase and in so doing change its template specificity from denatured to native DNA and alter other properties of the enzyme such as the response to salt concentration. It has not been shown that the effect of the factor has been on initiation rather than chain elongation, neither has any evidence been presented that the specificity of initiation has been either increased or changed. The postulate of Seifart (1970) that in vivo transcription takes place on native DNA, and therefore the purified enzyme should be able to transcribe native DNA, has led to the discovery of a factor which increases dramatically the ability of the extracted enzyme to utilise such a template. This raises a general question as to the integrity of the purified polymerases used by various workers and the possibility that they lack such specificity factors.
The factors that have been isolated have many properties in common with the sigma factor of bacteria, but until the specificity of initiation has been demonstrated then the two should not be equated.

The work presented in this thesis is based on studies of transcription, both in terms of the enzymes directly concerned and the RNA product, during the cell cycle and subsequent growth of artichoke explants. In order to analyse the biochemical changes of macromolecules during the cell division cycle, it is necessary to be able to provide adequate tissue which is at the same stage of development. Inevitably this involves the use of synchronous cell culture.

Certain problems, however, arise from the method used to synchronise the cells, and the efficiency of the synchronisation process itself may have a direct bearing on the interpretation of the results. Most synchronous cultures are imperfect in their degree of synchrony so that the finer details of the cell cycle become obscured by the variation between individual cells. Synchronised cells are usually obtained by either selection of cells at a particular stage of the cycle (often on a size basis) from an asynchronous culture and their growth as a separate culture; or by treatment of asynchronous cells to produce synchrony (James, 1966). Synchrony has been induced by blocking DNA synthesis using thymidine (Peterson and Anderson, 1964), fluorodeoxyuridine (Rueckert and Mueller, 1960; Littlefield, 1962), amethopterin (Rueckert and Mueller, 1960; Mueller and Kajiwara, 1966) and 5 aminouracil (Smith, Fussell and Kugelman, 1963; Wagenaar, 1966), with subsequent removal of the inhibitor. However, growth would not necessarily have been blocked by these treatments and may not be
synchronised. Results using these methods may therefore distinguish between those cellular events belonging to the DNA cycle and those of the growth cycle. The selection method of synchronisation will, on the other hand, entrain the growth cycle but not necessarily the DNA cycle and so the experimental technique used for synchronisation will tend to select changes in the growth cycle or changes in the DNA cycle.

A third method of synchronisation, often used for microorganisms and cultured mammalian cells, is by starvation when a culture is allowed to run into the stationary phase. Addition of new medium will re-start growth of the cells which may then grow synchronously for several divisions. This method depends on all the cells starting growth at the same time and from the same point in the cell division cycle. Using this method, the first division is often very protracted and may reflect changes not implicit in the actual cell cycle but rather consequential of this method of synchronisation, so that the second or subsequent cycles should really be studied rather than the first cycle after initiation of growth (Nagata, 1963, with E. coli). Various physiological triggers have been used to synchronise populations of cells; for example, single or multiple changes of light or temperature have been used to synchronise Chlorella (Tamiya, Iwamura, Shibata, Hase and Nichei, 1953) and Tetrahymena (Scherbaum and Zeuthen, 1954).

In addition to systems of induced synchrony there are certain examples of naturally synchronous groups of cells. Early embryonic development following fertilisation in certain animals may be synchronous; for example, in the holothurian Synapta digitata there are nine synchronous cycles (reviewed by Agrell, 1964). In plants, the divisions which form
the endosperm nuclei are synchronous (Bajer, 1958a, 1958b; Bajer and Mole-Bajer, 1954) as are those leading to the formation of the female gametophyte of the gymnosperms, (Erickson, 1964). Microsporogenesis of the liliaceous genera, Trillium and Lilium involves a slow synchronous meiosis (Stern, 1960, 1961; Hotta and Stern, 1963a, 1963b, 1965; and Dickenson and Heslop-Harrison, 1970) which has been used for cell cycle investigations. The interphase between the first and second meiotic divisions extends over a period of 21 days, and during this period fluctuations of RNA polymerase and many of the DNA synthesis enzymes have been followed. However, the isolated microspores cannot be cultured and all the analyses have been on whole anthers (including surrounding anther material) excised from the plant. This tissue is very specialised and the division meiotic, so that the situation described from analysis of this cell cycle is probably atypical.

There are certain tissues which, in dormancy, represent a synchronous population; for example, all the cells of the artichoke tuber are held at 2C level of DNA and when stimulated to divide by exogenous auxin treatment will do so with a high level of synchrony (Yeoman and Evans, 1967). In artichoke the first division shows the highest level of synchrony but is longer than subsequent divisions and is undoubtedly complicated by the effects of excision. Similarly, Roberts and Northcote (1970) partially synchronised a suspension culture of Acer pseudoplatinus by treatment with kinetin, whilst Wilson, King and Street (1971) have used a dilution shock treatment for synchronisation of a sycamore suspension culture. This latter treatment produced a synchrony that was maintained for up to 9 cell cycles but suffers from the disadvantage that the low cell densities used does not provide adequate material for biochemical investigations.
Under the conditions of growth used for the artichoke explants division is not accompanied by expansion and the daughter cells remain as twos and fours within the parent cell wall. Reduction in cell size leads to the production of a meristematic-like tissue composed of small non-vacuolate cells. One of the advantages of the artichoke system is that the explants can be prepared and cultured under sterile conditions which greatly facilitates the interpretation of radioactive precursor experiments.

RNA synthesis during the cell division cycle of artichoke exhibits a stepped increase (Yeoman and Mitchell, 1970), which has been suggested to be due to the synthesis of specific classes of RNA at various times (Fraser, 1968). It is potentially a system, like Xenopus embryo, which could demonstrate changes of synthesis of different classes of RNA. Several enzyme activities have been studied under two conditions of culture, one which induced only cell expansion (incubation in water, Masuda, 1966; or with auxin in the absence of nutrients, Flood, Rutherford and Weston, 1967) and the other which lead to synchronous cell divisions (auxin plus nutrients, Yeoman and Evans, 1967). This has allowed enzyme activity changes to be resolved into two classes; those related and those not related to cell division. The activity of peroxidase (Bastin, 1970), phenol oxidase (Bastin, 1968), acid phosphatase (Yeoman and Mitchell, 1970) and DNAase (Harland, 1971) increased under conditions of no cell division and probably represent a response to excision. The development of invertase activity associated with cell walls (Edelman and Hall, 1965) in washed artichoke discs was not associated with cell division but may be in response to the synthesis of endogenous gibberellic acid (Bradshaw and Edelman, 1969) and the leaching out of a protein inhibitor from the damaged cells (Bradshaw, Chapman and Edelman, 1970).
Under conditions of culture that led to DNA synthesis and cell division, changes of enzyme activities have been related to the replication of DNA since, during this period, there was a doubling of gene dosage per cell. Replication must also involve a transient re-organisation of the DNA and possibly secondary effects on the structure of the nucleus. It is therefore of interest to examine the relationship of DNA synthesis and changes of enzyme activities. Two groups of enzymes have so far been studied; those enzymes associated with DNA synthesis, e.g. thymidine monophosphate kinase (dTMP kinase), thymidine kinase (TdR kinase), (Harland, 1971), DNA polymerase (Jackson, 1971); and enzymes unrelated to DNA synthesis, e.g. ATP-glucokinase (ATP-GK) and glucose-6-phosphate dehydrogenase (G6PDH) (Yeoman and Aitchison, 1972).

The enzymes associated with DNA synthesis increased in activity at or just after the onset of DNA replication and continued to increase through the first division. These increases were dependent on cell division and were not observed when explants were incubated in the absence of 2,4-D (non-inductive conditions for cell division).

Both G6PDH and ATP-GK increased in activity before the onset of DNA replication, i.e. in G1 period, but again were dependent on culturing the explants in inductive conditions for cell division. However, the implication that the increased activity of these enzymes was not directly related to DNA synthesis was confirmed by the effect of 5-fluoro-deoxyuridine (FUDR) which inhibited DNA synthesis but not the increases of these enzyme activities.
These results indicate that there is no general obligate link between an increase of enzyme activity and DNA replication. This conclusion is clearly confirmed from the increases of enzyme activities observed under expansion growth conditions when there was no DNA synthesis. The enzymes concerned with DNA synthesis may represent a special case in which enzymic activities are related to DNA synthesis.

It is of particular interest to investigate the changes of RNA polymerase activities in the cell cycle, since this enzyme - although not related to DNA synthesis - is dependent on DNA for its activity. Its activity might therefore be expected to reflect the amount of endogenous template available in the absence of other control mechanisms.

Hotta and Stern (1965) followed the increase of RNA polymerase during the meiotic interphase of microspores of Lilium. The in vitro changes of RNA polymerase activity did not follow in vivo changes of the rate of RNA synthesis and were not reproducible in the following year using a different strain of Lilium. In vivo rates of RNA synthesis have been measured during the cell cycle of a variety of eucaryotic organisms by pulse-labelling with precursors, but interpretation of the data is complicated by differential rates of synthesis of various classes of RNA, and varying pool sizes. Prescott and Bender (1962) showed that RNA synthesis was continuous through most of the cell cycle of cultured mammalian cells but abruptly halted at mitosis. Transcription stopped in late prophase, (Doida and Okada, 1967) and was most easily explained by the chromosomes being highly condensed and the DNA unavailable for transcription. Das (1963)
showed that in root tips of both *Nigella* and *Allium* the rate of nucleolar RNA synthesis remained normal (as long as nucleoli were still present) and decreased when the chromosomes were condensed. In mitotic cells lacking distinct nucleoli, there was practically no RNA synthesis. Fan and Penman (1970) demonstrated that mitochondrial RNA synthesis continued during mitosis. In some cases the increase in the rate of RNA synthesis during the cell cycle was consistent with a gene dosage effect (Zetterberg and Killander, 1965; Pfeiffer and Tolmach, 1968). However, in other cases, there appeared to be a continuous increase in the rate of RNA synthesis through the cell cycle (Enger and Tobey, 1969; Scharff and Robbins, 1965; Terasima and Tolmach, 1963; Kim and Perez, 1965; Fujiwara, 1967; Warmsley and Pasternak, 1970).

Variations between results may represent *in vivo* fluctuations of pool sizes or genuine changes in the patterns of RNA synthesis, depending on growth conditions.

Although changes of total RNA synthesis during the cell cycle have been described, there is little data concerning the patterns of synthesis of various classes of RNA. RNA synthesis during the cell cycle of lower eucaryotes followed a similar pattern with one exception - in *Physarum*, where a sharp reduction in the rate of both RNA and protein synthesis was observed during the S period (Mittermayer, Braun and Rusch, 1964; Mittermayer, Braun and Rusch, 1966). This reduction, which at the moment is unexplained, appeared to be directly related to the RNA synthesis rather than uptake of the label, since isolated nuclei showed a similar pattern (Braun, Mittermayer and Rusch, 1966; Mittermayer, Braun and Rusch, 1966). The classes of
RNA transcribed varied as indicated by base composition changes (Cummins, Weisfeld and Rusch, 1966; Cummins and Rusch, 1967) and nearest neighbour frequency analysis. Studies on RNA synthesis during the cell cycle of various ciliates, e.g. Paramecium (Kimball and Perdue, 1962; Woodard, Gelber and Swift, 1961), Tetrahymena (Prescott, 1960) and Euplotes (Evenson and Prescott, 1970), have shown the same general patterns of continuous RNA synthesis throughout the cycle. However, ciliates differ from most eucaryotes by showing no arrest of RNA synthesis during nuclear division; this probably being related to the amitotic mode of division of the nucleus without chromosomal condensation. Euplotes represents an interesting exception in that DNA synthesis is restricted to two bands which move through the macronucleus and RNA synthesis occurred in all regions of the nucleus except these bands.

The accumulation of RNA has been determined during the cell cycle of cultured artichoke explants (Fraser, 1968; Mitchell, 1969) and has been shown to increase in a step-wise fashion. It has been suggested that each of these increases could represent the synthesis of a specific class of RNA and, indeed, tRNA appears to be preferentially synthesised during the final step at the end of the cell cycle (Fraser, 1968). The work presented in this thesis describes and correlates changes of the pattern of RNA synthesis and changes of the activities of the multiple RNA polymerases during the growth of artichoke explants.
A. Sterile Culture of Artichoke Explants

The tissue used during this study was isolated from tubers of a single clone of Jerusalem Artichoke (Helianthus tuberosus, L. var. Bunyards Round). Material of this variety was grown in the garden of the Botany Department, Kings Buildings, Edinburgh. The tubers were harvested in November, by which time they had attained their maximum size. Prior to harvesting, the tubers were protected from frost damage by a covering of straw around the bases of the plants. Most of the adhering soil was removed and tubers from individual plants were divided into groups of four or five and placed with some damp sand in numbered polythene bags. This enabled tubers derived from a single plant to be used in one or a set of experiments. The bags were stored at 4°C under more damp sand until required for use. Under these conditions of storage, the tubers remained dormant until May or June of the following year.

Explants were prepared from tubers of approximately the same size from a single plant; Robertson (1966) showed that the length of the first division cycle of the cultured explants was influenced by the size of the tuber from which they were derived. Any tubers showing signs of surface damage or infection were rejected. The selected tubers were scrubbed to remove adhering soil and surface-sterilised by immersion in a solution of sodium hypochlorite containing 2 to 3% (w/v) available chlorine, for 30 min. The tubers were then washed with sterile distilled water and transferred to the sterile room.
The sterile room contained two u-v lamps (Philips, tubular 1.5w.) left on whilst the room was unused, and a small positive pressure of filtered air reduced the inflow of contaminated air on entry. Conventional flaming techniques were used for routine sterilisation of instruments during use. Instruments and paper tissues were wrapped in aluminium foil and sterilised along with the glassware in sealed tin boxes at 150°C for 3hr.

The tubers were wiped dry with sterile tissues immediately prior to use, and the ends of each tuber removed, leaving a segment approximately 3cm. long. Cores of parenchyma tissue were removed using a 2mm. diameter stainless steel cannula. Up to fifteen cylinders of tissue, the long axes of which were parallel to that of the tuber, could be obtained from each tuber. These cylinders of tissue were then cut into segments 2.4mm long. Explants prepared from different tubers were randomised before distribution to the culture bottles. The explants were prepared under normal fluorescent lighting conditions, but transfer to the culture flasks was carried out in low intensity green light (Ilford bright green safe light < 1ft-c, Filter No. 909), since the inhibitory effect of light on cell division, (Fraser, 1968), is only observed after the addition of 2,4-dichlorophenoxyacetic acid (2,4-D) and during the first 9-12hr. of growth, (Davidson, 1971).

The medium used for culture of explants was based on that of Bonner and Addicott (1937) as modified by Yeoman, Dyer and Robertson (1965) and is shown in Table 2.1.
A stock solution of $10^{-3}M$ 2,4-D was prepared by dissolving 2.2mg. of 2,4-D in 10ml. of ethanol and this was diluted to give a concentration of $10^{-6}M$ on the final growth medium.

Table 2.1 Composition of Culture Medium for Growth of Artichoke Explants

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solution A</strong></td>
<td>MgSO$_4$·7H$_2$O</td>
<td>0.146mM</td>
</tr>
<tr>
<td></td>
<td>KNO$_3$</td>
<td>0.800mM</td>
</tr>
<tr>
<td></td>
<td>KCl</td>
<td>0.880mM</td>
</tr>
<tr>
<td><strong>Solution B</strong></td>
<td>KH$_2$PO$_4$</td>
<td>0.088mM</td>
</tr>
<tr>
<td><strong>Solution C</strong></td>
<td>FeCl$_3$</td>
<td>0.006mM</td>
</tr>
<tr>
<td></td>
<td>Ca(NO$_3$)$_2$</td>
<td>1.440mM</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>0.117M</td>
</tr>
</tbody>
</table>

Stock solution of A, B, and C were freshly prepared at intervals at 100 times the final concentration and stored at 0°C. The culture medium was prepared by mixing 10ml. of each stock solution; 40g. of sucrose and making up the volume to 1,000ml. with distilled water, which gave a final pH of 5.5.

Two conditions of culture were employed; one in the presence of sucrose, mineral salts, and 2,4-D, which promoted cell division, and one without 2,4-D which resulted in essentially no division. Culture medium, in the incubation flasks and distilled water, were autoclaved at 15 p.s.i. for 15 min.

The explants were added to the incubation flasks (70 explants per 250ml. roller bottle containing 10ml. of culture medium) and the mouth of the bottle was flamed and re-sealed. These cultural conditions satisfy the minimum volume of medium per explant described by Fraser (1968) and compares with that used by Davidson (1971) and Harland (1971).
The roller bottles were transferred in the dark to a special apparatus designed to revolve them at 2.5 rpm. The explants adhered to the walls of the bottle and passed through the culture medium once every revolution. The incubation conditions were maintained at 25°C ± 0.5°C in the dark, and samples were withdrawn aseptically in the sterile room in dim green light at the required times.

After use, glassware was washed in tap water and boiled for 20 min. in a solution of Calgon (sodium hexametaphosphate, 90 mg/l) and sodium metasilicate (800 mg/l) according to the method of Harding and Trebler (1947). After cooling and rinsing in tap water, the alkaline glassware was neutralised by immersion in 0.01 N HCl for at least 3 hr. This resulted in the deposition of a monolayer of metasilicate on the glass surfaces. After rinsing in tap water, the glassware was left overnight in distilled water, rinsed in distilled water and dried in a hot oven. The sterility of the cultured explants and the growth medium was routinely tested by spreading 0.25 ml of the growth medium onto a sterile malt agar plate. The plates were incubated for two days at 25°C and any bacterial colonies counted. In order to ensure that there was no partition of bacteria between the culture medium and explants, explants were occasionally withdrawn, aseptically homogenised in sterile distilled water, and plated out.

B. Analytical Methods

1. Cell Number Estimation

The total number of cells per explant was estimated by macerating a known number of explants in a known volume and determining the cell density obtained.
This method was developed by Brown and Rickless (1949) and modified by Yeoman, Dyer and Robertson (1965). At each sample time two replicates, each of 5 explants, were transferred to 3ml. aliquots of 5% chromic acid (5g. chromium trioxide in 100ml. of distilled water) and left for at least 24hr. at 0°C. The tissue was then macerated by repeated passage through a pasteur pipette. The macerate was mixed to prevent cells from settling and immediately a small volume of the cell suspension was placed beneath the coverslip of a Fuchs Rosenthall haemocytometer slide. The number of cells on six complete grids was determined using a hand tally counter, and a mean cell number per grid was calculated. One difficulty encountered with this method was that caused by the fragmentation of cells during excision of the explant and during maceration of the tissue. The standard procedure adopted was to count as a cell any fragment containing more than half of the cell wall.

The volume of the liquid overlying the grid was 3.2μl, and the total volume of the cell suspension was known so that the total number of cells per explant was obtained by multiplying the number of cells per grid by the volume of the cell suspension, in μl, and dividing by 3.2 times the number of explants used.

2. DNA Estimation

The DNA content of the tissue was estimated by the colour reaction of the deoxyribose component of the DNA with diphenylamine (Dische, 1930; Burton, 1956). For both the estimations of DNA content per explant and of chromatin preparations from artichoke, it was necessary to use a series of
organic solvent extractions, as described by Evans (1967) to remove interfering substances. The samples were extracted twice at 0 to 4°C with 5ml. of 5% trichloro-acetic acid (TCA) in methanol for 20min. followed by three x 15min. extractions in 0.05M formic acid in methanol. This was followed by extractions (15min.) in 5ml. of 80% ethanol, 100% ethanol, ethanol/ether (1:1 v/v), and ether, and the explants were finally air-dried. The DNA was hydrolysed by sequential treatments in 0.5N perchloric acid (PCA) at 70°C for 20min. Since the hydrolysis does not go to a defined end product, it was necessary to hydrolyse some standard DNA with each set of samples. Calf thymus DNA, at 200 μg/ml, was acidified with an equal volume of 1N.PCA and hydrolysed with the samples. A standard curve for 0 to 100 μg of DNA was prepared. Two volumes of diphenylamine reagent (containing 2g. Analar diphenylamine in 100ml. of glacial acetic acid, 1.5ml. of Analar concentrated sulphuric acid and 8mg. acetaldehyde was added immediately prior to use) was added to one volume of hydrolysate in a hard glass tube. The tubes were sealed with parafilm and incubated at 30°C for 18hr. Although the diphenylamine reaction will produce a colour with other sugars (Dische,1930) the ribose of the RNA present in the sample does not interfere (Fraser,1968). The intensity of the blue colour developed was measured using the SP500 spectrophometer and 4cm. light path cells. The optical density at 600 and 650nm. was recorded for each sample and the DNA content estimated from the calibration curve of ∆OD600-650 against μgDNA.
3. Estimation of Protein

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (1957). Aliquots of the protein solution to be estimated (containing approximately 20-200 \( \mu g \) protein) were precipitated with 5% TCA and stored overnight at 4°C. The precipitate was collected by centrifugation at 1500 x g for 10 min. and dissolved in 1ml. of 0.1N NaOH; five ml. of freshly-prepared alkaline copper tartrate, (2% \( \text{CuSO}_4 \cdot 5\text{H}_2\text{O} \), 4% Na-K tartrate, 4% \( \text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O} \) in 0.1N NaOH in 1:1:100 ratio) was added with thorough mixing. After 10 min. 0.5ml. of Folin Ciocalteaus' reagent (diluted 1:1 with water) was added and thoroughly mixed. The blue colour which developed was measured with an EEL colourimeter using filter 608 or ORI and protein content determined relative to a calibration curve containing 20-200 \( \mu g \) Bovine Serum Albumin (BSA, Calbiochem Fraction V).

4. Measurement of RNA

RNA from artichoke tissue was quantitatively estimated by two methods; one based on alkaline hydrolysis of RNA to ribonucleotides, and the other on the acid hydrolysis of both RNA and DNA (Evans, 1967). Both methods depend on the prior removal of non-nucleic acid u-v absorbing material from the artichoke tissue by a series of organic solvent washes as previously described for the estimation of DNA.
A modification of the Schmidt and Thannhauser Method (1945), was used to estimate the RNA content of solvent-extracted explants by incubating 30 explants in 2.0ml. of 0.3N KOH for 18hr. at 37°C. The pH of the alkali extract was adjusted to 2 with 0.5N PCA at 0°C and the resulting precipitate centrifuged down. The 260nm. OD of the supernatant was determined and used to calculate the RNA content of the solution on the basis that 31.7µg of RNA mononucleotides in 1ml. have a 260nm. OD of 1.0. Further extraction of the explants by 0.3N KOH for 30hr. at 37°C yielded little more ribonucleotide material (Fig.2.1).

The Schneider Method (1945) was used to estimate total nucleic acid (TNA) by hot acid hydrolysis, which yields a range of degradation products. The air-dried material was incubated with 0.5N PCA at 70°C for 20min. using 0.1ml. of 0.5N PCA per explant. Sequential extraction showed that two treatments were necessary to quantitatively extract the TNA (Fig.2.2). The acid hydrolysis does not reach a defined end point so that for its calibration a standard sample of nucleic acid must be incubated along with the samples of PCA. However, this procedure has not generally been applied but, instead, the total nucleic acid per explant was calculated on the basis that 40µg of acid hydrolysed TNA in 1.0ml. have a 260nm OD of 1.0, (Fraser,1968; Harland,1971). The RNA was estimated to represent 90% of the total nucleic acid by separation using gel electrophoresis (as shown in Fig.3.59).
Fig. 2.1. Extraction of RNA by KOH hydrolysis

15 explants (fresh tissue) were solvent extracted and hydrolysed in 1.5ml. of 0.3N KOH at 37°C. The supernatant was withdrawn after 18hr. (-----) and replaced by a further 1.5ml. of 0.3N KOH and the hydrolysis continued for 50hr. (-----). The supernatants were acidified with 0.5N PCA at 0°C to pH 2.0, the precipitate removed by centrifugation and the volume of the supernatant adjusted to 2.0ml. then scanned in an SP800 spectrophotometer.

Fig. 2.2 Extraction of total nucleic acid by acid hydrolysis

15 explants (fresh tissue) were solvent extracted and hydrolysed in 1.0ml. of 0.5N PCA at 70°C for 20min. (-----). The supernatant was removed and the explants reextracted as before (-----). A third extraction was made (-----) and the supernatants were scanned in an SP800 spectrophotometer.
The quantitative recovery of RNA by these two methods was comparable (Table 2.2).

<table>
<thead>
<tr>
<th>No. Explants</th>
<th>KOH Extraction RNA (µg/explant)</th>
<th>PCA Extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>$4.73^{+}\pm 0.17$</td>
<td>$5.37^{+} 0.17$</td>
</tr>
<tr>
<td>30</td>
<td>$4.42^{+}0.12$</td>
<td>$5.26^{+}0.11$</td>
</tr>
</tbody>
</table>

Values are given as the mean of two replicate extractions + half the range of the variation.

Purification of Ribonucleoside Monophosphates

Since alkali hydrolyses RNA specifically to ribonucleoside monophosphates, the product of alkaline hydrolysis can be purified further to eliminate any contaminating absorption not due to the nucleotides by ion exchange chromatography on Dowex-1 Cl⁻ (Smillie and Krosv, 1960). Dowex-1 Cl⁻ was washed for several hours with 6N HCl, followed by 0.8M NaCl in 2N HCl and finally with distilled water until the pH of the eluate was pH 4 and the 260nm OD was less than 0.05. Columns, 5cm x 0.6cm. diameter, gave a convenient flow rate without the necessity of applying pressure. Recovery of hydrolysed RNA from the Dowex-1 Cl⁻ column was checked with a sample of uridine monophosphate (UMP), the nucleotide which elutes last from the column under these conditions. The column was washed with 10ml. of distilled water, the sample of UMP (0.12µg/ml.) at pH6-8 was loaded, and the column washed with 10ml. of 10mM NaCl to reduce the 260nm OD of the eluate to zero.
The UMP was eluted from the column with 2 x 5ml. of 0.4M NaCl in IN HCl and the OD 260 of the eluate measured. The recovery of UMP was calculated to be 100% from measurement of the u-v absorption, after correction for the change of the absorption spectrum in acid. This method of purification of ribonucleoside monophosphates was used for the KOH hydrolysates of the artichoke explants. After acidification of the KOH hydrolysate to pH 2 with cold 3N PCA, the precipitated DNA, protein and potassium perchlorate, was removed by centrifugation at 1500 x g for 10min. The supernatant containing the ribonucleotides was neutralised to pH 6-8 by the addition of IN KOH and any further precipitate was removed by centrifugation. The extract was loaded onto a Dowex-1 Cl\textsuperscript{-} column and eluted as previously described. About 25% of the 260nm OD was removed by purification of the nucleotides on Dowex-1 Cl\textsuperscript{-} (Table 2.3).

Table 2.3  Estimated RNA Content per Explant, before and after Dowex-1 Cl\textsuperscript{-} Purification of KOH Hydrolysate

<table>
<thead>
<tr>
<th>No. Explants Used</th>
<th>Estimated RNA ((\mu g)/explant) Before Dowex-1 Cl\textsuperscript{-}</th>
<th>After Dowex-1 Cl\textsuperscript{-}</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>4.83 ± 0.17</td>
<td>3.38 ± 0.10</td>
</tr>
<tr>
<td>30</td>
<td>4.73 ± 0.11</td>
<td>3.25 ± 0.13</td>
</tr>
</tbody>
</table>

RNA was extracted from freshly-excised artichoke explants by KOH hydrolysis, and RNA content estimated before and after passage through a Dowex-1 Cl\textsuperscript{-} column. The u-v spectrum of the KOH hydrolysate derived from artichoke tissue (grown for 12.5hr.) was compared before and after purification through Dowex-1 Cl\textsuperscript{-} (Fig.2.3). The RNA content was therefore determined by hydrolysis in KOH, after sequential organic solvent extraction of the explants, and purification by Dowex-1 Cl\textsuperscript{-} exchange chromatography.
Fig. 2.3. The u-v spectrum of KOH hydrolysate before and after passage through Dowex-1 Cl⁻.

The KOH hydrolysate (5ml.) from 30 explants (grown for 12.5hr.) was obtained as described for Figure 2.1. The pH was adjusted to 6-8 by addition of 3N KOH and any precipitate removed by centrifugation. The supernatant was scanned in an SP800 spectrophotometer (-----) and then passed through a Dowex-1 Cl⁻ column. The column was washed with 10mM NaCl and then the ribonucleotides were eluted by 2 x 5ml. washes of 2.3% NaCl in IN HCl. The eluate was scanned in an SP800 spectrophotometer (-----).

Fig. 2.4 Changes of RNA content during the growth of explants

The RNA content per explant was estimated before (-----) and after (-----) Dowex-1 Cl⁻ chromatography as described in the text, during the growth of the explant.
The value for fresh artichoke tissue obtained by this method was $3.29 \pm 0.09 \mu g/\text{explant}$ compared with $4.78 \pm 0.15 \mu g/\text{explant}$ (Table 2.2) estimated by PGA hydrolysis. The extent of contamination of the hydrolysate, as estimated by Dowex-1 Cl$^-$ purification varied somewhat during the growth of the explants, reaching a maximum after 10-12.5 hr. incubation (Table 2.4).

Table 2.4  Contamination of the RNA KOH Hydrolysate during Culture of Explants

<table>
<thead>
<tr>
<th>Time of Incubation (hr.)</th>
<th>RNA Content ((\mu g/\text{explant}))</th>
<th>% Contamination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Dowex-1Cl$^-$</td>
<td>After Dowex-1Cl$^-$</td>
</tr>
<tr>
<td>0</td>
<td>5.40</td>
<td>3.17</td>
</tr>
<tr>
<td>2.5</td>
<td>4.80</td>
<td>2.99</td>
</tr>
<tr>
<td>5.0</td>
<td>6.50</td>
<td>3.58</td>
</tr>
<tr>
<td>7.5</td>
<td>7.00</td>
<td>3.89</td>
</tr>
<tr>
<td>10.0</td>
<td>8.40</td>
<td>3.46</td>
</tr>
<tr>
<td>12.5</td>
<td>9.30</td>
<td>4.05</td>
</tr>
<tr>
<td>15.0</td>
<td>9.10</td>
<td>4.29</td>
</tr>
<tr>
<td>17.5</td>
<td>8.85</td>
<td>4.64</td>
</tr>
<tr>
<td>20.0</td>
<td>9.87</td>
<td>5.23</td>
</tr>
<tr>
<td>22.5</td>
<td>9.30</td>
<td>5.37</td>
</tr>
<tr>
<td>25.0</td>
<td>8.50</td>
<td>5.26</td>
</tr>
</tbody>
</table>

The explants were incubated in the complete culture medium. For each value of RNA content/explant, 30 explants were removed aseptically from the culture medium and stored in methanol at -20°C until the RNA estimation was carried out as previously described. 20% division had occurred by 25 hr. Culture medium plated out on malt agar plates showed no detectable bacterial contamination.
The pattern of increase of RNA during this growth period was considerably altered by purification of the KOH hydrolysate. Whereas estimates based on direct KOH hydrolysis suggested an increase of RNA content in the first 12 hr. incubation, then a period without further increase, after purification, there appeared to be a linear increase over the whole period (Fig. 2.4).

The Effect of Tissue Homogenisation on the Recovery of RNA

Samples of 30 explants (fresh tissue) were homogenised in cold 5% TCA in a glass-in-glass homogeniser or Polytron. The homogeniser was washed with cold 5% TCA and the washing added to the initial homogenate. The total homogenate was allowed to precipitate for 1 hr. and then centrifuged for 10 min. at 1500 x g. The precipitate was washed and extracted as previously described, the precipitate being collected at each stage by centrifugation at 1500 x g for 10 min. The KOH hydrolysate was purified by Dowex-1 Cl− chromatography and the RNA content per explant calculated (Table 2.5).

<table>
<thead>
<tr>
<th>Sample</th>
<th>RNA Content/Explant (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass-in Glass</td>
<td>2.55 ± 0.15</td>
</tr>
<tr>
<td>homogenised</td>
<td></td>
</tr>
<tr>
<td>Polytron homogenised</td>
<td>2.20 ± 0.08</td>
</tr>
<tr>
<td>Intact explant</td>
<td>3.25 ± 0.13</td>
</tr>
</tbody>
</table>

The RNA was extracted from fresh tissue. The lower RNA recovery after homogenisation of the explants presumably results from losses during the organic solvent washes.
5. Measurement of the Rate of RNA Synthesis

The rate of incorporation of \(^3\)H-uridine into RNA was used as a measure of the rate of RNA synthesis. Since it has been shown (Jackson and Ingle, 1973) that disturbance to the tissue affects RNA synthesis, particularly as determined over short periods, experiments were designed to minimise any such disturbance or change of the physiological environment (e.g. transfer to a new medium). Roller bottles were set up containing the number of explants required for the various assays and measurements which, when removed, left 25 explants in the original incubation medium for the measurement of RNA synthesis. Sterile \(^3\)H-uridine (25 \(\mu\)C, 5.8C/m mole) was added in the dark to the explant culture and the incubation was continued for 1 hr. at 25°C in the dark. The explants were then removed, washed on a sieve with tap water and blotted dry to remove surface moisture. The RNA was extracted by alkaline hydrolysis as previously described and the specific activity of the nucleotides determined. It was necessary to establish that; RNA nucleotide purification, checked previously for 260 nm OD contamination, removed radioactive contaminants; the specific activity of the \(^3\)H-uridine did not affect uptake and incorporation of \(^3\)H-UTP and that the rate of incorporation of \(^3\)H-uridine was linear over the experimental period.

(i) Purity of nucleoside monophosphates

The elution from Dowex-1 Cl\(^-\) of an RNA hydrolysate prepared from 24 hr. tissue labelled for 1 hr. with \(^3\)H-uridine was examined. About 75% of the tritium label in the KOH hydrolysate eluted with the nucleotides from the column (Table 2.6).
Table 2.6  
**Elution of $^3$H-labelled Ribonucleotides from Dowex-1 Cl**

<table>
<thead>
<tr>
<th></th>
<th>c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial KOH hydrolysate</td>
<td>52,226</td>
</tr>
<tr>
<td>Elution from column:</td>
<td></td>
</tr>
<tr>
<td>Wash 1</td>
<td></td>
</tr>
<tr>
<td>Wash 2</td>
<td>967</td>
</tr>
<tr>
<td>Wash 3</td>
<td>6,587</td>
</tr>
<tr>
<td>Wash 4</td>
<td>743</td>
</tr>
<tr>
<td>0.4M NaCl in 1N HCl (5ml.)</td>
<td></td>
</tr>
<tr>
<td>Wash 1</td>
<td>32,313</td>
</tr>
<tr>
<td>Wash 2</td>
<td>6,109</td>
</tr>
<tr>
<td>Wash 3</td>
<td>1,490</td>
</tr>
</tbody>
</table>

25 explants (grown for 24hr.) were labelled for 1hr. as described in the text. RNA was extracted by KOH hydrolysis and purified by Dowex-1 Cl$^-$ chromatography. 0.5ml. aliquots were added to 4.5ml. of Triton-toluene scintillation mixture (2:1,W/V) and counted in an Intertechnique scintillation counter.

This was further confirmed by mixing the hydrolysate of $^3$H-labelled RNA with unlabelled UMP and eluting from a Dowex-1 Cl$^-$ column (Fig.2.5). The unlabelled UMP co-eluted with the major peak of $^3$H label confirming the identity of the label.

It was therefore necessary to include the Dowex-1 Cl$^-$ purification step in order to eliminate radioactive contaminants for the measurement of the incorporation of $^3$H-uridine into RNA.
Fig. 2.5  Co-elution of the Tritium label in the KOH hydrolysate with carrier UMP

The KOH hydrolysate of 25 explants grown for 17.5 hr. and incubated for 1 hr. with 25 μC of 3H-uridine of specific activity 5.8 C/m mole was mixed with 1.0 ml. of 0.3 mM uridine monophosphate (260 nm was 3.54) and chromatographed on Dowex-1 Cl⁻ as described for Fig. 2.3. The 260 nm OD (-----) and cpm (------) of the eluate (1.0 ml. fractions) was estimated.

Fig. 2.6  Kinetics of Uptake and Incorporation of 3H-uridine by explants

Flasks containing 60 explants (cultured for 24 hr.) in 5 ml. of culture medium were incubated for different periods with 25 μC of 3H-uridine (5.8 C/m mole). The RNA was extracted and purified through Dowex as previously described and the cpm incorporated into RNA measured (--------). Uptake was estimated by counting an aliquot of the first aqueous phase in the process of RNA extraction (--------).
Figure 2.5.

Figure 2.6.
(ii) Effect of Specific Activity of the $^3$H-Uridine on Uptake and Incorporation into RNA

Explants were incubated under two conditions: one in the presence of 25 $\mu$C of $^3$H-uridine of specific activity 290/m mole, (i.e. 0.86 mmoles uridine) and the other in the presence of 25 $\mu$C of $^3$H-uridine of specific activity 5.860/m.mole (0.86 mmoles $^3$H-uridine plus 3.6 mmoles of carrier uridine, i.e. in a total of 4.46 mmoles uridine). After 2hr. incubation, explants were washed with tap water, dried and 5 explants were homogenised in 2.5ml. of cold 5% TCA in a glass-in-glass homogeniser which was then washed with a further 2.5ml. of TCA(5%). The washing was combined with the initial homogenate and the RNA was allowed to precipitate for 1hr. at 0$^\circ$C. This was then centrifuged at 1500 x g for 10min. at 4$^\circ$C and an aliquot of the supernatant was counted as a measure of $^3$H-uridine uptake. Incorporation of $^3$H-uridine was measured as previously described.

Both the uptake and incorporation into RNA of the $^3$H-uridine were considerably stimulated by the increase in amount of uridine in the incubation (Table 2.7).
Effect of Specific Activity on the Uptake and Incorporation into RNA of $^3H$-Uridine by Explants

<table>
<thead>
<tr>
<th>Specific Activity</th>
<th>UPTAKE</th>
<th>INCORPORATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/explant</td>
<td>picomoles/explant</td>
</tr>
<tr>
<td>29 C/m mole (0.86m moles uridine)</td>
<td>5,270</td>
<td>0.205</td>
</tr>
<tr>
<td>5.8 C/m mole (4.46 m moles uridine)</td>
<td>28,492</td>
<td>5.500</td>
</tr>
</tbody>
</table>

30 freshly excised explants were incubated for 2hr. with $25 \mu C$ $^3H$-uridine with and without the addition of cold uridine.

In the absence of carrier uridine, approximately 40% of the total uptake (uptake + incorporation) was incorporated into RNA. Under these conditions, the incorporation is not likely to be an accurate measure of the rate of RNA synthesis.

In the presence of carrier uridine, uptake of radioactivity was five-times greater than in the absence of carrier, which, after correction for the lower specific activity, resulted in 25-times greater uptake. Similarly, incorporation of radioactivity into RNA was increased in the presence of carrier uridine and when corrected for specific activity represented an eightfold increase of incorporation.
(iii) Kinetics of Uptake and Incorporation of $^3$H-Uridine

The time course of uptake and incorporation of $^3$H-uridine into RNA was investigated using explants grown for 24 hr. in the culture medium (Fig. 2.6). The $^3$H-uridine was used at a specific activity of 5.8C/mmole, i.e., in the presence of added cold uridine. Under these circumstances, uptake was linear for 90 min. and incorporation into RNA for 120 min. During this 90 min. period, incorporation represented about 10% of uptake; an incubation period of 60 min. was chosen for measurement of the rate of RNA synthesis.

6. Preparation and Fractionation of RNA

The tissue was homogenised directly in 5 volumes of detergent medium (Kirby, 1965). The detergent medium contained 1% tri-iso propylphosphatidyl sulphate (TNS), 6% 4-aminosalicylate (PAS), 40 mM NaCl, 8 mM Tris HCl pH 7.4, and a drop of phenol to dissolve the PAS. Deproteinisation was effected by shaking with an equal volume of phenol mixture (o-phenol containing 10% m-cresol, 0.1% 8-hydroxy-quinoline, saturated with 0.01 M Tris HCl pH 7.4). The phases were separated by centrifugation at 1500 x g for 10 min. The phenol layer was discarded and the aqueous phase (and interface) was made to 0.5 M NaCl and re-extracted with phenol mixture. The aqueous phase was separated by centrifugation, removed, and re-extracted with an equal volume of phenol mixture. After centrifugation to separate the phases, the aqueous phase was removed and the nucleic acids precipitated by the addition of 2 volumes of ethanol and storage overnight at 0°C. The precipitated nucleic acid was collected by centrifugation at 1500 x g for 10 min. and taken up in 0.5% sodium dodecyl sulphate (SDS), dissolved in 0.15 M sodium acetate.
The nucleic acid was re-precipitated by the addition of two volumes of ethanol and washed twice by re-suspending in 80% ethanol containing 0.2% SDS. The precipitate was dissolved in electrophoretic running buffer containing 5% sucrose to give a concentration of approximately 1mg/ml.

Fractionation by gel electrophoresis

A stock solution, containing 15% acrylamide (Kodak, re-crystallised from chloroform) and 0.75% bisacrylamide (Kodak, re-crystallised from acetone), was used for the preparation of 2.2, 2.4, and 7.5% gels as shown in Table 2.8 (Loening, 1967).

<table>
<thead>
<tr>
<th>Gel concentration</th>
<th>2.2%</th>
<th>2.4%</th>
<th>7.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x buffer (ml.)</td>
<td>6.8</td>
<td>6.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Water (ml.)</td>
<td>22.0</td>
<td>18.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Stock acrylamide</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

The 5 x running buffer contained 180mM Tris, 150mM Sodium dihydrogen phosphate and 10mM disodium EDTA 2H₂O at pH 7.6-7.8 at room temperature.

The mixture of acrylamide, buffer, and water, was degassed under vacuum for 20sec. and 25 μl of N,N,N',N' tetramethylethylene diamine (TEMED) and 0.25ml. of a freshly-prepared 10% solution of ammonium persulphate were added to catalyse polymerisation. The solution was mixed gently by swirling and pipetted immediately into the gel tubes (6mm x 8.0cm) closed at the bottom with a PVC ring plus glass plug. Water was carefully layered on the top of the gel to produce a flat top and polymerisation was allowed to proceed at 25°C for 2hr.
In order to fractionate both the high molecular weight RNA i.e. ribosomal RNA (1.30 and $0.7 \times 10^6$ daltons) and low molecular weight RNA, i.e. 5S rRNA and 4S tRNA (3.5 and $2.5 \times 10^6$ daltons) split gels were used, composed of 5cm. of 2.2% gel formed on the top of 3cm. of 7.5% gel. The rate of polymerisation of these gels was reduced by working at 4°C and with half the concentration of the polymerising agents.

The advantage of leaving the polymerisation until after the composite gel was formed was that the interface so produced, caused less accumulation of ribonucleic acid migrating through the gel. After polymerisation the glass plug was removed from the bottom of the gel tube and the tubes were mounted vertically in an electrophoresis apparatus so that the top and bottom of the gels were immersed in the electrophoresis running buffer (5x diluted running buffer plus 0.2% SDS). Care was taken to remove all bubbles from the holes in the gel retaining rings. The gels were pre-run for 30min. at 50 volts to remove polymerisation catalysts and to allow SDS to enter the gel. The RNA (25 µl containing 25 µg nucleic acid) was loaded under a small voltage gradient in 5% sucrose to allow the RNA to enter the gels and counteract diffusion. The gels were run at 50V (about 5-6mA/tube) for 1½-4hr. depending on the degree of separation required. The length of each component of the split gels (2.2% and 7.5%) was calculated to allow the 4S and 5S RNA to penetrate the 7.5% gel and there be resolved whilst the ribosomal RNAs were resolved in the overlying 2.2% gel. This separation was achieved after 3.25hr. electrophoresis.
The gels were washed in distilled water for 30-60 min. and scanned in either a Joyce Loebl Chromoscan or Joyce Loebl Polyfrac u-v scanner coupled with a Servoscribe Recorder. Both were fitted with a medium pressure mercury lamp, and light at 265 nm was selected by use of an interference filter plus a liquid filter (paradimethylaminobenzaldehyde 10-12 mg/100 ml. methanol). A quantitative distribution of RNA components was estimated from the areas of the 260 nm OD peaks determined by weighing a tracing of the peak.

In order to line up the 260 nm OD scan and radioactive profile, the gel was injected with Indian Ink, usually immediately in front of the heavy and light ribosomal RNA peaks. The gel was frozen in an aluminium trough lying horizontally on powdered solid carbon dioxide. The length of the gel was fixed accurately, to be the same as that when scanned, by means of polystyrene blocks held in the aluminium trough. The frozen gel was then sliced on a Mickle gel slicer into 0.5 mm sections for radioactivity measurement. Slices containing $^{32}$P-labelled RNA were dried on every second frame of the non-emulsion side of 16 mm film and counted with a Geiger tube connected to a programme unit, scaler, and print-out. With $^3$H-uridine-labelled RNA, gel slices were placed in scintillation vials and the RNA hydrolysed by one of two methods. Slices were incubated in 10% piperidine containing 1 mM EDTA at 60°C for 48 hr. The piperidine was removed by opening the vials and allowing it to evaporate. Water (0.5 ml.) was added, followed by 4.5 ml. of Triton-toluene scintillator (1 part Triton X 100:2 parts toluene scintillator).
Alternatively, the slices were incubated with 1ml. of 1.0N NaOH for 3hr. at room temperature. Triton-toluene scintillator (9.0ml.) was added and the vial counted in a Packard scintillation counter. It was necessary to allow the white precipitate, formed on adding the scintillator, to dissolve before counting (10min.) (Table 2.9). The NaOH present in the aqueous phase did not interfere with the counting efficiency of the triton-toluene scintillator (Table 2.10).

Table 2.9  Counting Efficiency after Mixing the Triton-Toluene Scintillator and Aqueous Phase

<table>
<thead>
<tr>
<th>Minutes after Mixing</th>
<th>c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>193,303</td>
</tr>
<tr>
<td>2</td>
<td>252,925</td>
</tr>
<tr>
<td>3</td>
<td>315,908</td>
</tr>
<tr>
<td>4</td>
<td>351,006</td>
</tr>
<tr>
<td>5</td>
<td>356,139</td>
</tr>
<tr>
<td>6</td>
<td>354,391</td>
</tr>
<tr>
<td>10</td>
<td>341,413</td>
</tr>
<tr>
<td>15</td>
<td>341,048</td>
</tr>
<tr>
<td>20</td>
<td>341,242</td>
</tr>
<tr>
<td>30</td>
<td>341,209</td>
</tr>
</tbody>
</table>
Table 2.10  Effect of Alkali on the Counting Efficiency of Triton-Toluene Scintillator

<table>
<thead>
<tr>
<th>Sample</th>
<th>c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0N NaOH</td>
</tr>
<tr>
<td>1</td>
<td>343,713</td>
</tr>
<tr>
<td>2</td>
<td>350,791</td>
</tr>
<tr>
<td>3</td>
<td>288,704</td>
</tr>
<tr>
<td>Average</td>
<td>327,736</td>
</tr>
</tbody>
</table>

Triplicate samples of 1 μC $^3$H UTP in 1.0N NaOH or water were made up to 1ml., 9.0ml. of triton-toluene scintillator was added and the samples counted after 10min.

Optimal gain setting for the Packard using this scintillator was determined as 60-70% which gave an efficiency of counting tritium of about 15%.

C. RNA Polymerase Assay

The RNA polymerase assay used was based on that of O'Brien, Jarvis, Cherry and Hanson (1968), and depends on the incorporation of $^3$H-uridine triphosphate ($^3$H-UTP) into a TCA insoluble product.

The final assay system adopted, and details relating to some of the components of the assay, will be described in this Methods Section, while aspects of the development of the system will be dealt with in the Results Section.

Aliquots (0.2ml.) of RNA polymerase preparation were assayed in a final volume of 0.4ml. by incubation at 30°C for 10min. with a mixture containing 1.0 μmole MgCl$_2$, 0.5 μmole MnCl$_2$, 0.2 μmoles
each of CTP, GTP and ATP, 10 μ moles of Tris HCl pH 8.0, 0.2 μ moles dithiothreitol, and 2 μ C of ³H-UTP of specific activity 1.4C/m mole (i.e. containing 1.5 μ moles of UTP). A DNA template was included in the assay of soluble polymerase at 50 μ g/assay but not in assay of chromatin-bound activity. The reaction was terminated by the addition of 2.0 ml. of ice-cold 5% TCA containing 0.01M tetrasodium pyrophosphate and left for 1 hr. at 0°C to precipitate the nucleic acids. The contents of the reaction tube were then transferred to a GF/C glass-fibre 2.1 cm. disc (held in a Millipore filter apparatus) and the filter then was washed with 50 ml. of cold 5% TCA containing 0.01M pyrophosphate, followed by 5 ml. of 95% ethanol to remove the TCA (under gentle suction).

The filters were dried under an Ecko 250 W infra-red lamp for 10 min. to evaporate off any remaining TCA and counted by scintillation counting on either a Packard (60% gain) or an Inter-technique (pre-set tritium channel) scintillation counter. The scintillator used contained either 10 g. butyl PBD (2- (4'-t- butylphenyl) -5- (4'' biphenyl) -1,3,4-oxadiazole) (CIBA) in 2.5 litres of toluene or 12.5 g. of PPO (2,5-diphenyloxazole, Scintillation grade) (Packard), 0.75 g. dimethyl POPOP (Phenyloxozolyl-benzene, Scintillation grade) (Packard) in 2.5 litres of toluene, with no detectable difference in counting efficiency.

The time course of precipitation of TCA insoluble counts was investigated following the termination of the reaction (Fig. 2.7). After 2 hrs. there was a reduction in the counts incorporated into the TCA precipitate, probably due to the acid hydrolysis of the RNA.
Replicate RNA polymerase assays were terminated by the addition of 5% TCA (±0.01M sodium pyrophosphate) and stored at 0°C for different periods before filtering and washing as described in the text.

Aliquots (0.05ml from 5ml.) of the washing from the GF/C filters were counted to determine the efficiency of removal of excess $^3$H-UTP from the filters.
The efficiency of washing the GF/C filters was followed by counting aliquots of sequential 5ml. washes of TCA (Fig.2.8). A 50ml. TCA wash was adequate to remove all the free $^3$H-UTP from the TCA precipitate.

1. **Stability of $^3$H-uridine Triphosphate**

The stability of the $^3$H-UTP (Amersham) was examined with respect to storage at -20°C, and during the course of the assay. High voltage paper electrophoresis in 0.75% pyridine and 7.5% acetic acid was used to separate the $^3$H-UTP from the nucleoside mono and di-phosphate. Solutions were applied by microcaps as spots on the baseline to dry Whatman No.1 Paper, and a marker dye containing 0.5% orange G, 0.5% Acid Fuchsin in 50% ethanol, was also spotted.

Each spot was ringed with buffer to concentrate it, and the rest of the paper was carefully wetted. Excess buffer was removed by blotting and the loaded paper placed in the electrophoresis tank with each end in buffer and immersed totally in white spirit (non-conductive and heat-absorbant). The electrodes were so arranged that the nucleotides ran down the length of the paper towards the anode.

The electrophoresis was carried out at room temperature at a potential difference of 1000 volts for 1-3hr. and the progress of electrophoresis monitored by the separation of the marker dyes. When completed, the electrophoresis paper was removed from the tank and air-dried overnight. Irradiation with u-v light showed up the nucleotides as clear spots on a browned background.
The paper was cut up into strips and the radioactivity of each strip determined by scintillation counting. Unlabelled mono-, di-, and tri-phosphates were clearly separated after 1hr45min. Ethanol and aqueous stored (4 months at -20°C) <sup>3</sup>H-UTP were co-electrophoresed (i.e. loaded on the same spot) with uridine mono-, di- and tri-phosphate. Storage in aqueous solution resulted in greater than 50% conversion of labelled uridine triphosphate to the diphosphate whereas the integrity of the <sup>3</sup>H-UTP was maintained in ethanol (Fig.2.9). The degradation observed in aqueously stored <sup>3</sup>H-UTP was a result of radiation damage, whereas the integrity of the labelled nucleotide stored in the ethanol reflected the absorbance of the energy of radiation by the solvent molecules.

It was important when fractionating very small amounts of tritium labelled molecules (picomoles) by paper electrophoresis to co-electrophorese with a range of cold carrier molecules. When <sup>3</sup>H-UTP (1.7 picomoles) was co-electrophoresed with either 0.5 moles of carrier UMP, UDP, or UTP, the <sup>3</sup>H-UTP ran with, or just ahead of, the carrier, despite its difference in composition (Fig.2.10). Only when <sup>3</sup>H-UTP was co-electrophoresed with a mixture of the nucleotides did it run characteristically as UTP, i.e. overlying the UTP OD spot (Fig.2.10).

The purity of the Amersham <sup>3</sup>H-UTP was also checked by descending paper chromatography on Whatman No.1 Paper using isobutyric acid:water:ammonia: 0.1M EDTA (100:56:4.2:1.6). <sup>3</sup>H-UTP was co-chromatographed with the nucleoside mono-, di-, and tri-phosphates, both separately and together.
Fig. 2.9  **Analysis of the Tritium label in ethanol and aqueous stored samples of $^3$H-UTP**

Ethanol (.....) and aqueous (_____ ) stored samples of $^3$H-UTP (for 4 months at -20°C) were co-electrophoresed with uridine mono-, di- and tri-phosphate for 1hr. 50min. at 1000 volts and the distribution of the label determined. The location of the OD spots is shown on the figure.
Fig. 2.10  Co-electrophoresis of $^3$H-UTP with Uridine mono-
di- and tri- phosphate

$^3$H-UTP (ethanol-stored at -20°C) was co-electrophoresed with A) a mixture of uridine mono- (-----), di- (......), and tri- (-----) phosphate and B) with the uridine nucleotides separately, for 2hr. at 1000 volts. The location of the OD spots is shown for both cases and the distribution of the tritium.
Figure 2.10.
The chromatography paper was suspended in a tank with its upper edge held in a trough and the lower edge (serrated to ensure even flow down the paper of the solvent) hanging freely, with the atmosphere saturated with buffer. After equilibration of the paper for 3 hr., the running solvent was added to the upper trough and the chromatogram allowed to run for 18 hr. after which time it was removed and air-dried. OD spots were located by u-v absorption and the tritium distribution was determined by autoradiography. An X-ray plate was laid over the chromatography paper and held firmly in place by glass sheets for 7 days. After this time the plate was developed and tritium areas located as darkened spots. This method clearly fractionated picomole quantities of nucleotides and the distribution obtained of the tritium label was similar when co-chromatographed with carrier UMP, UDP, or UTP separately or together.

This method confirmed the integrity of $^3$H-UTP from Amersham. After storage for 4 months at -20°C, a second spot appeared running in the same position as UDP; thus confirming the electrophoretic analysis. The other three nucleotides (GTP, CTP and ATP) were analysed by both techniques and there was little contamination observed by mono- and di-phosphates in freshly-prepared solutions.

2. Preparation of DNA Templates for RNA Polymerase Assay

High molecular weight DNA was extracted from calf thymus gland tissue and artichoke (shoots) tissue. Calf thymus glands were frozen soon after removal from the animal and stored at -20°C prior to use.
A 10g. portion was cut into small pieces without thawing and homogenised in the Virtis for 30sec. at high speed, in 50ml/g of SSC (standard saline citrate), 0.15M NaCl and 0.015M sodium citrate. The homogenate was centrifuged at 3500rpm for 15-20min. at 0°C. The supernatant was discarded and the precipitate taken up in 50ml. of detergent medium containing 1% TNS, 6% PAP, 50mM NaCl, 10mM Tris pH 7.8, 10mM EDTA, and 6% butanol. 5M NaCl was added to bring the salt concentration of the solution to 0.5M. An equal volume of chloroform/amyl alcohol (25:1) was added and the mixture was shaken by gentle inversion of the tube (to avoid shearing the DNA). The phases were separated by centrifugation for 10-15min. at 0°C at 2000 x g and the aqueous phase removed and further deproteinised by gentle stirring with an equal volume of phenol mix. The aqueous layer was re-extracted by a further phenol treatment and the nucleic acids precipitated by the addition of 2 volumes of ethanol and storage at 0°C for 12hr.

The DNA was removed from the ethanol by either winding out of solution on a glass rod (with large quantities of DNA) or centrifugation (for small quantities of DNA). The precipitate was washed with 80% alcohol containing 0.2% SDS and dissolved in 0.1 x SSC; SDS and sodium acetate was added to a final concentration of 0.5% and 0.15M respectively. The DNA was precipitated by adding 2 volumes of ethanol and the pellet of nucleic acid obtained by centrifugation was dissolved in 0.1 x SSC, which was subsequently increased to SSC by the addition of 10 x SSC.
A DNAase free RNAase was prepared by heating RNAase (1mg/ml in SSC) to 80°C for 10min. to inactivate the contaminating DNAase. The nucleic acid preparation was incubated with the DNAase free RNAase for 2hr. at 37°C (at a final concentration of 100μg/ml). A solution of pronase was prepared (4mg/ml) and allowed to self-digest at room temperature for 10-15min. in order to remove any contaminating DNAase and then added to the DNA solution to a final concentration of 200μg/ml. After incubation at 37°C for 2hr. DNA was recovered by centrifugation at 12000 x g for 18hr. The supernatant was poured off and the tubes allowed to drain. The DNA was dissolved in 0.1 x SSC and stored frozen at -20°C.

Denatured DNA was prepared by heating a sample of native DNA in 0.1 x SSC at 100°C for 15min. and then rapidly cooling in ice. The denatured DNA was stored frozen at -20°C. It was noted that after protracted periods of storage, some gelatinization occurred in the DNA. These gel particles were removed by centrifugation at 2000 x g before use as template DNA, since use of such a heterogeneous sample resulted in high and variable background levels of radioactivity in the RNA polymerase assay.

Activated DNA was made by the method described by Aposhian and Kornberg (1962). Double-stranded DNA was dissolved in a solution of 5mM MgCl₂, 25mM Tris HCl pH 8.0 containing 0.5mg/ml BSA and 20μg/ml DNAase I (Worthington) to give a concentration of about 1mg/ml DNA. The reaction mixture was incubated for 30min. at 30°C and then heated to 65°C for 5min. to terminate the reaction.
This treatment introduced single-stranded nicks into the DNA template, effectively increasing the number of ends without decreasing the molecular weight.

The DNA was characterised by caesium chloride equilibrium centrifugation with a Beckman Model E analytical centrifuge. 3 \( \mu \)g of DNA (determined by OD) was made up to 0.77ml. with 0.1 x SSC, and 1g CsCl added; 1 \( \mu \)g of *Micrococcus lysodeikticus* DNA (buoyant density 1.731g cm\(^{-3}\)) was included as a marker. The density was adjusted to 1.707g/cm\(^{-3}\) by monitoring the refractive index (to be between 1.3995 and 1.4005). The sample was centrifuged at 44000rpm for 20hr. at 25\(^{\circ}\)C in the AN-F head and the distribution of DNA determined by photographing the absorption of u-v light on Ilford N\(_4\) E50 film. The photographs were scanned using the Joyce Loebl scanning microdensitometer. The molecular weight of the sample of DNA was estimated by the spill-over method described by Studier (1965). The Model E determinations were kindly performed by J.Sinclair.

**D. Preparation of Columns used in Polymerase Studies**

A variety of columns have been used to fractionate RNA polymerase from artichoke explants.

1. **Bio-gel, P Series**

   Bio-gel (Calbiochem) is a porous polyacrylamide gel, in which the degree of cross-linking can be controlled during its manufacture. The cross-linking restricts the extent of hydration when the gel is put in buffer, and hence controls the pore size of the gel.
Biogel P₄ (50-100 mesh) was used extensively to desalt enzyme extracts following \((\text{NH}_4)_2\text{SO}_4\) precipitations and to desalt eluates from columns eluted by salt gradients. The exclusion limit is 3,600 M\(_\text{ol}\) weight and the gel has a water regain value of 2.6g liquid/g.dry gel. The biogel was prepared for column chromatography by adding standard buffer (5% glycerol, 0.05M Tris HCl pH8.0, 0.5mM dithiothreitol and 5mM MgCl\(_2\)), and leaving overnight with stirring. Prior to use, approximately 20 bed volumes of buffer was pumped through the freshly-prepared column.

2. Phosphocellulose

1 vol. of P11 phosphocellulose powder (Whatman) was added to 15 volumes of 0.5N KOH and stirred for 30min. The slurry was poured into a large column (30 x 4cm.) and washed with distilled water until the eluate was pH8.0. The neutralised P11 was added to 15vol. of 0.5N HCl and stirred for 30min. The slurry was washed with distilled water as before until the pH of the eluate approached neutral, re-suspended in distilled water and titrated against KOH until the pH was 7.9 at 25°C. The columns (0.9 x 10cm.) were poured in the cold room at 4°C away from convection currents and equilibrated extensively with standard buffer (previously described).

3. Diethylaminoethyl Cellulose DE₃₂

A weighed quantity of DE₃₂ was stirred into 15vol. of 0.5N HCl and left for 30min. The supernatant was decanted and the slurry was washed in a column with distilled water until the pH of the eluate was 4. The DE₃₂ was then stirred into 15vol. of 0.5N NaOH and left for 30min.
After settling, the supernatant was poured off. The alkaline treatment was repeated and then the slurry was washed in a column until the eluate was approximately neutral. The DE32 cellulose was placed in the acid component of the buffer (0.5N HCl) and the pH checked to be below 4.5. The solution was stirred under vacuum (less than 10cm. of mercury) to remove dissolved carbon dioxide. This degassing step was essential in order to produce reproducible elution profiles of protein and RNA polymerase activities from the DEAE fractionation.

The alkaline component of the buffer (1M Trizma base) was added to adjust the pH to 8.0. The DE32 cellulose slurry was stirred into buffer (composed of 5% glycerol, 50mm Tris HCl pH8.0, 5mM MgCl₂ and 0.5mM dithiothreitol) so that the buffer to DE32 cellulose ratio was approximately 15-30ml/initial gm. DE32 cellulose. The supernatant was decanted and the slurry poured into a column. Buffer was passed through the column until the pH of the eluate was exactly the same as the buffer (approximately 2 litres). The equilibrated DE32 cellulose was then suspended in buffer so that there was about 6ml. buffer:1ml. of wet ion exchanger.

The slurry was allowed to settle in a suitable measuring cylinder and fines were removed by allowing a certain time for settling calculated by \( t = nh \) (where \( t \) is the time in minutes, \( h \) is the total height of the slurry in the measuring cylinder in cm., and \( n \) was chosen to be 2 as this removed sufficient fines to give the required flow rate for the column).
The "wet settled volume" was noted and the supernatant buffer (plus fines) was removed so as to leave the "wet settled volume" plus 20%. The slurry was made up with buffer to a final volume of 150% of the "wet settled volume". Freshly-prepared sodium azide (NaN₃) was added to the DE32 cellulose slurry to a final concentration of 0.01%, to prevent bacterial growth, and the ion exchanger was stored at +4°C until required for use. Prior to use, a column (10 x 0.9cm.) was prepared and washed for 2.5hr. with standard buffer. This served to remove the azide and pack and equilibrate the column.

4. DNA cellulose affinity chromatography

The affinity between RNA polymerase and DNA was exploited for the purification of the enzyme from a crude extract. Some of the problems involved in the synthesis of a suitable substrate for affinity chromatography, the binding and subsequent elution of the enzyme in question have been outlined by Cuatrecasas and Anfinson (1971).

There are two methods by which DNA can be bound to a suitable matrix. The DNA may be coupled by covalent bonds to agarose after activation by cyanogen bromide as described by Poonian, Schlabach and Weissbach (1971). Alternatively, DNA may be immobilised onto cellulose by u-v irradiation (Litman,1968). The latter method was used to synthesise native and denatured calf thymus DNA-celluloses.
20g. of cellulose powder CFI (Fibrous Powder, Whatman Column chromocinedia) was washed by stirring for 10min. in 400ml. of 1N HCl. The cellulose was then washed with distilled water until the eluate was approximately pH 7. This acid treatment of cellulose was repeated, the cellulose washed to neutral, and finally air-dried overnight. The native or denatured calf thymus DNA was prepared as described previously. The DNA and cellulose (1mg.DNA to 1g.cellulose) were kneaded together with a spatula to achieve adequate mixing, then spread thinly over the surface of a 100ml. evaporating dish and allowed to air-dry overnight. The DNA-cellulose mixture was freeze-dried for 4hr. and then irradiated for 45min. at 10cm. with a u-v source delivering 1500erg./mm²/40sec. i.e. with 90,000erg. The DNA bound to the cellulose was estimated by washing a weighed amount of the irradiated DNA-cellulose with standard buffer until all the unbound DNA had been removed. The 260nm. OD of the washings was monitored and from this the unbound DNA could be estimated on the basis that 1mg/ml.DNA absorbs 25 OD units. Hot PCA hydrolysis of the washed DNA-cellulose gave an estimate of bound DNA (as previously described for the estimation of TNA from plant tissue).

Lysphilisation alone, without the u-v treatment, bound 94% of native DNA to the cellulose and 71% of the denatured DNA. After u-v irradiation, 92% of the native and 73% of the denatured DNA was bound to the cellulose (Table 2.11), it was therefore concluded that the u-v treatment did not substantially enhance binding of the DNA to the cellulose.
## Table 2.11  Efficiency of binding of DNA to Cellulose

<table>
<thead>
<tr>
<th>Input DNA mg/g cellulose</th>
<th>mg DNA/g cellulose</th>
<th>Unbound DNA removed by washing</th>
<th>Bound DNA estimated by PCA hydrolysis</th>
<th>%Bound (estimated from PCA hydrolysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native lyophilised</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.63</td>
<td>0.46</td>
<td>2.48</td>
<td></td>
<td>94</td>
</tr>
<tr>
<td>Native lyophilised + u-v treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.63</td>
<td>0.20</td>
<td>2.40</td>
<td></td>
<td>92</td>
</tr>
<tr>
<td>Denatured lyophilised</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.97</td>
<td>1.26</td>
<td>2.12</td>
<td></td>
<td>71</td>
</tr>
<tr>
<td>Denatured lyophilised + u-v treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.97</td>
<td>0.74</td>
<td>2.15</td>
<td></td>
<td>73</td>
</tr>
</tbody>
</table>

The DNA bound by these treatments was stable to elution of the column by the salt gradient used in the chromatographic procedure.
RESULTS

SECTION I: RNA Polymerase from Artichoke Tissue

The assay conditions used for the estimation of RNA polymerase activity have been described. The conditions were derived from investigations of the individual factors involved in the assay of either chromatin-bound or soluble activities.

1. Preparation and Properties of Chromatin-bound RNA Polymerase activity

Chromatin was prepared by the method described by Huang and Bonner (1962). The artichoke tubers (for bulk preparation) were peeled, weighed and sliced into cold homogenising medium containing 0.05M Tris HCl pH8.0, 50mM β mercaptoethanol, 5mM MgCl₂, 5% glycerol, 1mg/ml. pyrrolidine vinyl phosphate (PVP), (30g tissue:50ml homogenising medium), and the tissue was homogenised for 5sec. at full speed of the polytron homogeniser. The homogenate was filtered through 1 layer of miracloth and the liquid squeezed out of the residual matter. The filtrate was centrifuged at 10,000 x g for 10min. at 0°C. The supernatant was discarded and the gelatinous chromatin (pellet 1) was scraped from an underlying layer of dark-coloured material. The chromatin was re-suspended in a glass-in-glass homogeniser (Kontes C) in 10ml. of wash medium, containing 0.05M Tris HCl pH8.0 and 50mM β mercaptoethanol, and then centrifuged at 10,000 x g for 10min. to re-pellet the chromatin (pellet 2). The pellet was taken up in 2ml. of wash medium, layered on 19ml. of 75% glycerol containing 50mM β mercaptoethanol and centrifuged at 40,000 x g in a SW30 swing out rotor for 2hr.45min. at 4°C. The purified chromatin pellet (pellet 3) was suspended in 2ml. of suspending medium (0.15M Tris HCl pH8.0 and 0.5mM dithiothreitol) and assayed for RNA polymerase activity, as described in the Methods, without the addition of exogenous DNA.
The Kinetics of Incorporation of $^3$H-UTP

The kinetics of incorporation of $^3$H-UTP (1.3C/m mole) by the chromatin-bound polymerase activity was investigated over a 30min. incubation of 30°C (Fig.3.1). After a short initial lag the rate of incorporation was reasonably linear for 10min. before flattening off. The possibility that the decrease in rate of incorporation was due to removal of $^3$H-UTP from the incubation medium, was investigated by high voltage electrophoretic analysis of medium after 0 and 15min. incubation.

Aliquots of incubation medium were co-electrophoresed with uridine mono-, di- and triphosphate (Fig.3.2a). The label was present predominantly as UTP with some UDP in the 0 time medium, and after 15min. incubation the label was found in UTP and UMP with about 50% still as UTP.

The 0 and 15min. incubation media were mixed and electrophoresed together to check that no interfering substance was produced during the course of the incubation which affected the electrophoretic mobility of the nucleotides (3.2b). The distribution of tritium observed was essentially similar to the sum of the separate fractionations indicating no production of contaminants during incubation. The decrease of rate of incorporation after 10min. cannot be explained by lack of UTP in the medium and may reflect on inherent instability of the enzyme or perhaps a saturation of available template with newly-synthesised RNA which is not released under these conditions of assay.

Routinely, a 10min. incubation was used as a measure of the rate of incorporation.
Fig. 3.1 Kinetics of Incorporation of $^3$H-UTP into a TCA insoluble Fraction by chromatin-bound RNA polymerase

The incorporation is expressed as cpm. incorporated above background (i.e. the zero time value, which was 110cpm). Each value shown is the average of three measurements. The specific activity of $^3$H-UTP used was 1.30/cmmole (i.e. 2$\mu$C added plus 1.35 $\mu$moles carrier UTP).

Fig. 3.3 Dependence of chromatin-bound RNA polymerase activity on UTP concentration of the assay medium

The UTP concentration of the assay medium was varied by the addition of cold UTP to 2 $\mu$C of $^3$H-UTP.
Fig. 3.2  Analysis of the Tritium label in the assay medium before and after incubation with the chromatin preparation. Aliquots of the 2,000xg supernatant from zero (-----) and 15 min. (---------) incubations of the assay medium with chromatin were co-electrophoresed A) separately and B) together with a mixture of the three uridine phosphates for 1 hr. 50 min. at 1000 volts. The locations of OD spots of the marker nucleotides are shown and the distribution of tritium shown as a histogram.
Figure 3.2.
b) Substrate Dependency

The dependency of the rate of incorporation of $^3$H-UTP by chromatin-bound RNA polymerase on substrate concentration was investigated. RNA polymerase requires the presence of a DNA template and all four nucleotides for activity; $^3$H-UTP was chosen to study as it was present in the assay at one thousandth of the concentration of the other nucleotides and would therefore be rate-limiting. The initial rate of incorporation was determined at various levels of UTP by varying the specific activity, maintaining 2 $\mu$C/assay and adding carrier UTP to the reaction mixture. The rate of the reaction, measured as picomoles UTP incorporated per min. was plotted against the UTP concentration in the assay (Fig.3.3), and over the range of UTP concentrations shown the RNA polymerase was not fully saturated by substrate although there was a marked flattening of the curve after 0.5 $\mu$m moles UTP, which was $10^3$ to $10^4$ times the calculated $K_m$ (calculated from a Lineweaver-Burk plot of the results shown in Fig.3.3) for UTP of this enzyme.

The dependence of $^3$H-UTP incorporation upon the presence of the other 3 nucleotides was examined in order to confirm the identity of the RNA polymerase reaction. The results in Table 3.1 confirm the necessity of the presence of all four nucleotides.
Table 3.1  Effect of Omission of Nucleotides from the Assay of Chromatin-bound Polymerase Activity

<table>
<thead>
<tr>
<th></th>
<th>cpm</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (all four nucleotides)</td>
<td>850</td>
<td>100</td>
</tr>
<tr>
<td>Minus GTP</td>
<td>89</td>
<td>11.5</td>
</tr>
<tr>
<td>Minus CTP, ATP, GTP</td>
<td>77</td>
<td>9.0</td>
</tr>
</tbody>
</table>

All the media used in the preparation and assay of the chromatin were either autoclaved or freshly prepared to reduce possible bacterial contamination. Without these precautions, the percentage inhibition due to the omission of various nucleotides was much less. Activities are expressed as cpm incorporated above background (105cpm) and are averages of replicate assays.

Although the chromatin-bound polymerase preparation was active in the absence of exogenous DNA, presumably utilising DNA of the chromatin as template, it was of interest to examine the response of this activity to added DNAs. The addition of native or denatured calf thymus DNA (both at 50 μg) to the assay inhibited or stimulated the chromatin-bound activity respectively (Table 3.2).
Table 3.2  Effect of Exogenous DNA on Chromatin-bound Polymerase Activity

<table>
<thead>
<tr>
<th>Exogenous DNA added</th>
<th>cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>258</td>
</tr>
<tr>
<td>50 μg native calf thymus DNA</td>
<td>95</td>
</tr>
<tr>
<td>50 μg denatured calf thymus DNA</td>
<td>356</td>
</tr>
</tbody>
</table>

Activities are expressed as cpm incorporated above background (1000cpm) and are averages of replicate assays.

This data suggests that there was competition between exogenous and endogenous DNA template for the enzyme, and that the denatured DNA was more efficient than, and native DNA less efficient than, the endogenous template.

c) Dependence on Enzyme Level

A characteristic property of enzymic reactions is that in non-limiting conditions the rate of reaction is proportional to the amount of enzyme present. This was confirmed for chromatin-bound RNA polymerase by incubating increasing quantities of the chromatin preparation under constant assay conditions (Fig.3.4). This approach makes the assumption that the amount of enzyme bound to the chromatin is limiting the rate of reaction and not the availability of template. In the presence of excess enzyme on the chromatin, addition of further chromatin would only give a measure of template availability.
RNA polymerase activity as a function of chromatin concentration in the assay medium

The polymerase activity is expressed as cpm incorporated above background (105 cpm) and corrected for $^3$H absorption by protein according to Fig. 3.5. Each point is the average of replicate assays.

Counting Efficiency of Tritium in the presence of chromatin

Aliquots of chromatin were added to replicate RNA polymerase assays immediately after termination of the reaction. The radioactivity detected after filtering and washing is expressed as a percentage of the control assay which contained 250 $\mu$g chromatin protein. Each point is the average of replicate determinations.
It was, however, necessary to correct for self-absorption of the tritium, which increased as more chromatin was added. Therefore, after termination of the reaction by the addition of cold 5% TCA, increasing amounts of chromatin were immediately added, and the samples were washed and filtered by the normal method. The effect of this added chromatin on the efficiency of tritium counting is shown in Fig.3.5. Over the range 250 to 750 μg total protein (i.e. 0-500 μg protein added) there is a reasonably linear reduction of counting efficiency with increasing protein added of 12.5%/100 μg and this estimate was used to correct the data presented in Fig.3.4. The corrected data demonstrated a linear relationship between the rate of incorporation of $^{3}$H-UTP and the amount of chromatin added.

d) Effect of Divalent Ions

The conditions for optimal polymerase activity vary with different tissues especially with respect to the nature and concentration of divalent ions. The effect of a range of manganese and magnesium concentrations varied both independently (in the absence of the other ion), and in combination on the activity of chromatin-bound RNA polymerase was therefore examined. In the absence of magnesium, increasing concentrations of manganese up to 2.5 mM (1 μmole per assay) stimulated RNA polymerase activity, and at higher concentrations inhibited the activity (Fig.3.6). At the optimal concentration of manganese, RNA polymerase activity was 150% that of the control which contained 2.5 mM $\text{Mg}^{2+}$ and 0.625 mM $\text{Mn}^{2+}$ (as defined in Methods). The optimal concentration of magnesium (in the absence of manganese) was also 2.5 mM (Fig.3.6) and under these conditions the polymerase activity was slightly less than the control.
Fig. 3.6  Effect of Manganese and Magnesium on the activity of chromatin-bound RNA polymerase

Polymerase activity is expressed as a percentage of control incorporation (415cpm) measured above a background of 100cpm, which was assayed in 2.5mM magnesium and 0.625mM manganese. Each point is the average of four determinations using chromatin from two preparations; the range shown indicates the variation between assays and preparations. The effect of manganese in the absence of magnesium is shown by and the effect of magnesium in the absence of manganese by ——.

Fig. 3.7  The Interaction of magnesium and manganese ions on the activity of chromatin-bound RNA polymerase

The effect of increasing levels of manganese added to the assay is shown in the absence of magnesium (——) and the presence of 0.625mM (——), 1.25mM (—–) and 2.50mM (-----) magnesium. Each point is the average of four determinations and is expressed as a percentage of control incorporation (320cpm) above background. The background was 115cpm.
Figure 3.6.

Figure 3.7.
Interaction of manganese and magnesium was investigated by determining polymerase activity in the presence of a range of manganese concentrations at 4 different levels of magnesium (Fig. 3.7). The presence of magnesium in the assay reduced the optimal concentration of manganese from 2.5mM to 1.25mM and reduced the stimulation from 165 to 100% of the control. At low levels of manganese (less than 1.25mM) increasing magnesium levels stimulated the polymerase activity to 100% of the control value. At higher levels of manganese (greater than 1.25mM) the inhibition of activity was unaffected by the different levels of magnesium used, so that this inhibition does not seem to be a simple function of ionic concentration. The chromatin-bound polymerase appears to be predominantly manganese dependent and is partially inhibited by magnesium. Paradoxically, at low manganese concentrations, magnesium stimulates the polymerase activity.

e) Effect of Monovalent Ions

Both procaryotic and eucaryotic RNA polymerases have been assayed in the presence of a high concentration of monovalent ions. The activities of the different eucaryotic polymerases have been shown to be affected by monovalent salt concentration which has also been shown to be important in the release of newly-synthesised RNA from the template and subsequent re-initiation.
Both ammonium sulphate and potassium chloride stimulated polymerase activity (Fig.3.8); with ammonium sulphate the optimum concentration was 62.5mM which stimulated the activity to 210% of the control (incubated in the absence of added salt), whereas with potassium chloride, a much higher concentration was required (300mM) to optimally stimulate the polymerase activity (to 160% of the control). Both salts inhibited activity at higher concentrations. The kinetics of incorporation indicated an increased rate of synthesis in high salt which remained linear at 25min. by which time the control was flattening (Fig.3.9).

f) Regulators of RNA polymerase Activity

The intracellular level of polyamines has been postulated as a control mechanism for RNA polymerase activity in both eucaryotes and procaryotes. Since the level of intracellular spermidine has been correlated with that of RNA in many systems, the in vitro effect of spermidine on chromatin-bound RNA polymerase was investigated (Fig.3.10). Spermidine increasingly inhibited the polymerase activity with increasing concentration and at 11.5mM inhibited 50%. The toxin, αamanitin, extracted from Amanita phalloides specifically inhibits only one of the eucaryotic RNA polymerases (polymerase II). Fig.3.11 shows that the chromatin-bound activity was partially inhibited (by 20%) by 0.1μg amanitin added per assay, a level which completely inhibited the activity of soluble polymerase II (see Page 78).
The effect of monovalent cations on the activity of Chromatin-bound RNA polymerase

The effect of increasing levels of (a) ammonium sulphate and (b) potassium chloride on chromatin-bound RNA polymerase activity is shown. Activities are expressed as a percentage of cpm incorporated, above background (350cpm), of the control in the absence of added salt. Background was 98cpm.

Fig. 3.9 Effect of high salt on the kinetics of chromatin-bound RNA polymerase

Replicate assays were incubated in the absence (-----) and presence (—) of 0.32 M KCl. The activities shown are the average of replicate assays and are expressed as cpm incorporated above background (100cpm).
Fig. 3.10  **Effect of spermidine on chromatin-bound RNA polymerase activity**

Each point shown is the average of replicate assays and is expressed as a percentage of cpm incorporated above background of the control in the absence of spermidine (420cpm). Background was 110cpm.

Fig. 3.11  **Effect of α-Amanitin on chromatin-bound RNA polymerase activity**

The values shown are the average of replicate assays and expressed as cpm incorporated above background. The activities were assayed under standard conditions in the absence of additional KCl. In the presence of 0.33M KCl the percentage inhibition by 2.5μg αamanitin per assay was also 20%.
Figure 3.10.

Figure 3.11.
Preparation and Properties of Soluble RNA Polymerase Activity

The soluble RNA polymerase activity was derived directly from the supernatant of the initial 10,000 x g centrifugation of the tissue homogenate as described for the preparation of chromatin. The soluble activity is probably nuclear in origin, as, during the course of homogenisation, the nuclei were disrupted.

The kinetics of incorporation of $^3$H-UTP followed a similar course to that of the chromatin-bound activity, being reasonably linear for 10 min, followed by a reduction in the incorporation rate (Fig. 3.12). The standard assay time for soluble polymerase activity was therefore set at 10 min at 30°C.

The relationship between the rate of incorporation and the amount of enzyme was linear (Fig. 3.13) after making correction for the absorption due to increased protein, as described for chromatin-bound RNA polymerase.

Incubation of the polymerase in an assay medium containing DNAase (7 µg DNAase per assay) reduced the incorporation by 60%. If the DNAase was pre-incubated with the assay medium at 30°C for 20 min, incorporation was completely inhibited, presumably by removal from the assay of the DNA template (Table 3.3).
Fig. 3.12  Kinetics of Incorporation of $^3$H-UTP by soluble RNA polymerase activity

Values shown are the average of replicate assays and are expressed as cpm. above the zero time value (50±10 cpm.). The protein content per aliquot of the preparation used was 500 μg.

Fig. 3.13  The rate of Incorporation of $^3$H-UTP as a function of the amount of the soluble polymerase preparation added to the assay

Values shown are the average of replicate assays and are uncorrected for counts lost due to absorption by protein (-----). A correction was made by measuring absorption of cpm. by increasing amounts of protein added after termination of the RNA polymerase reaction to give the corrected values of incorporation (-----).
Figure 3.12.

Figure 3.13.
Table 3.3  Requirement for Substrates for Soluble RNA Polymerase Activity

<table>
<thead>
<tr>
<th>Assay Medium</th>
<th>cpm</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td>Minus GTP</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>Minus GTP, CTP, ATP</td>
<td>48</td>
<td>19</td>
</tr>
<tr>
<td>Plus phosphate (30μ moles/assay)</td>
<td>243</td>
<td>97</td>
</tr>
<tr>
<td>Plus pyrophosphate (20μ moles/assay)</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>DNAase (7 μg/assay) added with polymerase</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>DNAase (7 μg/assay) preincubated with the assay medium for 20min. at 30°C</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

All activities are expressed as cpm incorporated above background (75cpm) per 100μg DNA added and are the average of replicate assays.

The dependence of activity on the presence of various nucleotides is shown in Table 3.3. Inclusion of 75μM phosphate did not inhibit the reaction of all, whereas 50μM pyrophosphate almost totally inhibited the soluble RNA polymerase activity. The RNA polymerase reaction proceeds by cleaving pyrophosphate from the nucleoside triphosphate during the formation of the phosphodiester bond. Increasing the concentration of pyrophosphate presumably inhibited the reaction by favouring the reverse direction of the equilibrium.
a) **Effect of Template on the Soluble Activity**

The soluble RNA polymerase was stimulated 20-30 fold by the addition of denatured calf thymus DNA, either intact, sonicated or activated but only 2-fold by native calf thymus DNA (Table 3.4).

### Table 3.4: Effect of Exogenous DNA on Soluble Polymerase Activity

<table>
<thead>
<tr>
<th>Exogenous DNA added</th>
<th>cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>Native artichoke DNA</td>
<td>25</td>
</tr>
<tr>
<td>Native calf thymus DNA</td>
<td>23</td>
</tr>
<tr>
<td>Denatured artichoke DNA</td>
<td>260</td>
</tr>
<tr>
<td>Denatured calf thymus DNA (new)</td>
<td>309</td>
</tr>
<tr>
<td>Denatured calf thymus DNA (stored)</td>
<td>415</td>
</tr>
<tr>
<td>Activated Denatured calf thymus DNA (stored)</td>
<td>420</td>
</tr>
<tr>
<td>Sonicated Denatured calf thymus DNA (stored)</td>
<td>412</td>
</tr>
</tbody>
</table>

All activities are expressed as cpm incorporated above background (75 cpm) per 100 μg DNA added and are the average of replicate assays. Sonicated DNA was prepared by 4 x 30sec. sonication of denatured calf thymus DNA (at 6 microns peak to peak) in a MSE sonicator at 0°C. Stored DNA was kept at -20°C for 4 months and was prepared from a different sample of calf thymus. The size of the native calf thymus DNA was estimated to be $18 \times 10^6$ daltons and that of denatured calf thymus DNA as $7 \times 10^6$ daltons.
The polymerase appears to be fairly specific for single-stranded template, but not dependent on the size of the DNA strands or on the nature of the ends. Artichoke DNA was as effective as calf thymus DNA, indicating no specificity of the RNA polymerase with regard to the origin of the DNA used. The polymerase activity was saturated by 25 \( \mu g \) denatured calf thymus DNA per assay (Fig.3.14); at higher concentrations there was an apparent decrease of activity which was at least partially due to absorption of \( ^3H \) counts by the DNA. Counting efficiency was estimated by adding DNA after polymerase activity had been terminated by TCA precipitation (Fig.3.15); the efficiency was reduced by \( 3\% \) per 150 \( \mu g \) DNA added per assay.

b) **Effect of Divalent Ions on Soluble Polymerase Activity**

In order to test the effect of divalent cations on soluble polymerase activity it was necessary to remove the magnesium (5mM) present in the homogenising medium. This was achieved by using a 35-65\% (NH\(_4\))\(_2\)SO\(_4\) cut from the 10,000 x g supernatant (see Page 88). The precipitate was dissolved in buffer containing 5\% glycerol, 0.05M Tris HCl pH 8.0, 0.05M \( \beta \) mercaptoethanol and dialysed against 2 changes of this buffer, each for 1 hr. This enzyme preparation was assayed in a range of concentrations of magnesium or manganese in the absence of the other ion. (Fig.3.16).

In the presence of the optimal concentrations of either divalent cation (1.25mM) the activity was only 60-70\% of the control (containing 2.5mM magnesium and 0.625mM manganese); both ions inhibited at higher concentrations, manganese being more inhibitory than magnesium.
Fig. 3.14  The effect of Denatured calf-thymus DNA on the activity of soluble RNA polymerase activity

Increasing amounts of denatured calf-thymus DNA was used as template; activities are expressed as cpm above background (45cpm) and are the results of replicate assays.

Fig. 3.15  Effect of DNA on Counting Efficiency

Increasing amounts of DNA were added after termination of replicate assays containing 50µg DNA and 400 µg soluble fraction protein which incorporated 350cpm above background (38cpm).
**Figure 3.14.**

**Figure 3.15.**
Fig. 3.16  Effect of Manganese and Magnesium on the activity of soluble RNA polymerase

The enzyme extract was prepared by taking a 35-65% (NH₄)₂SO₄ cut from the 10,000 x g supernatant fraction. The (NH₄)₂SO₄ precipitate was washed briefly, dissolved in 5ml. of 5% glycerol, 50mM Tris HCl pH8.0, 50mM mercaptoethanol, and extensively dialysed against this buffer. Each point shown is the result of replicate assays and the activities are expressed as a percentage of the control (containing 0.625mM manganese, 2.5mM magnesium) which incorporated 400cpm above background (50cpm). Effect of manganese is shown by \( V \) and magnesium by \( \_V \).

Fig. 3.17  Effect of Potassium chloride on soluble RNA polymerase activity

Each value shown is the average of replicate assays and activities are expressed as a percentage of the control (0mM KCl, 0.625mM MnCl₂, 2.5mM MgCl₂) which incorporated 270cpm above a background of 40cpm.
c) **Effect of KCl on Soluble Polymerase Activity**

The response of soluble RNA polymerase to increasing levels of KCl differed from that of the chromatin-bound polymerase, showing increasing inhibition with KCl concentration (Fig. 3.17) with 85% inhibition at 0.31M KCl, a level which caused maximum stimulation of the chromatin-bound activity.

d) **Effect of Regulators on Soluble Polymerase Activity**

When tested against a range of spermine concentrations, soluble polymerase activity was increasingly inhibited with increasing concentration. At low levels of spermidine, however, there was a small stimulation of activity (at 1.0mM), whilst at higher concentrations spermidine inhibited in the same way as spermine (Fig. 3.18).

The toxin, αamanitin, partially inhibited the activity of soluble polymerase with 50% inhibition at 0.1 µg added per assay and very little further inhibition at 100-fold greater concentration of αamanitin (Fig. 3.19). As in the case of chromatin-bound polymerase the partial inhibition of activity observed here suggests the existence of multiple activities in the extract with different responses to αamanitin.

e) **Product of Soluble RNA polymerase**

The product of soluble RNA polymerase was examined by gel electrophoresis. Tritiated UTP with a specific activity of 29C/m mole was used to increase the labelling of the nascent RNA chains.
Fig. 3.18  Effect of spermidine and spermine on soluble RNA polymerase activity

Each value shown is the average of replicate assays and activities are expressed as a percentage of the control (with no added polyamines) which incorporated 375cpm. above background (50cpm) ——— = response to spermidine and ——— denotes the response to spermine.

Fig. 3.19  Effect of αamanitin on soluble RNA polymerase activity

Each value shown is the average of replicate assays and activities are expressed as a percentage of the control (minus αamanitin) which incorporated 420cpm. above background (38cpm).
After various times of incubation, aliquots of the assay mixture were removed and the reaction terminated by the additions of SDS to 1% concentration. The time course of the reaction was followed by precipitating and washing 0.1ml. aliquots of the assay mixture by the standard method. The newly-synthesised RNA was fractionated on 2.4% polyacrylamide gels (Fig.3.20) which fractionated a peak of \(^3\)H-RNA of about 110,000MW (approximately 7-8S) estimated from the mobility of the 1.3 and 0.7 x 10\(^6\) RNA peaks. (Bishop, Claybrook and Spiegelman, 1967). The 260nmOD scan showed that there was a rapid and progressive decrease in the size of the denatured template DNA, presumably due to the presence of a very active DNAase in the extract (Harland, 1971). The ribosomal RNA peaks, derived from the ribosomes present in the enzyme preparation, were superimposed on the absorption of the denatured calf thymus DNA. The peak of absorption, of estimated molecular weight 180,000 daltons, behind the \(^3\)H peak, probably represents aggregated protein present in the enzyme preparation. The total incorporation into \(^3\)H RNA by the polymerase was estimated from the area under the \(^3\)H peak and agreed with the data from TCA precipitation. The decrease of activity observed after long incubation times possibly reflects loss of template from the reaction mixture.

3. Relative Activities of Chromatin-Bound and Soluble Polymerases

The chromatin-bound and soluble activities that have so far been described differ in their DNA dependency, divalent and monovalent ion response. An analysis of the quantitative contribution of these two activities in this tissue is somewhat complicated by the probable existence of multiple polymerase activities within each
Soluble polymerase activity was assayed using 3H-UTP at 29C/m mole (20 x normal specific activity). Aliquots were removed at various times (after 5 min. for the figure shown here) and the reaction terminated by making the aliquots of the assay medium to 1% SDS, and the RNA synthesised was fractionated on 2.4% polyacrylamide gels run for 1 hr. 50 min. The OD scan is denoted --- and the distribution of the radioactivity as a histogram.

Fig. 3.21 Elution Profile of protein and RNA polymerase activity from P4 Biogel
The 35-65% (NH4)2SO4 fraction from the 10,000 x g supernatant was dissolved in 5 ml. of standard buffer and eluted through a P4 Biogel column (10x0.9 cm.) with standard buffer. Fractions of 2 ml. were collected and assayed for RNA polymerase activity, values shown include background counts and are plotted as a histogram. Elution of protein was followed by the 280 nm OD trace (continuous line).
Figure 3.20.

Figure 3.21.
of the fractions (for example: as in the soluble fractions from rat liver, sea urchin, calf thymus, *Blastocladiaella* and coconut endosperm) and by the individual properties of the multiple activities. The quantitative contribution of these two fractions of RNA polymerase activity was estimated from fresh tissue, assaying each under optimal conditions (with regard to DNA and monovalent ion concentration). The degree of cross-contamination of the soluble fraction by chromatin-bound activity was estimated by assaying the 10,000 x g supernatant in 0.25M KCl and in the absence of exogenous DNA. Under these conditions (optimal for chromatin-bound activity) there was no significant incorporation. The level of incorporation (40cpm.), observed in the absence of exogenous DNA in Fig.3.14, was reduced to zero when assayed in the presence of 0.125M KCl. There was, therefore, little contamination of the soluble RNA polymerase by chromatin-bound activity. It was more difficult to estimate contamination of the chromatin-bound activity by soluble polymerase, but by assaying at high salt (0.25M KCl) in the absence of exogenous DNA, the contribution of any contaminating soluble polymerase was reduced as far as possible. Recovery of chromatin-bound activity in the second and third centrifugation steps was approximately in proportion to the recovery of DNA which can be taken as a measure of chromatin recovery (Table 3.5).
The total activity recovered from 7.6% of fresh tissue expressed
of the chromatin-bound activity.

Table 7.6

<table>
<thead>
<tr>
<th>Ac</th>
<th>%H</th>
<th>gel</th>
<th>0.25M KOH</th>
<th>0.2% TNP</th>
<th>10% 3000</th>
<th>6.3% 3000</th>
<th>Total Recovery %</th>
<th>Activity %</th>
<th>Acceptor Activity %</th>
<th>Amount of DNA incorporated (cpm incorporated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17</td>
<td>31</td>
<td>-</td>
<td>0.0000</td>
<td>-</td>
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</tr>
<tr>
<td>2</td>
<td>14</td>
<td>27</td>
<td>-</td>
<td>0.0000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
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<td>06</td>
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<td>15</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>02</td>
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</tr>
<tr>
<td>9</td>
<td>00</td>
<td>11</td>
<td>-</td>
<td>0.0000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: All activities are expressed as cpm incorporated above.
The figures represent total cpm. incorporated for each fraction from 45g. fresh tissue assayed in the absence of KCl. The values in brackets are the total activities when assayed in high salt (250mM) KCl. The total activities measured under optimal conditions are underlined.

4. Solubilisation of Chromatin-Bound Activity

The chromatin-bound polymerase activity was partially solubilised by a variety of treatments and the response of this solubilised activity to high salt and exogenous DNA was determined.
The purified chromatin was incubated for 90min. at 4°C in standard buffer (5% glycerol, 50mM Tris HCl pH8.0, 50mM β mercaptoethanol, and 5mM MgCl₂), standard buffer minus magnesium, and standard buffer (minus magnesium) plus 10mM EDTA. The results (Table 3.7) indicated that the removal of magnesium from the buffer resulted in no solubilisation of the activity, whereas incubation in the presence of EDTA solubilised 40% of the total activity which retained its salt-stimulated characteristic. The total recovery of activity after EDTA treatment was 40% more than that recovered after incubation with standard buffer. This probably reflects the removal of inhibitory proteins from the chromatin by EDTA.
Table 3.7  Solubilisation of Chromatin-bound RNA Polymerase Activity

<table>
<thead>
<tr>
<th></th>
<th>Total Activity cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assayed at 0 KCl</td>
</tr>
<tr>
<td>1. Standard buffer</td>
<td></td>
</tr>
<tr>
<td>10,000xg. pellet:</td>
<td></td>
</tr>
<tr>
<td>supernatant:</td>
<td>0</td>
</tr>
<tr>
<td>2. Standard buffer</td>
<td></td>
</tr>
<tr>
<td>minus magnesium</td>
<td></td>
</tr>
<tr>
<td>10,000xg. pellet:</td>
<td>0</td>
</tr>
<tr>
<td>supernatant:</td>
<td></td>
</tr>
<tr>
<td>3. Standard buffer</td>
<td></td>
</tr>
<tr>
<td>minus magnesium</td>
<td></td>
</tr>
<tr>
<td>plus 10mM EDTA</td>
<td></td>
</tr>
<tr>
<td>10,000xg. pellet:</td>
<td>2,850</td>
</tr>
<tr>
<td>supernatant:</td>
<td>4,380</td>
</tr>
</tbody>
</table>

Purified chromatin was incubated for 90 min. at 4°C with the buffers shown; (standard buffer was 5% glycerol, 5 mM Mg$^{2+}$, 0.5 mM dithiothreitol, 50 mM Tris HCl pH 8.0). Magnesium was added back after treatments 2 and 3 to give 5 mM Mg$^{2+}$ in the preparation. All activities are expressed as cpm incorporated above background (85 cpm.). The 10,000 x g pellets were assayed in the absence of exogenous DNA and supernatants assayed in the presence of 50 μg/assay denatured calf thymus DNA.

b) High Salt Treatment

Purified chromatin was re-suspended in standard buffer or standard buffer in 0.3 M KCl and incubated for 30 min. at 4°C. The chromatin was re-pelleted by centrifugation at 10,000 xg for 10 min. and the pellet and supernatant assayed for polymerase activity at 0.3 M KCl in the absence of exogenous DNA. At this salt concentration any polymerase activity was was salt-inhibited would make a negligible contribution to the assay.
Recovery of activity was nearly 100% after this treatment (indicating no substantial change of characteristics of the polymerase activity to salt-inhibition) and 61% of the activity was found in the 10,000 x g supernatant (Table 3.8).

The solubilised polymerase activity had retained its salt-stimulated characteristic and was not dependent on exogenous DNA.

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Total Activity cpm</th>
<th>% in Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Chromatin incubated in standard buffer: 10,000xg pellet supernatant</td>
<td>15,000</td>
<td>0</td>
</tr>
<tr>
<td>2. Chromatin incubated in 0.3M KCl for 30 min. at 4°C: 10,000xg pellet supernatant</td>
<td>5,743</td>
<td>61</td>
</tr>
<tr>
<td>3. Chromatin incubated in 0.3M KCl (as above) and sonicated for 2 min: 10,000xg pellet supernatant</td>
<td>4,300</td>
<td>72</td>
</tr>
</tbody>
</table>

Purified chromatin was incubated for 30 min. at 4°C with either standard buffer or standard buffer in 0.3M KCl. An aliquot of the salt-treated chromatin was sonicated with an MSE Sonicator for 4 x 30 sec. at 0°C. The chromatin was then re-pelleted by centrifugation at 10,000 x g for 10 min. All values shown for activities are the average of replicate determinations and expressed as cpm. incorporated above background (103 cpm/assay). All assays were in the presence of 0.3M KCl with no exogenous DNA added.
c) **High Salt Sonication Treatment**

Purified chromatin was incubated for 30min. at 0°C in 0.3M KCl and sonicated for 4 x 30sec. at 0°C in an MSE sonicator. The chromatin was re-pelleted and the pellet and supernatant assayed as in the high salt treatment previously described. This treatment resulted in 72% of the activity being found in the supernatant (Table 3.8) which was salt-stimulated and independent of exogenous DNA. Attempts were made to fractionate this activity by DEAE cellulose chromatography after desalting, but all the activity eluted through the column in the wash and was not bound.

d) **DNAase Treatment**

A purified chromatin preparation was incubated with DNAase at 10 μg/ml in 0.2M KCl for 1.5hr. at 0°C and then the chromatin re-pelleted by centrifugation at 10,000 x g for 10min. The supernatant and pellet were assayed for polymerase activity in presence or absence of KCl with added template DNA (Table 3.9).
Table 3.9  Solubilisation of Chromatin-bound RNA
Polymerase Activity by Treatment with DNAase

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Total Activity cpm</th>
<th>% in Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard buffer</td>
<td>50,000</td>
<td>0</td>
</tr>
<tr>
<td>DNAase treatment:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10,000xg pellet</td>
<td>7,400</td>
<td></td>
</tr>
<tr>
<td>10,000xg supernatant:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>assayed-KCl</td>
<td>9,000</td>
<td>62</td>
</tr>
<tr>
<td>assayed+KCl</td>
<td>12,000</td>
<td></td>
</tr>
</tbody>
</table>

Purified chromatin was incubated for 90min. with 10 μg/ml (final concentration) DNAase (RNAase free) at 4°C. The chromatin was re-pelleted by centrifugation at 10,000xg for 10min. and the pellet assayed at high salt (0.25M KCl) with no exogenous DNA added. The supernatant was assayed in the presence of 10μg denatured calf thymus DNA plus or minus KCl (0.25M).

Activities are expressed as before (background was 62cpm).

Recovery of activity after DNAase treatment was 39% with 62% of the recovered activity in the supernatant. Both activities were stimulated by the addition of KCl at 0.25M. The supernatant was desalted by F₄ biogel treatment and loaded on a DEAE cellulose column. All the activity washed through the column and was not bound to the ion exchanger. The poor recovery of activity after DNAase treatment is probably due to contamination of the assay with DNAase which has previously been shown to inhibit the activity.
The solubilisation of the chromatin-bound polymerase activity described here gives a 10,000 x g supernatant activity which is salt-stimulated and not dependent on exogenous DNA and which is not bound to DEAE cellulose as was the soluble activity described previously.

5. **Purification of Soluble RNA Polymerase**

The 10,000 x g supernatant of the initial tissue homogenate was fractionated by ammonium sulphate precipitation. Weighed amounts of finely-ground ammonium sulphate, as calculated from the nomogram of Dixon (1953), were added slowly with stirring at 0°C. The pH was maintained constant at pH 8.0 by the addition of 0.1N NaOH as necessary.

The protein was allowed to precipitate for 20 min. at 0°C and then pelleted by centrifugation at 10,000 x g for 10 min. The supernatant was removed, further fractionated by the addition of more \((\text{NH}_4)_2\text{SO}_4\), and the precipitate recovered by centrifugation. The precipitates were washed briefly, dissolved in and dialysed against standard buffer for 3 hr. with 4 external buffer changes. Each \((\text{NH}_4)_2\text{SO}_4\) fraction was assayed for RNA polymerase activity and the protein content determined. The recovery of activity exceeded 100% (Table 3.10), probably because the fractions were assayed in the presence of less protein with consequent greater counting efficiency. The best purification was in the 45-55% \((\text{NH}_4)_2\text{SO}_4\) saturated fraction, which represented a 4 to 5-fold increase in specific activity and contained 50% of the initial total activity. For preparative work, the 35-65% \((\text{NH}_4)_2\text{SO}_4\) saturation fraction was used, which gave a 3-5 fold purification and ensured that a representative sample of the polymerases present was taken.
Table 3.10 Fractionation of Soluble RNA Polymerase by (NH₄)₂SO₄ Precipitation

<table>
<thead>
<tr>
<th>(NH₄)₂SO₄ Concentration (% saturation)</th>
<th>Activity cpm/0.1ml aliquot</th>
<th>Volume (ml)</th>
<th>Total Activity (10³cpm)</th>
<th>Protein (μg)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (cpm/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Supernatant</td>
<td>306</td>
<td>50.0</td>
<td>153</td>
<td>215</td>
<td>107.5</td>
<td>1.42</td>
</tr>
<tr>
<td>0-25%</td>
<td>43</td>
<td>12.5</td>
<td>5.4</td>
<td>16</td>
<td>2.0</td>
<td>2.70</td>
</tr>
<tr>
<td>25-35%</td>
<td>531</td>
<td>6.0</td>
<td>31.9</td>
<td>192</td>
<td>11.5</td>
<td>2.75</td>
</tr>
<tr>
<td>35-45%</td>
<td>1041</td>
<td>6.0</td>
<td>62.5</td>
<td>250</td>
<td>15.0</td>
<td>4.16</td>
</tr>
<tr>
<td>45-55%</td>
<td>1249</td>
<td>6.0</td>
<td>74.9</td>
<td>196</td>
<td>11.8</td>
<td>6.35</td>
</tr>
<tr>
<td>55-65%</td>
<td>391</td>
<td>6.0</td>
<td>23.5</td>
<td>70</td>
<td>4.2</td>
<td>5.6</td>
</tr>
<tr>
<td>65-75%</td>
<td>130</td>
<td>6.0</td>
<td>7.8</td>
<td>51</td>
<td>3.0</td>
<td>2.6</td>
</tr>
<tr>
<td>75-100%</td>
<td>20</td>
<td>6.0</td>
<td>1.2</td>
<td>8</td>
<td>0.5</td>
<td>2.4</td>
</tr>
</tbody>
</table>

All activities are expressed as previously described and are the average of replicate assays (background was 76cpm).

The ammonium sulphate fractionation served not only to purify the RNA polymerase but also to concentrate the extract (greater than ten-fold) for subsequent column chromatography and to remove the β mercaptoethanol (50mM) present in the homogenising medium, which interfered with the Folin-Lowry protein estimation and had a high u-v absorption, which introduced difficulties in detecting protein in the chromatographic fractionation. The standard buffer in which the 35-65% (NH₄)₂SO₄ fraction was dissolved contained 0.5mM dithiothreitol which was adequate to maintain the reduced state of the sulphydryl groups on the enzyme, but which neither interfered in the protein estimation nor significantly absorbed u-v.
An alternative method for the concentration of the extract was the Amicon column eluate concentrator (CEC1) which utilized the principle of ultra-dialysis with a positive pressure of 70-100 psi across a supported dialysis membrane (UM10). However, 3 hr. ultra-dialysis was necessary to produce an adequate concentration, after which time much of the RNA polymerase activity had been lost.

The ammonium sulphate precipitation necessitated the introduction of a further step to remove any residual ammonium sulphate, which strongly inhibited RNA polymerase activity. This could be achieved by 3 hr. dialysis against standard buffer, but was more quickly achieved by passage through a P₄ biogel column. The 35-65\% (NH₄)₂SO₄ fraction was washed briefly, dissolved in standard buffer, and loaded on a 15 cm. P₄ biogel column (prepared as described on Page 61). The protein was washed through the column with standard buffer (40 ml/hr) and detected in the eluate by absorption at 280 nm using an LKB Uvicord II Detector (0.5 mm dia. flow cell) linked to an LKB Chopper Bar Recorder. The eluate was collected in 2 ml. fractions which were assayed for both protein and polymerase activity (Fig. 3.21). For preparative use, the first 6 ml. of protein-containing eluate was collected and further fractionated by chromatography on DEAE cellulose.

DEAE cellulose was prepared as described in the Methods, and the slurry - stored in standard buffer plus 0.01\% sodium azide - was poured to form a column 10 x 0.9 cm. and washed with standard buffer for 2.5 hr. (40 ml/hr). The desalted P₄ eluate was loaded onto the DEAE column and the column was then washed with standard buffer until the protein concentration in the eluate, as detected by 280 nm. OD was negligible. This required 30 ml. of buffer.
The column was then eluted with a linear gradient of 0 to 0.5M \((\text{NH}_4)_2\text{SO}_4\) in a total volume of 60mL. Fractions of 2mL were collected and assayed for polymerase activity (Fig.3.22). The elution profile from DEAE cellulose showed two peaks of activity eluting at 0.125M and 0.3M \((\text{NH}_4)_2\text{SO}_4\), termed polymerase I and II respectively. There was no polymerase activity detectable in the initial wash from the column. The associated profile of 280nm OD showed major peaks in the wash (of unbound protein) and at approximately 0.125M and subsequent minor peaks between 0.35M and 0.5M \((\text{NH}_4)_2\text{SO}_4\). (Fig.3.22).

The successful fractionation of the RNA polymerase activity by DEAE cellulose chromatography was dependent on adequate degassing of the slurry during its preparation. Storage of the slurry in an open container resulted in the absorption of atmospheric carbon dioxide and consequent alteration of both the protein and polymerase activity elution profiles. This alteration of the 280nm OD profile was time dependent, with the 0.125M salt peak decreasing with increasing time of storage (Fig.3.23). Associated with this change of protein elution profile was a loss of resolution of polymerase I and II, with only one peak of activity eluting at approximately 0.25M \((\text{NH}_4)_2\text{SO}_4\) (Fig.3.24). By degassing the stored slurry immediately prior to use the resolution of the polymerase activities I and II, and the normal protein profile, were restored. The possibility that the change in resolution of the DEAE column was due to a component of the standard buffer, in which it was stored, was vitiated by storing the slurry in water and equilibrating the ion exchanger with the buffer immediately prior to use. The profile so obtained was similar to that obtained from exchanger stored in buffer (see Fig.3.23) thereby eliminating changes due to buffer alone.
Fig. 3.22  **Elution profile of Protein and RNA polymerase activity from DEAE cellulose**

The desalted fraction, from P_{i}Biogel, containing the polymerase activity was loaded onto a DE32 column (10x 0.9cm.) and washed with standard buffer until the 280nm OD was zero, then with a linear gradient of (NH_{4})_{2}SO_{4} 0 to 0.5M. Fractions of 2ml. were collected and assayed for RNA polymerase activity (histogram).

Fig. 3.23  **Change of protein elution profile from DE32 with storage of the ion exchanger**

The RNA polymerase preparation was chromatographed as before, and the 280nm. OD scan of the eluate from a linear gradient of ammonium sulphate (0 to 0.5M) followed, from DEAE cellulose stored for a) 0 days b) 5 days c) 10 days d) 20 days.
Figure 3.22.

Figure 3.23.
RNA polymerase activity was prepared as previously described and chromatographed on DEAE cellulose that had been stored for 1 month at 4°C. in standard buffer. The RNA polymerase activity (expressed as a histogram) of the eluate was assayed and the 280nm OD followed (continuous line).

The desalting step (P4 Biogel) was omitted in the preparation of the RNA polymerase extract and the preparation loaded directly onto DEAE cellulose. The elution of RNA polymerase activity (shown as a histogram) and the 280nm OD of the eluate (continuous line) was followed.
Figure 3.24.

Figure 3.25.
In the absence of complete desalting of the enzyme preparation, prior to DEAE chromatography, there was a large peak of polymerase activity in the wash after loading the sample, and only one peak of activity at 0.3M (NH₄)₂SO₄ (Fig. 3.25). When this initial activity was re-run on a second DEAE column, after desalting, it bound to the DEAE and was eluted at 0.125M (NH₄)₂SO₄ (Fig. 3.26). This anomaly was resolved by ensuring complete removal of (NH₄)₂SO₄ from the preparation after (NH₄)₂SO₄ fractionation by means of a P₄ biogel column. When this procedure was followed, the profile of enzymic activity shown in Fig. 3.22 was obtained, with no activity in the wash after loading the extract, i.e. all the polymerase activity was bound to the column.

In order to examine the properties of RNA polymerases I and II it was necessary to remove the (NH₄)₂SO₄ with which they were eluted, since results from studies with total soluble polymerase indicated that at these concentrations of (NH₄)₂SO₄ (0.125 to 0.3M) the polymerase activity would be substantially inhibited. Accordingly, three 2ml fractions from the 0.125M and 0.3M (NH₄)₂SO₄ peaks (polymerases I and II respectively) were combined and desalted on 5 x 0.9cm P₄ biogel columns. The protein eluate was collected and used to characterise the properties of polymerase activities I and II.

In order to ensure that polymerase activities I and II were not merely artifacts of chromatographic fractionation, each purified desalted activity was re-chromatographed on DEAE cellulose. Both polymerases I and II were quantitatively eluted at their characteristic molarities (0.125M and 0.3M respectively). (Figs. 3.27; 3.28).
Fig. 3.26  Rechromatography of RNA polymerase activity in the wash-through obtained from loading the preparation in salt.

The wash-through obtained after loading an RNA polymerase preparation in salt was desalted by passage through P_{4} biogel and re-run on DEAE cellulose. RNA polymerase activity is shown as a histogram and the 280nm OD by a continuous line.

Fig. 3.27  Rechromatography of RNA polymerase I activity

The fractions containing RNA polymerase I activity were pooled and desalted by passage through a P_{4} Biogel column (5 x 0.9 cm.), and then re-run on DEAE cellulose. RNA polymerase activity is shown as a histogram and protein (280nm OD) as a continuous line.
Figure 3.26.

Figure 3.27.
Fig. 3.28 Rechromatography of RNA polymerase II activity

The fractions containing RNA polymerase II activity were pooled and desalted by passage through a P₄ column (5 x 0.9 cm.) and then re-run on DEAE cellulose. RNA polymerase activity is shown as a histogram, and protein (280 nm OD) as a continuous line.

Fig. 3.29 Stability of RNA polymerase I and II activities

RNA polymerase activities I (○) and II (●) prepared as described in the text, were stored in the salt in which they were eluted from the DE32 column in standard buffer at 0°C. and assayed at the times shown. Each point is the average of replicate assays and is expressed as a percentage of the control which incorporated 950 cpm for polymerase I and 1230 cpm for polymerase II (assayed immediately after preparation) above background (40 cpm.).
Figure 3.28.

Figure 3.29.
In order to confirm that both activities were true polymerases, the effect of omission of various nucleotides was investigated (Table 3.11).

Table 3.11 Dependence of Polymerases I and II on Nucleotides

<table>
<thead>
<tr>
<th>Assay Conditions</th>
<th>Polymerase I % Control</th>
<th>Polymerase II % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>-GTP</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>-GTP,CTP,ATP</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>+DNAase</td>
<td>25</td>
<td>40</td>
</tr>
<tr>
<td>+RNAase</td>
<td>82</td>
<td>83</td>
</tr>
</tbody>
</table>

Polymerase I and II activities were prepared as described in the text. DNAase (RNAase free) and RNAase at 50 μg/ml were included in the assays as shown. Activities are expressed as a percentage of control which incorporated 650cpm for polymerase I and 520cpm for polymerase II above a background of 80cpm.

The activity of each polymerase was reduced by about 80% by the omission of GTP and further reduced when all three unlabelled nucleotides were omitted. Inclusion of RNAase inhibited the activity of both polymerase I and II by less than 20% (Table 3.11). The relative stability of the polymerase product to RNAase digestion was probably due to the product remaining on the template as a DNA-RNA hybrid. DNAase treatment produced more marked inhibition of the polymerase activities, the degree of inhibition probably reflecting the extent of hydrolysis of the DNA template as well as the DNA-RNA hybrid formed in the reaction.
The purified RNA polymerases were unstable, and had to be freshly prepared for each experiment. Storage at 4°C in 5% glycerol (in standard buffer) resulted in the rapid loss of activity (Fig.3.29); both polymerases had a half life of 4-5hr.

a) **Effect of Divalent and Monovalent Ions**

The multiple activities fractionated from other eucaryotic tissues have been shown to differ in their response to various ions in such a way that certain ionic environments have been shown to favour one polymerase rather than the other. The effect of mono- and divalent ions and their interaction was determined for polymerases I and II. Both polymerases were optimally stimulated by 1.25mM manganese in the absence of magnesium (Fig.3.30). Polymerase II showed greater inhibition at higher manganese levels. The maximum activity of both polymerases was similar to that of the control, containing 2.5mM magnesium and 1.25mM manganese. This contrasted with the effect of manganese in the absence of magnesium on total soluble polymerase when manganese was unable to substitute fully for both ions. This discrepancy may be explained by contamination of polymerase activity I and II with magnesium which was present throughout their extraction and purification. In the preparation of total soluble activity, the ammonium sulphate precipitate was dissolved in buffer minus magnesium and desalted in the absence of magnesium so that magnesium contamination would be substantially lower. Magnesium, in the absence of manganese, stimulated incorporation by polymerase I to 100% of the control level when present at 2.5mM (Fig.3.31).
Maximum activity of polymerase I was therefore obtained in the presence of either magnesium or manganese independently. Polymerase II, on the other hand, although stimulated to control (manganese 1.25mM; magnesium 2.5mM) level of activity by manganese alone, was not substantially stimulated by increasing levels of magnesium (Fig.3.31). Interaction of magnesium and manganese ions on polymerase I and II activities was investigated by assaying the activities in the presence of 2.5mM magnesium and a range of manganese concentrations (Fig.3.32).

Polymerase I was not further stimulated by manganese, and at higher concentrations was inhibited indicating that polymerase I activity was dependent on only one divalent ion which may be either manganese or magnesium. Polymerase II was stimulated considerably by 1.25mM manganese when assayed in the presence of 2.5mM magnesium and characteristically was inhibited at higher concentrations of manganese. These results indicated that polymerase II required only manganese for optimal activity. However, since the enzyme was extracted and purified in buffers containing magnesium, the sites for binding magnesium could have all been filled and it is not possible therefore to rule out a requirement for both magnesium and manganese.

Both polymerase I and II activities were inhibited by monovalent cations (Fig.2.33). The concentration of (NH₄)₂SO₄ which gave 50% inhibition of the two polymerases was 0.44±0.04M for polymerase I and 0.14±0.01M for polymerase II, which indicated that polymerase II was more sensitive to increased ammonium sulphate concentration than polymerase I.
Fig. 3.32  **Effect of manganese in the presence of 2.5mM magnesium on the activity of RNA polymerase I and II**

Each value shown is the average of replicate assays and activities are expressed as a percentage of an assay containing 2.5mM magnesium and 1.25mM manganese which incorporated 1400cpm. for polymerase I and 2000cpm. for polymerase II above background (50cpm.).

\[ \odot = \text{polymerase I activity and } \bigcirc = \text{polymerase II activity.} \]

Fig. 3.33  **Effect of Ammonium sulphate on the activity of RNA polymerase I and II**

Each value shown is the average of replicate assays and activities are expressed as a percentage of the control (containing no ammonium sulphate) which incorporated 1010cpm. for polymerase I and 908cpm. for polymerase II above background (45cpm.).

\[ \odot = \text{polymerase I activity and } \bigcirc = \text{polymerase II activity.} \]
Figure 3.32

Figure 3.33
b) **Specificity for Template DNA**

The specificity of each polymerase activity for template DNA was examined, assaying the polymerases on native and denatured calf thymus DNA. Polymerase I transcribed denatured DNA, and was saturated by 10 \( \mu g \)/assay, but there was less than 10% of this activity when assayed on native template (Fig.3.34). Polymerase II was active on both native and denatured template, but, expressed per \( \mu g \) DNA, the activity was four times greater on denatured DNA.

Since the template specificity of the enzyme may be effected by divalent ion content and concentration in the assay, polymerase II, which transcribed both native and denatured DNA, was assayed on both templates at a range of divalent ion concentrations. In the presence of 2.5mM magnesium and 0 to 5mM manganese (Fig.3.35) the relative efficiency of polymerase II on native and denatured DNA was not changed, indicating that the manganese:magnesium ratio was not important in controlling the template specificity of polymerase II.

The ability of polymerase II to utilise both native and denatured DNA as template could be explained on the basis of a mixture of polymerase activities each with an individual polymerase requirement. The broadness of the polymerase II peak from the DEAE column gave some suggestion of multiple polymerases. This postulate was tested by assaying each fraction from the polymerase II peak (from DEAE) on both native and denatured template (Fig.3.36). The two activity profiles on denatured and native DNA showed a single broad coincident peak, and there was no resolution detectable with reference to the template specificity.
Fig. 3.34  Effect of native and denatured calf thymus DNA template on the activity of RNA polymerase I and II

Each value shown is the average of replicate assays and activities are expressed as a cpm. incorporated above background (40cpm.)

○ = polymerase II assayed on denatured template, ■ = polymerase II assayed on native template; ○ = polymerase I on denatured template and □ = polymerase I assayed on native template.

Fig. 3.35  Effect of manganese in the presence of 2.5mM magnesium on polymerase II activity assayed on native and denatured calf thymus DNA

Each value shown represents the average of replicate assays and activities are expressed as cpm. incorporated above background (50cpm.)

○ = assayed on denatured DNA template and □ = assayed on native DNA template.
Fig. 3.36 Assay of polymerase II fractions from DEAE cellulose on native and denatured template

Polymerase II activity was prepared as previously described and each fraction (2ml) eluted from DEAE cellulose between 0.2M and 0.4M ammonium sulphate was assayed on native (- - - - - - ) and denatured (-----) DNA. Activities are expressed as cpm incorporated above background of 45 cpm.

Fig. 3.37 Effect of a) cycloheximide, b) rifampicin c) αamanitin on the activities of polymerase I and II.

Each point shown is the average of replicate assays and the activities are expressed as a percentage of control, which incorporated 2,800 cpm for polymerase I and 2,400 cpm for polymerase II above background (50 cpm).

⊙ = polymerase I and ○ = polymerase II
Figure 3.36.

Figure 3.37.
Taken together with the evidence from re-chromatography of the activities (Fig. 3.28), it would appear that polymerase II is a single homogenous polymerase activity active on denatured template and to a lesser extent (25%) on native DNA.

c) Regulators of Polymerase Activities I and II

Various inhibitors have been included in the in vitro assay in an attempt to find responses specific to the two activities. Rifampicin inhibition is normally restricted to procaryotic RNA polymerase where the inhibitor binds to the core enzyme, but it has been demonstrated to inhibit the extranucleolar polymerase III of Blastocladiella (Horgen and Griffen, 1971). αamanitin has been shown to specifically inhibit polymerase activity II from both animal and plant sources. While it is generally accepted that cycloheximide inhibits protein synthesis in eucaocytes, it has been reported to also act directly on RNA synthesis.

There was some inhibition (18%) of polymerase I activity at high concentrations of cycloheximide (40 μg/ml) but no inhibition of polymerase II activity at this level, as shown in Fig. 3.37a. However, there was some inhibition of both activities by higher concentrations of rifampicin (Fig. 3.37b), which could reflect some bacterial contamination of the preparations or some chemical effect unrelated to the normal action of rifampicin.

αamanitin completely inhibited polymerase II at 0.25 μg/ml. but had no inhibitory effect on polymerase I at 25 μg/ml. (Fig. 3.37c).
This finding is in agreement with all the published data on the effect of αamanitin, and allows an estimate to be made of the relative proportions of polymerase I and II in the total soluble polymerase. For example: the 50% inhibition of total soluble activity from fresh tissue by αamanitin (Fig. 3.19) therefore indicates an approximate 1:1 ratio of the polymerase I and II activities.

6. Specific Activities of Enzyme Preparations

The specific activities of the chromatin-bound, soluble, I and II polymerases were calculated and compared with values from other published work of RNA polymerase activities (Table 3.12).
### Table 3.12 Specific Activities of Plant RNA Polymerases

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatin-bound (Artichoke) (in absence of salt)</td>
<td>19.6</td>
</tr>
<tr>
<td>Chromatin-bound (Artichoke) (in presence of salt)</td>
<td>30.0</td>
</tr>
<tr>
<td>Chromatin-bound (Soybean)</td>
<td>584</td>
</tr>
<tr>
<td>Chromatin-bound (Pea)</td>
<td>56-120</td>
</tr>
<tr>
<td>Total soluble (Artichoke)</td>
<td>0.375</td>
</tr>
<tr>
<td>Polymerase I (Artichoke)</td>
<td>184</td>
</tr>
<tr>
<td>Polymerase I (Maize)</td>
<td>300</td>
</tr>
<tr>
<td>Polymerase II (Artichoke)</td>
<td>340</td>
</tr>
<tr>
<td>Polymerase II (Maize)</td>
<td>300</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>$4.53 \times 10^3$</td>
</tr>
</tbody>
</table>

Specific activities are expressed as picomoles of UMP incorporated in 10min. incubation at 30°C per mg. protein. DNA, when present, was in excess (50μg/assay). Values for chromatin-bound activity are based on protein estimations, and published values based on DNA content are modified on the basis of the composition of chromatin being 1 unit of DNA: 2.5 units of protein.

2. McComb, McComb and Duda, 1970
3. Strain et al, 1971
4. Burgess, 1969
Alternative Methods of Purification and Fractionation of Soluble RNA Polymerase

a) Phosphocellulose Chromatography (Whatman P11)

The column was prepared as described in the Methods and washed immediately prior to use with 100ml. of standard buffer (40ml/hr). The precipitate from a 35-65% (NH₄)₂SO₄ cut was dissolved in 4ml. of standard buffer and dialysed for 3hr. against 4 changes of 250ml. of this buffer. The enzyme extract was loaded onto the P11 column, washed with 30ml. of standard buffer, followed by elution with a linear gradient of ammonium sulphate, 0 to 0.5M in 60ml. Fractions of 2ml. were collected and assayed for polymerase activity (Fig.3.38). At pH 0, polymerase activity was not bound to the phosphocellulose column and eluted in the wash.

b) DNA Cellulose Columns

The preparation of native and denatured DNA cellulose has already been described. The dried DNA cellulose was washed thoroughly with standard buffer and poured to form 6 x 0.9cm. column. Buffer was pumped through this column (40ml/hr) for 2.5hr. until the 260nm OD of the eluate was zero.

For successful application of affinity chromatography, it was necessary to remove from the enzyme preparation any endogenous substrate which could compete with the substrate present on the column, resulting in non-binding of the enzyme to the column (Cuatrecasas and Anfinson, 1971). Two methods have been used for the removal of DNA from a crude tissue extract; treatment with a nuclease; or precipitation of the DNA with polyethylene glycol (Alberts and Herrick, 1971).
Fig. 3.38  Elution profile of protein and RNA polymerase from Phosphocellulose P11
The 35-65% ammonium sulphate cut was washed briefly, taken up in standard buffer, (5ml.) and loaded on to a P11 column (10 x 0.9cm) equilibrated with this buffer. Standard buffer was pumped through (40ml/hr) until the 280nm OD was zero (continuous line) and then the column was eluted with a linear gradient of 0 to 0.5M ammonium sulphate. Fractions (2ml.) were assayed for RNA polymerase (shown as a histogram) and activities are expressed as cpm. above background (45cpm.).

Fig. 3.39  Precipitation of a) protein and b) DNA by PEG 6000 and salt
The percentage of protein and DNA remaining in the 10,000 x g supernatant is shown after treatment with increasing concentrations of PEG 6000 in the presence of OM NaCl (▼), 0.5M NaCl (▼), 1.0M NaCl (■) for 30min. at 0°C.
If a nuclease is used, it is of course necessary to inactivate its activity before chromatography on DNA cellulose. This may be done by chelation of the divalent ion present, but since the activity of RNA polymerase is also dependent on the presence of the divalent ion this is not feasible. Alternatively, *Micrococcus* nuclease, (which specifically requires calcium), could be used and this enzyme can be readily inactivated by the specific chelating agent, ethylene glycol-bis-(aminoethyl ether) tetraacetic acid (Spiegelman, Burny, Das, Keydar, Schlom, Travnik and Watson, 1970).

The alternative method for removal of DNA was by precipitation of the DNA with polyethylene glycol (PEG, 6000) and salt. A range of PEG and salt concentrations was tested to determine the efficiency of DNA precipitation (Fig. 3.39). Above 5% PEG and 1.0M NaCl, 100% of the DNA was precipitated after 30min. at 0°C. The effect of this treatment on soluble protein was determined using Bovine Serum Albumin (BSA fraction V), (Fig. 3.39). The results using purified DNA and protein indicated that 100% of the DNA was precipitated with virtually no protein by treatment with 5% PEG and 1.0M NaCl.

Both PEG precipitation and DNAase methods were used for removal of DNA in the preparation of the artichoke extract for DNA cellulose chromatography. Treatment with PEG resulted in the complete loss of RNA polymerase activity from the supernatant. Treatment of the soluble polymerase with DNAase (Sigma DN-EP RNAase free, 166 μg protein) for 10min. at 30°C removed all detectable DNA in the extract (by Burton's Method, 1956).

Chelation of magnesium by EDTA and DNA cellulose chromatography resulted in the complete loss of measurable polymerase activity in the column eluate.
An alternative means of removing endogenous DNA from the extract was by centrifugation at 120,000 × g for 2 hr. at 4°C, after which 77% of the activity was left in the supernatant, which was free from DNA. The 120,000 × g supernatant was passed through a DNA cellulose column (40 ml/hr), the column washed with standard buffer and eluted with a linear gradient of 0 to 0.5 M (NH₄)₂SO₄. Denatured DNA cellulose bound 50% of the polymerase activity which eluted as a broad peak at approximately 0.2 M (NH₄)₂SO₄ (Fig. 3.40). The specific activity of this polymerase was in excess of 2,600 picomoles UMP incorporated/mg. protein/100 μg DNA present. (No accurate specific activity could be determined as the levels of protein were below that detectable using Folin estimation). No activity was eluted from the native DNA cellulose column (Fig. 3.41). The DNA per g. cellulose was estimated before and after the fractionation, and it was found that subsequent to chromatography all the DNA had been removed from the column. This presumably reflected the presence of the DNAase in the extract which was very active in the standard buffer containing 5 mM magnesium. The conditions of binding the polymerase to the DNA cellulose were obviously not optimal, but the denatured DNA cellulose column did result in a very high single step purification.
Fig. 3.40  Elution profile of protein and RNA polymerase activity from denatured DNA cellulose

The 120,000 x g supernatant (40ml.) was pumped through a denatured DNA cellulose column at 40ml/hr., followed by standard buffer until the 280nm OD was zero (continuous line). The column was then eluted with a linear gradient of 0 to 0.5M ammonium sulphate and 2ml. fractions were collected which were assayed for RNA polymerase activities (shown as a histogram) expressed as cpm. above background (60cpm.).

Fig. 3.41  Elution profile of protein and RNA polymerase activity from native DNA cellulose

The 35-65% ammonium sulphate cut was washed briefly, taken up in standard buffer (5ml.) and loaded onto a column (5 x 0.9cm.) of native DNA cellulose and eluted as described previously. RNA polymerase activities (shown as a histogram) are expressed as cpm. above background (55cpm.).
Figure 3.40

Figure 3.41
SECTION II: RNA Synthesis During the Growth of Artichoke Explants

The activity of chromatin-bound and soluble polymerases, and in vivo RNA synthesis, measured by total RNA accumulation and by $^3$H-uridine incorporation, have been determined through the cell division cycle and for longer culture periods. Cell division was monitored by following the increase of cell number per explant and the sterility of the system was checked at each sample time. Results obtained from flasks containing bacterial contamination were discarded. The increase of total RNA measured during the first cell division cycle was 250%, which agreed with other workers (Yeoman and Mitchell, 1970). However, the levels of RNA/explant were very much lower. The RNA extracted from explant tissue was purified by Dowex-1 Cl$^-$ chromatography to remove other u-v absorbing contaminants and this may account for the lower values determined here when compared with previous work.

1. Modification of Polymerase Assay Procedures

The techniques that have been developed in the previous section were adapted for use with small amounts of material available when studying growth of explants, particularly with respect to the cell division cycle.

The tissue (10 to 25 explants) was homogenised by 6 strokes in a small glass-in-glass Kontes homogeniser in 1.0ml. of homogenising medium. In order to avoid losses on filtration, the size of the miracloth filter was reduced to a 2cm. disc which was supported in a small porcelain Buchner filter funnel. This was pre-wetted with homogenising medium and the apparatus held in a Buchner flask connected to a water pump.
A polypropylene centrifuge tube fitted below the neck of the funnel in the Buchner flask, so that the filtrate was collected directly into the centrifuge tube. The homogeniser was rinsed with 0.5ml. of homogenising medium and this was filtered as above. The combined filtrates were centrifuged at 10,000 x g for 10 min. at 0°C and the supernatant assayed for polymerase activity as described previously for soluble activity. The precipitate was washed with 1.0ml. of homogenising medium and centrifuged as before, then re-suspended in 1.0ml. of homogenising medium in the glass-to-glass homogeniser and assayed for chromatin-bound activity as described previously. It was essential to have quantitative recovery of both polymerase activities for following their changes during growth of the explants. This was tested by homogenising 10, 25 and 50 explants and assaying the resultant polymerase activities.

Both soluble and chromatin-bound activities were linear over this range of material (Fig. 3.42); with this procedure chromatin-bound activity represents 17.5% of the total activity which is comparable to 19% found for bulk preparations (Page 81).

The activity of the soluble polymerase was followed during a culture period of 25 hr. in medium containing 2,4-D. Ten explants were aseptically removed from the culture medium every 2 hr. and the total soluble activity assayed. At the same sample times the cell number per explant was determined and 0.05ml. of the culture medium was aseptically removed and plated out on nutrient agar (3g. bacto-beef extract, 5g. bacto-peptone, and 15g. of bacto-agar per litre water). There was a progressive loss of soluble polymerase activity during the first 12 hr. by which time the level of incorporation was essentially background (Fig. 3.43).
Fig. 3.42  Relationship of explant number to chromatin-bound and soluble RNA polymerase activities

Each point shown is the average of replicate assays and activities are expressed as cpm. incorporated above background (measured for each point).

\[ \text{\textbullet} = \text{total soluble polymerase activity and } \text{\textbox{m}} = \text{chromatin-bound activity} \]

Fig. 3.43  Changes of soluble RNA polymerase activity through the cell cycle

The soluble RNA polymerase activity (—\textbullet—) was measured as described in the text, and the activities (averages of replicate assays) expressed as cpm. incorporated above background (65cpm.) per explant. The 330 nmOD of the extract was measured in an SP800 spectrophotometer. (—○—)
Figure 3.42.

Figure 3.43.
The development of a yellow colour, with a peak absorption at 330nm, was associated with the ageing of the explants, the intensity of which (shown also in Fig.3.43) was inversely related to the decrease of soluble polymerase activity. Since 50% cell division occurred between 24 and 28hr. and the system was sterile throughout (no bacterial colonies were observed on nutrient agar) the loss of soluble polymerase activity cannot be attributed to contamination.

The progressive decrease of soluble polymerase activity could be explained by the production of some inhibitory substance which, after homogenisation, inhibited the in vitro activity, or by a real decrease in soluble polymerase activity. To differentiate between the two possibilities a mixed extract from fresh and 30hr. tissue was assayed (Table 3.13).

Table 3.13 Detection of an Inhibitor of RNA Polymerase in aged tissue

<table>
<thead>
<tr>
<th>Source of Soluble Polymerase Preparation</th>
<th>Activity (cpm/explant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh tissue</td>
<td>435</td>
</tr>
<tr>
<td>30hr. tissue</td>
<td>65</td>
</tr>
<tr>
<td>Fresh + 30hr. tissue mixed in assay</td>
<td>105</td>
</tr>
<tr>
<td>Fresh + 30hr. tissue extracted together</td>
<td>15</td>
</tr>
</tbody>
</table>

10 explants each of fresh and 30hr. tissue were extracted separately and together. 0.1ml. aliquots of each extract was assayed in a final volume of 0.4ml. Where fresh and 30hr. tissues were mixed in the assay, 0.1ml. of each extract was used. Activities were expressed as previously described (background was 85cpm.).
A preparation of soluble polymerase activity from a mixture of the explants and assay of a mixture of separately isolated preparations, indicated that activity of fresh tissue was strongly inhibited by the preparation from 30 hr grown tissue. It was therefore necessary to prevent this inhibition of soluble polymerase activity. The initial successful isolation of polymerase activity from artichoke (both DNA polymerase, J.F. Jackson, 1971, and RNA polymerase) depended on the prevention of browning of the extract, i.e. inhibition of the very active phenol oxidase system. This problem was therefore considered in more detail, and the effectiveness of combinations of PVP and β-mercaptoethanol, in inhibiting the phenol oxidase system, measured indirectly by activity of RNA polymerase, was investigated with both fresh and cultured tissue. PVP at 1 mg/ml in the homogenising medium increased RNA polymerase activity of the fresh tissue by about 60%, and increased the activity from cultured explants from essentially zero activity (above background) to 600 cpm per explant (Fig. 3.4). The higher level of β-mercaptoethanol (0.25M) increased the measured activity from cultured explants but had little effect on preparations from fresh tissue. For all subsequent work PVP (1 mg/ml) and β-mercaptoethanol (0.25M) was included in the homogenising medium.
Effect of PVP, and β-mercaptoethanol in the extraction medium on the activity of soluble RNA polymerase from fresh and cultured artichoke explants

Fresh explants (Figure A) and explants grown for 15 hr. in the presence of 2,4-D (Figure B) were homogenised in a range of PVP concentrations at two levels of mercaptoethanol (\( \nu = 0.05 \text{M} \) and \( \nu = 0.25 \text{M} \)).

The soluble polymerase activity was assayed in duplicate and the results are expressed as cpm incorporated per explant above background (65 cpm.).

Changes of chromatin-bound RNA polymerase activity during the first cell division cycle.

Activities (averages of replicate assays) are expressed as cpm incorporated above background (50 cpm.) and time of incubation is shown as a fraction of the cell cycle (of 30-32 hr.). The figure is compiled from three overlapping experiments (shown as □, ■ and ○) in which the cell cycle times are similar.
Figure 3.44.

Figure 3.45.
2. Chromatin-bound and Soluble Polymerase Activity during the Growth of Explants

Due to the length of the cell cycle (time to first division as measured by increase of cell number) the experiments were divided into overlapping periods and the results combined to give the final figures. This procedure was only applied when the length of the cell cycles in the experiments were similar. The enzyme activities have been expressed on an explant basis since the determination of specific activity (activity per mg. protein) for crude extracts could be a misleading parameter if total protein varied independently of enzyme activity.

The changes of chromatin-bound RNA polymerase which occurred in the presence of 2,4-D are summarised in Fig.3.45. The time of culture is expressed as a fraction of the length of the cell division cycle, as determined by cell counts. The results shown are from 3 separate experiments, and indicated that after a lag of 3hr. (approximately 10% of the first cell division cycle), there was a rapid rise of activity which peaked after 12 to 15hr. (0.4 to 0.5 of the cycle), at 3-4 times the initial activity. This activity decreased to a minimum about 20hr. (0.7 cycle), when it was only twice the initial level, then increased through the rest of the cell cycle. The low level of 0.7 cycle was quite repeatable and a constant feature of the pattern of chromatin-bound polymerase activity (Fig.3.46) and appears to be associated with the start of the period of DNA synthesis (S period) as calculated on the basis that S + mitosis lasted 16hr. (Mitchell, 1967).
Fig. 3.46  Changes of Chromatin-bound RNA polymerases activity
during the first cell cycle

The results shown here are compiled from two overlapping experiments
(■ and □) and expressed as previously described. Background for
polymerase assay was 52cpm.

Fig. 3.47  Changes of chromatin-bound RNA polymerase activity
and cell number during the growth of explants for 80hr. in the
presence and absence of 2,4-D

Activities (averages of replicate assays) of chromatin-bound RNA
polymerase are expressed as cpm. incorporated per explant above back-
ground (60cpm.) and are denoted as ■ when grown in the presence of
2,4-D and □ in the absence of 2,4-D. Cell numbers are shown as ● for
explants grown in the presence of 2,4-D and ○ in the absence of 2,4-D.
Figure 3.46.

Figure 3.47.
The activity of chromatin-bound polymerase was compared in explants cultured in the presence and absence of 2,4-D for 80 hr. (Fig. 3.147). In the presence of 2,4-D, the initial increase of activity seen in the first cell cycle decreased slightly in the remainder of the period. Since the cell number increased 4-fold, the polymerase activity per cell in 80 hr. explants was considerably lower than in fresh tissue (about 50% of the initial value).

In the absence of 2,4-D, the activity showed a similar increase during the initial stages reaching 85% of the level of 2,4-D treated explants at 20 hr. and thereafter decreased until by 80 hr. there was only 22% of the level found in fresh tissue.

The activity of the total soluble RNA polymerase showed a pattern similar to chromatin-bound polymerase during the first cell division cycle (Fig. 3.48). There was an increase of soluble polymerase activity of about 50% during the first part of the cycle (up to 30% of the cycle), followed by a decrease of activity to a minimum, slightly higher than that in fresh tissue, at 70% of the cycle. In the final part of the cell cycle there was a small increase of activity through mitosis. Similar data from another experiment - shown in Fig. 3.49 - confirms these results. The pattern of soluble polymerase activity was therefore similar to that of chromatin-bound polymerase although the magnitude of the changes was less. The maximum increase of soluble activity was 150% of the level measured in fresh tissue, whereas the change of chromatin-bound activity was 370% over the same period.
Fig. 3.48 Changes of soluble RNA polymerase during the first cell cycle and during the early growth in the absence of 2,4-D

Activities (averages of replicate assays) of soluble RNA polymerase are expressed as cpm. incorporated above background (48 cpm.) per explant and time of incubation for the explants is shown as a fraction of the cell cycle (30 hr) or alternatively at 3 hr intervals for growth in the absence of 2,4-D. Changes of soluble polymerase in the presence of 2,4-D are denoted ▼ and ▼ in the absence of 2,4-D.

Fig. 3.49 Changes of soluble RNA polymerase activity during the first cell cycle

The results shown are expressed as previously described. Background for polymerase assay was 45 cpm.
Fraction of cell cycle

Figure 3.48.

Figure 3.49.
Culture of explants in the absence of 2,4-D gave rise to a similar pattern of soluble polymerase activity (Fig.3.48). There was an initial increase in activity reaching a maximum after 10hr. culture lower in magnitude to that observed in the presence of 2,4-D; thereafter there was a progressive reduction of soluble polymerase activity.

A comparison of soluble polymerase activities in explants cultured in the presence and absence of 2,4-D over longer periods is shown in Fig.3.50. In the presence of 2,4-D, soluble polymerase activity showed a stepped increase of activity, although the magnitude of the increase was less than that of cell number, so that the level of enzyme per cell decreased by more than 50% during the growth of the explant. In the absence of 2,4-D the activity decreased from 10 to 25hr. and then remained at this low level until 80hr.

The percentage of polymerase activity that was bound to chromatin, calculated from these results and shown in Fig.3.51, increased when explants were cultured in the presence of 2,4-D from 20% in fresh tissue to almost 50% during the cell cycle. There was a drop in the percentage of activity bound to chromatin at 70% through the cell cycle. Explants cultured in the absence of 2,4-D (Fig.3.51) showed a similar increase of the proportion of polymerase activity bound to chromatin. However, there was no detectable drop at the time corresponding to 70% of the cell cycle. Over a longer period of growth (80hr.), in the presence and absence of 2,4-D, the chromatin-bound activity increased to 50% of the total, and decreased only slowly with growth to about 40% after 80hr. whereas the chromatin-bound polymerase activity in explants cultured without 2,4-D showed a similar increase but then decreased rapidly until it represented only 7% of the total activity (Fig.3.52).
Fig. 3.50  Changes of soluble RNA polymerase and cell number during the growth of explants for 80hr. in the presence and absence of 2,4-D

Activities of soluble RNA polymerase (average of replicate assays) are expressed as cpm incorporated above background (48cpm) per explant and are denoted ■ for explants grown in the presence of 2,4-D and □ in the absence of 2,4-D.

Fig. 3.51  Changes of the percentage of total activity bound to the chromatin during the first cell division cycle and during culture in the absence of 2,4-D

Chromatin-bound polymerase activity, expressed as a percentage of the total polymerase activity, was calculated from the data previously shown and followed through the first cell division cycle of 30hr. ( ■ ) and during culture in the absence of 2,4-D ( □ ) for 30hr. (each division = 3.0hr.)
Figure 3.50.

Figure 3.51.
The total polymerase activity (chromatin-bound and soluble activity) was calculated through the cell cycle (Fig. 3.53). In the presence of 2,4-D there was a large initial increase of activity, which showed a substantial reduction at 70% of the cycle (to almost the original level of activity), and subsequently recovered to the level of maximum activity. In the absence of 2,4-D, the initial rise of polymerase activity was reduced and after a time, equivalent to 60% of the cycle (20hr.), fell substantially below the levels of activity of fresh tissue. Over longer periods of incubation in the presence of 2,4-D the total polymerase activity reached a level about twice that found in fresh tissue, (Fig. 3.54), at which time the cell number had doubled. However, during the subsequent increase of cell number, there was no further increase of polymerase activity so that by 80hr. the polymerase activity per cell had been reduced to half of the original level. In the absence of 2,4-D, the total polymerase activity was increasingly reduced after 20hr. until it was substantially below that of fresh tissue and only one-third of the level per explant as in the 2,4-D treated tissue.

3. RNA Synthesis In Vivo

Changes of the in vitro rate of RNA synthesis, as indicated by polymerase activity, were compared with measurements of the in vivo rates of synthesis of RNA. The accumulation of total RNA measured during the cell cycle showed an initial increase of 50% during the first quarter of the cell cycle followed by a flattening off and then a second increase similar in magnitude to the first about halfway through the cell cycle. A final increase in the last quarter of the cell cycle of RNA resulted in an overall increase of 250% during the cell cycle (Fig. 3.55).
Fig. 3.52 Changes of the percentage of total activity bound to the chromatin during growth of explants for 80 hr. in the presence and absence of 2,4-D

The results shown were calculated from data previously described. The percentage of total activity bound to the chromatin from explants grown in the presence of 2,4-D is shown by ■ and □ in the absence of 2,4-D.

Fig. 3.53 Changes of total RNA polymerase activity during the first cell cycle and culture in the absence of 2,4-D

The results shown are calculated from data previously described (i.e. chromatin-bound plus soluble activities expressed as cpm incorporated above background.)

■ = total polymerase activity from explants grown in the presence of 2,4-D and □ from explants grown in the absence of 2,4-D.
Figure 3.52.

Figure 3.53.
The results shown are calculated from data previously described. □ denotes changes of total activity from explants grown in the presence of 2,4-D and □ from explants grown in the absence of 2,4-D.

The total RNA per explant (▼), rate of \(^3H\)-uridine uptake (□) and \(^3H\)-uridine incorporation into RNA (●) was determined in duplicate as detailed in the text and the average values shown, during the first cell division cycle (33 hr.).
Figure 3.54

Figure 3.55
The incorporation of $^3$H-uridine into RNA was used as a measure of the rate of synthesis. Both uptake of $^3$H-uridine into the tissue and its incorporation into RNA commenced after a lag period of 3 hr. following excision of the explants and culture in the 2,4-D containing medium. The uptake and incorporation increased in parallel during the next 8 hr. after which time the uptake remained constant until 60% of the cell cycle, when it abruptly decreased and then increased to the previous level after 70% of the cell cycle. Incorporation into RNA decreased after 40% to a minimum at 60% of the cell cycle, followed by a slow increase until at the end of the cycle, the rate of incorporation was approaching the maximum rate observed earlier. This measurement of RNA synthesis showed a similar reduction after 70% of the cycle (20 hr. culture) to that observed with chromatin-bound and, to a lesser extent, the soluble polymerase activities. At all times the uptake of uridine was 10-20 times greater than that incorporated into RNA, so it was unlikely that, after the initial lag phase, uptake was limiting incorporation. A repeat of this experiment showed very similar changes of uptake and incorporation (Fig. 3.56).

In the absence of 2,4-D there was an initial rise in the rate of uptake and incorporation into RNA of $^3$H-uridine for 10 hr. (40% of the cell cycle). The magnitude of uptake and incorporation was comparable to that seen in explants grown in the presence of 2,4-D (Fig. 3.56). After 10 hr., both uptake and incorporation of $^3$H-uridine decreased progressively.
Fig. 3.56 Changes in the rate of $^3$H-uridine uptake and incorporation into RNA during growth in the absence and presence of 2,4-D.

The rate of $^3$H-uridine uptake (■) and incorporation into RNA (○) was followed during the growth of explants in the presence of 2,4-D and the absence of 2,4-D (□ for uptake and ○ for incorporation). Cell cycle time was 35 hr. and each division on the abscissa is 3.5 hr.

Fig. 3.57 Changes of nucleic acid content per explant during growth in the presence and absence of 2,4-D

The RNA content (sum of 1.3 x $10^6$ and 0.7 x $10^6$ RNA molecules) per explant was estimated by weighing tracings of u-v gel scans of nucleic acid preparations from cultured explants grown in the presence (■) or absence (□) of 2,4-D, similarly the DNA content was estimated after growth in the presence (○) or absence (○) of 2,4-D.
During the first 15 hr. of explant culture the patterns of uridine uptake and incorporation into RNA and the pattern of chromatin-bound and soluble polymerase activities were similar in both the presence and absence of 2,4-D. It was only after this period, which coincided with the onset of DNA synthesis in 2,4-D-treated tissue, that there were detectable differences between the two treatments. The changes observed during the first 15 hr. of culture may therefore be only incidentally rather than causally related to cell division.

The distribution of the classes of RNA transcribed at various times during the culture of the explants was determined by pulse labelling with $^{32}$P phosphate for 1 hr. and fractionation of the total RNA by gel electrophoresis. In order to get sufficient radioactivity incorporated into the RNA during the short labelling period, it was necessary to use as high a specific activity as possible without causing radiation damage to the tissue (Jackson and Ingle, 1973). Explants were therefore cultured in growth medium minus phosphate to examine the effects of this medium on division, total RNA and DNA (Table 3.14).
Table 3.14  Effect of Phosphate on the Growth of Explants

<table>
<thead>
<tr>
<th>%Division</th>
<th>+PO₄</th>
<th>-PO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40</td>
<td>42</td>
</tr>
<tr>
<td>RNA/µg/explant</td>
<td>5.60</td>
<td>5.65</td>
</tr>
<tr>
<td>DNA/µg/explant</td>
<td>1.34</td>
<td>1.53</td>
</tr>
</tbody>
</table>

Explants were grown in the presence of 2,4-D in medium plus and minus phosphate. Cell number, RNA and DNA content per explant were determined after 24hr.

The results confirmed that exogenous phosphate was not necessary for normal growth during the first cell division cycle.

At four points, chosen in the cell cycle to coincide with changes of total RNA, 0.75mCi of sterile $^{32}$P orthophosphate, in $6 \times 10^{-6}$M carrier phosphate, was added in the dark to each flask containing 100 explants in 15ml. The final concentration of $^{32}$P was 50 µCi/ml. in the growth medium.

The flasks were incubated for 1hr. and then the explants removed, washed, and the total nucleic acid prepared and fractionated by gel electrophoresis on 2.2 and 7% gels (Fig. 3.58). The synthesis of each class of RNA (2.5 x $10^6$ daltons precursor rRNA, 1.3 x $10^6$ rRNA, plus 1.4 x $10^6$ precursor rRNA, 0.7 x $10^6$ rRNA, polydisperse, 5S RNA and t-RNA) was expressed as a percentage of total newly-synthesised RNA (Table 3.15).
Table 3.15 Distribution of Newly-synthesised RNA during the Cell Division

<table>
<thead>
<tr>
<th>Time of Incubation (hr.)</th>
<th>Total RNA (measured as peak areas)</th>
<th>2.5x10^6 precursor rRNA</th>
<th>1.3x10^6 rRNA</th>
<th>0.7x10^6 rRNA</th>
<th>5S RNA</th>
<th>t RNA</th>
<th>Polydisperse RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10-11</td>
<td>405</td>
<td>10</td>
<td>35</td>
<td>16</td>
<td>0.5</td>
<td>6</td>
<td>33</td>
</tr>
<tr>
<td>13-14</td>
<td>480</td>
<td>9</td>
<td>36</td>
<td>11</td>
<td>0</td>
<td>4</td>
<td>39</td>
</tr>
<tr>
<td>22-23</td>
<td>802</td>
<td>10</td>
<td>37</td>
<td>15</td>
<td>1</td>
<td>3.5</td>
<td>33</td>
</tr>
</tbody>
</table>

Explants (100/flask) were incubated for 1hr. with 0.75mCi of 32P in 6μM carrier phosphate at the times shown in complete growth medium plus 6μM phosphate. Total nucleic acid was prepared and fractionated on 2.2% and 7.0% polyacrylamide gels. The distribution of various components was calculated from the relative areas of the radioactivity peaks (see Fig.3.58); the results from each sampling time were comparable on an explant basis. (* including 1.4 x 10^6 precursor rRNA).

During the first hour of culture there was no detectable synthesis of RNA as measured by incorporation of 32P label. At the other 3 times in the first cell cycle, the distribution of the newly-synthesised classes of RNA was quite constant with about 60% of ribosomal RNAs, 35% polydisperse, and 5% tRNA synthesised at specific times during the cell cycle.
Fig. 3.58  Synthesis of nucleic acids during the cell division cycle

Nucleic acid was prepared from explants grown for: A) 22hrs. B) 13hr.
and C) 10hr. in 2,4-D containing medium and labelled for 1hr. with
$^{32}P$-orthophosphate (750 $\mu$C/15ml.). Samples were fractionated by
polyacrylamide gel electrophoresis (2.2% gels run for 3hr. at 6mamps/
tube). The continuous line represents the $E_{265}$ and the histogram
represents the radioactivity as counts/100sec./0.5mm slice.
Figure 3.58.
The distribution of the classes of RNA synthesized was examined over a longer period of culture in the presence (Fig. 3.59) and absence of 2,4-D (Fig. 3.60). In the presence of 2,4-D, the DNA content per explant, measured as the area of the 260nm OD peak, increased linearly after 36hr., which was in agreement with the increase of cell number. Total rRNA (measured by 265nm OD peak area) showed an initial increase, in the absence of DNA synthesis, and then a second increase, up to 48hr., thereafter the level of total rRNA remained constant (Fig. 3.57). This data on accumulation was confirmed by the rates of incorporation of $^{32}$P into RNA (Table 3.16). There was an increase of incorporation up till 48hr. after which time it ceased.
Table 3.16 Distribution of Newly-synthesised RNA during 80hr. Culture of Explants in the Presence of 2,4-D

<table>
<thead>
<tr>
<th>Time of Incubation (hr.)</th>
<th>TOTAL RNA (measured as peak areas)</th>
<th>% of total newly-synthesised RNA</th>
<th>2.5x10⁶ precursor RNA</th>
<th>1.3x10⁶ rRNA</th>
<th>0.7x10⁶ rRNA</th>
<th>5S RNA</th>
<th>tRNA</th>
<th>Polydisperse RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>103</td>
<td>5</td>
<td>21</td>
<td>10</td>
<td>2</td>
<td>5</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>373</td>
<td>13</td>
<td>23</td>
<td>9</td>
<td>2</td>
<td>6</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>920</td>
<td>8</td>
<td>23.5</td>
<td>20</td>
<td>2</td>
<td>6</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>772</td>
<td>8</td>
<td>23.5</td>
<td>8</td>
<td>3</td>
<td>6</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>853</td>
<td>8</td>
<td>23</td>
<td>11</td>
<td>3</td>
<td>7</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>78</td>
<td>319</td>
<td>7</td>
<td>25</td>
<td>7</td>
<td>2</td>
<td>7</td>
<td>53</td>
<td></td>
</tr>
</tbody>
</table>

Explants (100/flask) were incubated for 1hr. with 0.75mCi of 32P in 6 μM carrier phosphate at the times shown after growth in complete medium + 6 μM phosphate. Total nucleic acid was prepared and fractionated on split gels of 2.2% and 7.0% polyacrylamide. The distribution of various components was calculated from the relative areas of the radioactivity peaks (see Fig.3.59); the results from each sampling time were comparable on an explant basis.

(* Including 1.4 x 10⁶ precursor RNA)

The distribution of 32P in the various classes of RNA remained reasonably constant up to 80hr. growth. Ribosomal RNAs represented 45% of the total RNA synthesised, polydisperse 50%, and tRNA about 5%. The relationship between transcription and processing of rRNA remained constant as determined by the ratio of label in 2.5 x 10⁶ precursor RNA and 1.3 or 0.7 x 10⁶ rRNA. When the rate of accumulation of total RNA decreased after 48hr. there was a general reduction of synthesis of all classes of RNA in proportion.
Nucleic acid was prepared from explants grown for A) 78hr., B) 48hr., and C) 23hr. in the presence of 2,4-D and labelled for 1hr. with $^{32}$P-orthophosphate (750 $\mu$Ci/15ml.). Samples were fractionated by polyacrylamide gel electrophoresis (2.2% and 7.0% split gels run for 3hr. 15min. at 6mamp/tube). The continuous line represents $E_{265}$ and the histogram represents the radioactivity as counts/min/0.5mm slice.

Fig. 3.59  Nucleic acid synthesis during the culture of explants in presence of 2,4-D
In the absence of 2,4-D, there was no significant increase in DNA, whilst the total RNA increased in the first 24 hr. to the same extent as in the 2,4-D-treated tissue, and thereafter was constant or decreased slightly (Fig. 3.57). This was supported by measurements of the rates of synthesis by incorporation of $^{32}$P which increased up to 23 hr. and then decreased (Table 3.17).

Table 3.17 Distribution of Newly-synthesised RNA during 80 hr. Culture of Explants in the Absence of 2,4-D

<table>
<thead>
<tr>
<th>Time (hr.)</th>
<th>TOTAL RNA (measured as peak area)</th>
<th>% of total newly-synthesised RNA</th>
<th>Polydisperse RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5x10$^6$ precursor rRNA</td>
<td>1.3x10$^6$ rRNA</td>
<td>0.7x10$^6$ rRNA</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------------</td>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>10</td>
<td>70</td>
<td>7</td>
<td>21.5</td>
</tr>
<tr>
<td>23</td>
<td>1150</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>36</td>
<td>458</td>
<td>8</td>
<td>11</td>
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<tr>
<td>48</td>
<td>367</td>
<td>6.5</td>
<td>7</td>
</tr>
<tr>
<td>57</td>
<td>243</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>78</td>
<td>338</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Explants (100/flask) were incubated for 1 hr. with 0.75 mCi of $^{32}$P in 6 μM carrier phosphate at the times shown, after growth in complete medium + 6 μM phosphate. Total nucleic acid was prepared and fractionated on split gels of 2.2% and 7.0% polyacrylamide. The distribution of various components was calculated from the relative areas of the radioactivity peaks (see Fig. 3.60); the results from each sampling time were comparable on an explant basis. (* Including 1.4 x 10$^6$ precursor RNA)
Fig. 3.60  Nucleic acid synthesis during the culture of explants in the absence of 2,4-D

Nucleic acid was prepared from explants grown for A) 78hr., B) 48hr., and C) 23hr. in the absence of 2,4-D and labelled for 1hr. with $^{32}$P-orthophosphate (750 µC/15ml.). Samples were fractionated by polyacrylamide gel electrophoresis (2.2% and 7.0% split gels for 3hr.15min. at 6mamp/tube). The continuous line represents $E_{265}$ and the histogram represents the radioactivity as counts/4min/0.5mm slice.
Figure 3.60.
Up to 23hr. the distribution of the RNAs synthesised was identical to the 2,4-D-treated tissue and after this time, although the rate of total RNA synthesis decreased, there was a differential inhibition of rRNAs and tRNA more than polydisperse RNA which increased from 50 to 80% of the total synthesised.

This estimate of polydisperse RNA may include some rRNA degradation products but, since much of the increase in this class of RNA was of high molecular weight RNA, the rRNA contamination may be negligible. The major effect on rRNA was on the processing of the $2.5 \times 10^6$ precursor rRNA, since the precursor was still transcribed in the same amount throughout. The processing of the precursor through to rRNA and 5S RNA was co-ordinately repressed. There was a relatively small decrease of transcription of precursor rRNA which may be a feed-back effect from the processing of the molecule.

4. **Effect of α-Amanitin on the Synthesis of Classes of RNA**

Since it has been shown that α-amantin inhibits *in vitro* polymerase II (nuclear enzyme, presumably responsible for the synthesis of polydisperse RNA), the effect of its addition to the growth medium of the explants was investigated. Preincubation with α-amantin resulted in the same overall rate of total RNA synthesis as measured by $^{32}$P-orthophosphate incorporation but reduced the percentage of the label in the polydisperse RNA from 50 to 45% (Table 3.18). The small reduction in the synthesis of this class of RNA does not correspond to the total inhibition of polymerase II activity seen *in vitro* and probably reflects difficulty in uptake of α-amantin by the explants.
Table 3.18: Effect of Incubation with α-Amanitin on the Synthesis of Various Classes of RNA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of total RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Precursor RNA</td>
</tr>
<tr>
<td>+α-Amanitin</td>
<td>4</td>
</tr>
<tr>
<td>-α-Amanitin</td>
<td>4</td>
</tr>
</tbody>
</table>

Explants, grown for 80hr, were treated with 1 μg/ml. α-amanitin for 1 hr, and then incubated with $^{32}$P-orthophosphate for 1 hr to label the newly-synthesised classes of RNA. The distribution of classes of RNA synthesised was investigated by fractionation by gel electrophoresis of the total RNA as previously described.
SECTION III: Demonstration of a factor intermediate in the Response of Chromatin-bound Polymerase to the 2,4-D treatment

In several cases, a 2,4-D binding protein has been implicated as an intermediary in the response of chromatin-bound polymerase to the addition of 2,4-D to a tissue (e.g. Hardin, O'Brien, and Cherry, 1970; Matthyse, 1970; Venis, 1971). In order to examine the possibility that such a system exists in artichoke, the response of chromatin to 2,4-D added either to the assay medium or to the homogenising medium was determined. When 2,4-D was added directly to the assay medium at 10^{-4} M (Fig. 3.61), the chromatin-bound polymerase activity was inhibited. However, when chromatin was isolated from replicate samples of fresh explants in homogenising medium containing 2,4-D (at 10^{-4} M), the level of chromatin-bound polymerase was stimulated by up to two fold (Fig. 3.61).

This has been interpreted as evidence for a factor (lost during the preparation of chromatin) which binds or is activated by 2,4-D and which then combines with or activates the chromatin-bound RNA polymerase. A third possibility exists for the action of this 2,4-D-activated factor, and that is it may cause increased binding of soluble polymerase to the chromatin.

In order to further investigate the role of this factor, explants that had been grown in the presence and absence of 2,4-D for 6 days were extracted in media with and without the addition of 10^{-4} M 2,4-D. The chromatin-bound activity of explants grown in the absence of 2,4-D was stimulated by two-fold when extracted in the presence of 10^{-4} M 2,4-D, and there was a decrease of polymerase activity from the soluble fraction (Table 3.19). This result supports the theory that the 2,4-D factor could function by binding soluble polymerase to the chromatin resulting in an increased level of chromatin-bound polymerase activity. The chromatin-bound polymerase activity from explants grown
Fig. 3.61  **Effect of 2,4-D present in the homogenising medium and the assay medium on chromatin-bound polymerase activity**

Replicate extractions from 100 explants in 10ml. homogenising medium containing various levels of 2,4-D were made, and the RNA polymerase activity of the chromatin extracted was assayed (-----). Chromatin extracted in the absence of 2,4-D was assayed at different levels of 2,4-D (-----) for RNA polymerase activity. Each point shown is the average of duplicate extractions and assays and activities are expressed as a percentage of the control incorporation above background (80cpm.) in the absence of 2,4-D. The control incorporated 560cpm.
Concentration of 2,4-D M.

Fig. 3.61.
in the presence of 2,4-D is only slightly stimulated when isolated in the presence of 2,4-D in comparison with the level of activity when isolated in the absence of 2,4-D, possibly because after 6 days' growth in 2,4-D containing medium, the system is saturated and no longer responds to 2,4-D. However, the soluble activity was also reduced when extracted in the presence of 2,4-D compared to isolation in the absence of 2,4-D so that this effect may not be related to the stimulation of chromatin-bound activity.

Table 3.19 Effect of Isolation of Chromatin in the presence and absence of 2,4-D from Explants grown for 6 days in the presence and absence of 2,4-D

<table>
<thead>
<tr>
<th>Growth Conditions of Explants</th>
<th>Extraction Medium</th>
<th>RNA Polymerase</th>
<th>Total Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2,4-D</td>
<td>-2,4-D</td>
<td>Chromatin-bound</td>
<td>2417</td>
</tr>
<tr>
<td></td>
<td>+2,4-D</td>
<td>Chromatin-bound</td>
<td>5375</td>
</tr>
<tr>
<td></td>
<td>-2,4-D</td>
<td>Soluble</td>
<td>6950</td>
</tr>
<tr>
<td></td>
<td>+2,4-D</td>
<td>Soluble</td>
<td>5590</td>
</tr>
<tr>
<td>+2,4-D</td>
<td>-2,4-D</td>
<td>Chromatin-bound</td>
<td>14,810</td>
</tr>
<tr>
<td></td>
<td>+2,4-D</td>
<td>Chromatin-bound</td>
<td>16,320</td>
</tr>
<tr>
<td></td>
<td>-2,4-D</td>
<td>Soluble</td>
<td>21,100</td>
</tr>
<tr>
<td></td>
<td>+2,4-D</td>
<td>Soluble</td>
<td>12,170</td>
</tr>
</tbody>
</table>

All activities are the average of duplicate determinations and are expressed as cpm incorporated above background (85cpm for chromatin bound activity and 60cpm for soluble RNA polymerase activity).
RNA polymerase activity has been demonstrated in a number of plant tissues either in the soluble (10,000 x g) fraction, e.g. maize (Stout and Mans, 1967; Strain, Mullinix and Bogorad, 1971) or bound to the chromatin fraction, e.g. from soyabeans (O'Brien, Jarvis, Cherry and Hanson, 1968; Holm, O'Brien, Key and Cherry, 1970); sugar beet (Duda and Cherry, 1971; Dunham, Jarvis, Cherry and Duda, 1971); pea (McComb, McComb and Duda, 1970), hazel (Jarvis, Frankland and Cherry, 1968), and cucumber (Johnson and Purves, 1970). The initial reports suggested no chromatin-bound activity from maize and no soluble activity from soyabeans. However, this distinction may not be so clear; for example, Duda and Cherry (1971) refer to a rather large amount of soluble activity in the supernatant from sugar beet and some insoluble polymerase activity was noted from maize extracts (Stout and Mans, 1967). In the present studies, both soluble and chromatin-bound RNA polymerase activities have been isolated from artichoke tissue. Both activities possess the properties of an RNA polymerase, in that the activity is dependent on the presence of a DNA template (endogenous or exogenous), all four ribonucleoside triphosphates and a divalent cation. Although the chromatin-bound activity utilizes endogenous DNA, the activity is stimulated by the addition of denatured DNA but inhibited by native DNA. Presumably, therefore, the chromatin-bound polymerase activity can bind onto exogenous DNA, which may or may not be as efficient a template as the endogenous DNA. The divalent ion requirement of the chromatin-bound polymerase activity is predominantly for manganese, in that addition of magnesium does not stimulate the reaction except at suboptimal manganese concentrations. In this result, the artichoke chromatin-bound polymerase is similar to E. coli polymerase (Chamberlin and Berg, 1962).
Magnesium, in the presence of manganese, inhibits the polymerase as does either ion at high concentration (greater than 2.5mM). The inhibition of activity by magnesium at optimal manganese concentrations does not appear to be a direct effect of ionic strength, since the combined concentration (of Mn\textsuperscript{2+} and Mg\textsuperscript{2+}) results in a greater activity of the polymerase than the same concentration of either ion alone. Soyabean chromatin-bound polymerase, however, requires both manganese and magnesium ions for optimal activity.

Monovalent ions stimulate artichoke chromatin-bound polymerase by 160 and 210% (potassium chloride and ammonium sulphate respectively). Comparable stimulation was not observed with soyabean chromatin-bound activity. This stimulation may well be due to the removal of histones from the chromatin by the salt concentrations used (300mM KCl and 62.5mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}).

The kinetics of nucleotide polymerisation by RNA polymerase of artichoke chromatin is similar to that reported in other plant systems; after an initial linear rate, the incorporation flattens off after about 10min. This may be a general property of chromatin-bound RNA polymerases, as it has been reported in the other cases described. It cannot be attributed to loss of \textsuperscript{3}H-UTP from the incubation medium (Fig.3.2) and may be a result of an inherent instability of the enzyme or lack of release of the newly-synthesised RNA chains. Richardson, (1966) has shown that there is no release of newly-synthesised RNA chains when E. coli polymerase is incubated in a reaction medium in low salt; the RNA remaining bound to the DNA. In higher salt (0.20M KCl) the RNA chains were released and the rate of reaction remained linear for longer. The kinetics of incorporation of the artichoke chromatin-bound polymerase showed a similar continued increase in high salt.
Such conditions are not, however, compatible with accurate transcription from chromatin, since chromatin is minimally soluble in the salt range 0.2-0.4M and higher salt concentrations disrupt the protein-nucleic acid association (Georgiev, Ananieva and Kozlov, 1966). The specific activity of chromatin-bound RNA polymerase measured in high salt was 30 picomoles UMP incorporated in 10 min. incubation at 30° C per mg. protein, compared with 56 picomoles UMP mg. protein of pea polymerase and 58% of soyabean polymerase (Table 3.1.2).

Observed correlations between spermidine and RNA levels, together with an in vitro stimulation of RNA polymerase by spermidine has led to the suggestion that polyamines may act as an intracellular regulator of RNA polymerase activity. However, spermidine inhibits RNA polymerase activity of artichoke chromatin at all concentrations used. This is in agreement with the results from soyabean chromatin (O'Brien, Jarvis, Cherry and Hanson, 1968). The extent of the inhibition (about 50% at 12.5mM spermidine) was very similar in both cases. The activity of chromatin polymerase was only slightly inhibited by α-amanitin. Since this drug has been shown to inhibit polymerase II but not polymerase I in eucaryotic tissues, e.g. artichoke (this thesis), maize (Strain, Mullinix and Bogorad, 1971), coconut endosperm (Mondal, Mandal and Biswas, 1970), sea urchin and rat liver (Roeder and Rutter, 1969), calf thymus (Chambon et al., 1970), this partial inhibition could suggest that the chromatin-bound activity was composed predominantly of polymerase I type.

The chromatin-bound activity could be limited by the availability of template or by the amount of the polymerase itself. The stimulation on adding exogenous denatured DNA suggests that it is the template that is limiting, although it may be argued that the purified DNA added is a more efficient template which the endogenous polymerase can bind onto and utilise.
Conversely, the addition of E.coli polymerase to chromatin preparations results in a 6-10 fold stimulation of incorporation, which has been interpreted as excess template being available for transcription.

The polymerase activity in the 10,000 x g supernatant is presumably of nuclear origin, since the nuclei of artichoke are extremely fragile and rupture easily during homogenisation of the tissue. By comparison, the soluble polymerases from both animals and coconut endosperm are extracted from purified nuclei. An RNA polymerase activity has been isolated from mitochondrial preparations from artichoke, but from an estimate of its quantitative contribution it is unlikely to contribute greatly to the total polymerase activity isolated from the tissue. The soluble polymerase was sensitive to DNAase, RNAase and pyrophosphate, but was not inhibited by phosphate. The product of the reaction was identified by acrylamide gel electrophoresis as a reasonably discrete molecule of 120,000M.W. Since this polymerase activity has been shown to consist of at least two separate activities, interest in the properties of the total soluble activity stems only from the necessity to define optimal assay conditions. The enzyme can utilise single-stranded DNA as template and shows no specificity as to the origin of the DNA (artichoke or calf thymus). Double-stranded DNA is only 10% as efficient as single-stranded DNA. Introduction of more ends into the DNA by sonication or DNAase I-treatment did not increase the efficiency of the template, so that the enzyme appears to bind onto single-stranded DNA and not to polymonucleotide chain ends in the template, assuming that these would have been limiting the activity prior to sonication or DNAase-treatment. This contrasts somewhat with the result of Stout and Mans(1967), working with total soluble polymerase from maize, who found that denaturation of the DNA did not substantially increase its template efficiency.
Unlike the chromatin-bound polymerase, the soluble activity required the presence of both cations ($\text{Mg}^{2+}$ and $\text{Mn}^{2+}$) for full activity but this may only reflect the multiple polymerase activities in this fraction.

Increasing monovalent salt concentration inhibited the soluble polymerase activity, with 50% inhibition at 125mM KCl. This agrees well with the data for maize soluble polymerase (Strain, Mullinix and Bogorad, 1971), although Stout and Mans (1967) reported that the activity was dependent on 50mM (NH$_4$)$_2$SO$_4$. The inhibition of the soluble artichoke polymerase by salt contrasts with the salt stimulation of the chromatin-bound activity.

Polyamines, (spermine and spermidine), inhibited the soluble polymerase except at low concentrations of spermidine where there was some stimulation of the activity. The activities were assayed on denatured DNA, whereas the stimulations seen with E. coli polymerase occurred only on native DNA. Regulation of artichoke polymerase activity by polyamine concentration cannot be completely dismissed but finds little supporting evidence from this work. The specific activity of the total soluble polymerase was very low (0.375 pmoles UMP incorporated per mg. protein) since no purification had been attempted.

The drug $\alpha$-amanitin at 0.25 $\mu$g/ml. inhibits total soluble activity by 50%. This suggests an equal contribution of polymerase I and II activities to the total soluble activity, since this concentration of $\alpha$-amanitin is known to completely inhibit polymerase II with no effect on polymerase I.
Chromatin-bound and soluble activities differ in their response to DNA, manganese and magnesium, and monovalent salt. This raises the question of whether or not the two activities are the same enzyme in different locations or two different enzymes. On the basis of the αamanitin data, the soluble activity would represent a mixture of 50% of polymerase I and II, whereas the chromatin-bound activity represents 80% polymerase I and 20% polymerase II. The difference of the fractions in their salt response may only reflect the complex nature of chromatin; the observed stimulation of this fraction, for instance, could be related to the removal of inhibitory histones from the DNA so increasing template availability rather than a direct effect on the RNA polymerase. Exogenous DNA added to chromatin produces a similar qualitative response to that of soluble polymerase, i.e. more efficient utilisation of the denatured template, and argues in favour of a certain lability in polymerase binding to the endogenous DNA. Although it has been possible to solubilise the chromatin-bound activity to some extent so that it is recovered in the supernatant fraction (10,000 x g), the activity still retains its salt stimulation. This activity is not DNA dependent and so solubilisation, in this context, probably only resulted in the shearing of the chromatin into smaller pieces without disruption of its structure. While it has not been possible to show conclusively that chromatin-bound and soluble polymerase activities are different, the available evidence allows the interpretation that the chromatin-bound activity may be the same enzyme as found in the soluble fraction but associated with the chromatin.
The fractionation of two enzyme activities from DEAE cellulose columns is consistent with recent findings from both animal and plant tissues, e.g. rat liver (Roeder and Rutter, 1969), maize (Strain, Mullinix and Bogorad, 1971). The failure of Stout and Mans (1967), to resolve two activities from maize may be related to the lability and smaller quantities of polymerase I in this tissue. A low yield of polymerase I, assumed to be of nucleolar origin by comparison with rat liver (Roeder and Rutter, 1970a), may result from incomplete nucleolar breakage during the homogenisation, yielding high ratios of polymerase II to polymerase I. Inadequate degassing during the preparation of the DEAE cellulose resulted in loss of resolution of the two polymerases (Fig. 3.2) and this could explain the results of Stout and Mans.

The activities of polymerase I and II differed in their response to divalent cations. Polymerase I was stimulated to maximum activity by either manganese or magnesium, whereas polymerase II was stimulated only by manganese. It is possible that all the magnesium binding sites have been saturated with magnesium during the extraction and purification of the enzyme in magnesium buffers. These results are intermediate between those from animal tissue, where the RNA polymerases show maximal activity with Mn$^{2+}$ (Roeder and Rutter, 1969; Sugden and Sambrook, 1970), and those from maize where Mg$^{2+}$ produces maximum activity for both polymerases (Strain, Mullinix and Bogorad, 1971). At divalent ion concentrations higher than the optimum, both of the polymerase activities are inhibited in both artichoke and maize; manganese being more strongly inhibitory than magnesium in both cases.
Monovalent ions (KCl) inhibited both polymerases in artichoke, polymerase II being more sensitive to salt (50% inhibition at 140mM) than polymerase I (50% inhibition at 440mM). The two maize polymerase activities are equally inhibited by KCl or (NH₄)₂SO₄ (50% inhibited at 125mM), whereas rat liver and sea urchin polymerase I is 50% inhibited at 75mM, and polymerase II at about 150mM (NH₄)₂SO₄. The significance of this variation will remain obscure until a wider range of tissues has been analysed for multiple polymerase activities.

Much importance has been attached to the template specificities of the multiple polymerases isolated from eucaryotic tissues. One of the points of interest has been the recognition and preferential transcription of homologous template DNA. Neither polymerase I nor II from artichoke tissue exhibited any specificity towards artichoke DNA in preference to calf thymus DNA (in the same state, e.g. native or denatured) although maize DNA was utilised most efficiently by the multiple maize polymerases (Strain, Mullinix and Bogorad, 1971). On the basis of the procariotic model, the specificity for transcription of DNA may reside in a factor or factors associated with the polymerase. During purification it is possible that such a factor could be lost with the concomitant loss of template specificity. However, even in the crude supernatant fraction, before any purification, the total soluble activity did not exhibit any preferential transcription of artichoke rather than calf thymus DNA. It would however be of interest to test the template specificity of each polymerase against specific parts of the genome, e.g. nucleolar and nucleoplasmic DNA. This has been demonstrated using Xencopus polymerases I and II, which show an affinity for nucleolar and nucleoplasmic DNA respectively (Tocchini-Valentini and Crippa, 1970).
The situation with respect to the utilization of native versus denatured DNA is more complex. Artichoke polymerase I will utilise only denatured DNA, whilst polymerase II utilises both denatured and native DNA, but the latter with only 25% efficiency of the denatured template. It was not however possible to further fractionate polymerase II on the basis of template requirements. The polymerase II activity (as eluted from DEAE cellulose) was coincident when assayed on native or denatured DNA. Furthermore, utilisation of either template by polymerase II was unaffected by divalent ion concentration. In maize (Strain, Mullinix and Bogorad, 1971) polymerase I (eluting at 0.08M) prefers native DNA and polymerase II can be resolved into fractions IIIa (eluting at 0.18M), and IIb (eluting at 0.22M), which prefer denatured and native DNA respectively. On rechromatography of peaks IIIa and IIb on DE-52, activity IIIa again eluted as a symmetrical peak at 0.18M, but IIb, which previously eluted at higher ionic strength, eluted at 0.18M also, and had all the properties of peak IIIa, including its preference for denatured DNA as template. This alteration of IIb to IIIa could also be effected by glycerol-gradient centrifugation and has been explained by the loss of a specific factor that enables the polymerase II to read native DNA. The partial loss of such a factor during the preparation of artichoke polymerase II and total loss from polymerase I, or contamination of the second peak of activity with this factor, could account for the observed template requirements. Attempts have been made to explain the template requirements of multiple polymerases by postulating contamination of one or both of the peaks by ribonuclease H (Stein and Hausen, 1970a). Ribonuclease H is an RNAase which specifically hydrolyses RNA when it is hybridised to DNA. Thus, if the product of an RNA polymerase activity is a DNA-RNA hybrid (e.g. when assayed on a denatured template) then the product will be hydrolysed. However, if the product of the reaction is free RNA (e.g. assayed on native template), it would not be hydrolysed.
However, there is no evidence for the existence of ribonuclease II in plant tissues and different patterns of elution from DEAE would have to be postulated for different tissues to account for variation of the observed template requirements. Polymerases I and II from sea urchin can utilise both native and denatured DNA, but are saturated at lower concentrations of denatured DNA (Roeder and Rutter, 1969). The relative activity of the calf thymus polymerase II on native and denatured DNA is also a function of salt concentration (Jacob, Sajdel and Munro, 1968). Whilst the effect of salt on polymerase II activity on native and denatured DNA was not considered directly, the effect of salt on total soluble activity on the two templates was investigated. There was a similar inhibition of polymerase activity with increasing salt when assayed on either native or denatured template, so it is unlikely that the template requirement of polymerase II was being influenced greatly by high levels of salt. As expected, neither polymerase I nor II was inhibited by cycloheximide or rifampicin; however, α-amanitin (0.25 μg/ml.) inhibited totally polymerase II, whilst having no effect on the activity of polymerase I. This is in agreement with all of the other results of the response of multiple eucaryotic polymerases.

The specific activities of the artichoke polymerases I and II were similar to that reported for maize (Strain, Mullinix and Bogorad, 1971); polymerase I was 184 pmoles/mg. protein (compared to 300 pmoles/mg. protein for maize polymerase I) and polymerase II was 240 pmoles/mg. protein (compared to 300 pmoles/mg. protein for maize polymerase II).

Both chromatin-bound and soluble polymerase activities increased when artichoke explants were cultured for 20 hr. under conditions which did not lead to cell division (-2, 4-D). After longer times of incubation in the absence of 2, 4-D, both activities decreased drastically.
The percentage of activity bound to the chromatin increased from 30 to 50% during the first 20hr; then decreased to 6% after 60hr incubation. Loss of histones from chromatin of artichoke tuber discs on ageing in water has been reported by Kamińska and Masuda, 1968, and this would lead to an increase of template DNA available for transcription. High salt concentrations have been shown to dissociate chromosomal proteins and consequently increase the template activity of the chromatin (Marushige and Bonner, 1966). This initial rise of activity therefore appears to be consequential of the excision and culture process rather than being involved in the process of cell division. Similar changes of chromatin-bound activity have been observed in sugar beet (Duda and Cherry, 1971) and pea tissue (McComb, McComb and Duda, 1970).

Culture of explants under conditions leading to cell division (+2,4-D) resulted in an initial increase of soluble and chromatin-bound activities very similar to that of the non-dividing system. However, in the presence of 2,4-D the activities were sustained throughout the rest of the first cell division cycle with the exception of a transient decrease, of both activities, at 70% of the cell cycle. The ratio of chromatin-bound to soluble activity increased over this period from 30 to 50% in the first 20hr. with a transient decrease to 35% after 22hr incubation (70% of cycle). The drop of polymerase activity, which is also correlated with a drop of 3H-uridine uptake and incorporation into RNA, coincides with the calculated start of DNA synthesis. This situation is exceptional in comparison with other synchronised eucaryotic cell cultures where RNA synthesis increases linearly throughout the cell cycle, but is similar to the changes of RNA synthesis observed in the cells and isolated nuclei of Physarum (Mittermayer, Braun and Rusch, 1964; Braun, Mittermayer and Rusch, 1966).
This interphase fall of the in vivo and in vitro rates of RNA synthesis may perhaps be associated with DNA replication. Longer periods of culture in 2,4-D resulted in a maintenance of the elevated level of chromatin-bound activity and a further increase of the soluble polymerase activity. Consequently, the total polymerase activity per explant increased and the ratio of chromatin-bound to soluble activity slowly decreased to about that level found in fresh tissue (Fig. 3.52). However, when expressed on a cell basis, both the chromatin-bound and soluble activity had decreased to about 50% of the level found in fresh tissue. Over this period of culture, in the presence of 2,4-D, both the total protein and total RNA content per cell decreased (Yeoman and Mitchell, 1970). Therefore, the increase in polymerase activity is not an essential feature of the cell cycle since subsequent cycles are achieved with lower amounts of polymerase per cell. This initial increase of polymerase activity is not stimulated by 2,4-D, but the maintenance and the subsequent increase is dependent on the presence of 2,4-D.

In many cases that have been studied, treatment with 2,4-D has been shown to lead to an increase of endogenous polymerase activity and RNA content, e.g. soya bean (O'Brien, Jarvis, Cherry and Hanson, 1968), pea internodes (GA₃-treated), (McComb, McComb and Duda, 1970), cucumber (Johnson and Purves, 1970). This raises the question of whether the increased activity is as a result of increased polymerase activity itself or increased template availability. These possibilities can be discriminated by the addition of excess E.coli polymerase to measure the availability of template DNA.
The validity of this method is dependent on showing that *E. coli* polymerase is transcribing the same part of the genome as the endogenous polymerase and is not initiating synthesis at sites unavailable to the endogenous polymerase. By competitive hybridisation studies it has been shown that the RNA transcribed from chromatin by *E. coli* RNA polymerase is similar to the *in vivo* transcription product (Smith, Church and McCarthy, 1969; Paul and Gilmour, 1968). Under the conditions used in these hybridisation studies only the transcription product from the highly repetitive part of the genome would hybridise to the DNA (Britten and Kohne, 1968), and this may represent only a small portion of the total RNA. Differences of initiation specificity between eucaryotic and *E. coli* polymerases might be expected to result in different transcription products on eucaryotic chromatin. This, however, does not seem to be the case as RNA transcribed from mouse kidney chromatin by *E. coli* or mouse polymerase, competes similarly against hybridised homologous *in vivo* RNA. It would seem therefore that *E. coli* polymerase transcribes the same part of the genome but at a more rapid rate than the endogenous polymerase.

The increase of chromatin-bound polymerase activity after 2,4-D-treatment was examined by O'Brien, Jarvis, Cherry and Hanson (1968). Addition of *E. coli* polymerase to chromatin (treated and control) stimulated incorporation indicating that template availability was not limiting in either case. The 2,4-D treatment resulted in a higher level of endogenous polymerase activity, implying since template was not limiting, there had been an increase of polymerase activity. If there had been no change in the template availability of the chromatin, addition of excess *E. coli* polymerase would be expected to saturate the chromatin (treated and untreated) to the same extent. However, this was not the case since 2,4-D treated chromatin maintained a higher level of incorporation with saturating *E. coli* polymerase than untreated chromatin so that the template availability must have been increased.
From this work the conclusion must be drawn that 2,4-D-treatment increased both template availability and endogenous polymerase activity. When expressed as a percentage of the control value, the effect of 2,4-D on template availability was small compared with the effect on polymerase activity.

Changes of template availability and endogenous polymerase activity have been followed during washing of sugar beet discs, (Duda and Cherry, 1971). The RNA polymerase activity increases 7-fold over 24 hr, then declines with further washing, and the DNA template availability follows a similar pattern. Like the soyabean response to 2,4-D, the sugar beet exhibits a co-ordinated change of endogenous polymerase activity and template availability. Gibberellin induced RNA synthesis in dwarf peas (McComb, McComb and Duda, 1970) appears to involve only an increase of RNA polymerase activity without any change of template availability; however, very small amounts of chromatin were used to assay for template changes and any such changes could have been obscured.

The stimulation of endogenous chromatin-bound RNA polymerase by hormones may be a result of de novo synthesis of a specific chromatin-bound polymerase, activation of a pre-existing chromatin-bound polymerase by the synthesis of an initiation factor, or more simply it may represent binding of the soluble nuclear polymerase to the chromatin, possibly by a binding factor. Previous work has only considered changes of the chromatin activity in isolation from other polymerase activities that may exist in the nucleus. There are indications from experiments that homogenisation in 2,4-D containing buffers resulted in the increase of activity of the chromatin-bound polymerase by 2-3 fold.
This stimulation could result from binding of the soluble polymerase or activation of a chromatin-bound polymerase via a 2,4-D binding protein intermediate. This intermediate must be postulated since 2,4-D added to the assay of chromatin in vitro results in an inhibition of polymerase activity. The increase of the ratio of chromatin-bound to soluble activity with growth supports the theory that the stimulation of RNA synthesis may be a result of increased binding of polymerase to the chromatin. Maintenance of a high ratio of chromatin to soluble activity was dependent on the presence of 2,4-D.

The relative importance of the chromatin-bound activity was suggested by the correlation of its changes with the synthesis and accumulation of RNA (compare Figures 3.45 and 3.55) under conditions which induced cell division; however, changes of soluble activity were less dramatic and only weakly followed those of chromatin-bound activity and total RNA synthesis. In the absence of 2,4-D, the decrease of in vivo RNA synthesis occurred after 10 hr. compared with 15 hr. for the in vitro chromatin-bound polymerase activity, indicating that some mechanism other than the change of activity of chromatin-bound polymerase was responsible for the reduction of in vivo RNA synthesis. Since these measurements were made in the same experiment the relative timings of the decrease of in vivo RNA synthesis and chromatin-bound activity are significant. However, certain reservations must be held about the calculation of in vivo rates of RNA synthesis based on the incorporation of a precursor. The apparent rate of accumulation of radioactive precursor into RNA will depend on a number of factors; the rate of uptake of the label, e.g. for the first 3 hr. of incubation of the explants very little 3H-uridine is taken up.
Secondly, variation of the size of precursor pools would result in differences of the measured $^3\text{H}$-uridine incorporation, e.g. Hotta and Stern (1965) showed the inducibility of thymidine kinase so that both thymidine and thymidine monophosphate pool sizes will vary widely. Thirdly, the rate of degradation of the RNA molecule will alter the estimate of $^3\text{H}$-RNA synthesised and, finally, recycling of the ribonucleotides will reduce the rate of incorporation of $^3\text{H}$-uridine into RNA. The rate of synthesis of RNA was very similar when estimated by $^3\text{H}$-uridine incorporation and by $^{32}\text{P}$-orthophosphate incorporation into RNA and so was accepted as a reasonable estimate.

The stepped increase in total RNA observed during the cell cycle (Fig.3.55) was not related to the synthesis of specific classes of RNA at particular times. The pattern of RNA synthesis was very similar at 10, 13 and 22 hr. through the cycle, timed to coincide with the major increases of total RNA. At these times, 10% of the radioactivity was incorporated into precursor rRNA ($2.5 \times 10^6$ daltons), 50% into rRNAs, 35% into polydisperse RNA, and 5% to transfer RNA. During longer periods of culture in the presence of 2,4-D, the pattern of RNA synthesis remained reasonably constant and similar to that observed during the first cell cycle (except that in these experiments polydisperse represented 50% of the total). However, culture of the explants in the absence of 2,4-D leads to a drastic change of the pattern of RNA synthesis. The synthesis of precursor rRNA is reduced by 30% after 36 hr. incubation, whilst the appearance of label in rRNA is inhibited by more than 4-fold, compared to the incorporation into precursor rRNA and rRNA per cell of the 2,4-D-treated explants.
This is correlated with the reduced accumulation of total RNA in the absence of 2,4-D at this time. This reduction of rRNA synthesis, as expressed both by accumulation of rRNA and the incorporation of label into rRNAs, appears therefore to be mediated by a post-transcriptional control mechanism; synthesis of the precursor molecule is only reduced by 30% (possibly by feed-back inhibition) whilst processing of the molecule is substantially inhibited. Suboptimal temperature has a similar effect in inhibiting rRNA synthesis in HeLa cells (Stevens and Amos, 1971) by inhibiting the processing of the 45S precursor molecule, resulting in an apparent accumulation of the precursor.

A similar response to growth inhibition of pea roots has been described (Jackson and Ingle, 1973). The incorporation into polydisperse RNA is not similarly affected, with the result that expressed as a percentage of total incorporation it increases from 50% to 80%.

The culture of artichoke explants in either the presence or absence of 2,4-D has been shown to lead to an increase of RNA synthesis. A similar increase of RNA synthesis has been observed during the ageing of washed sugar beet slices (Duda and Cherry, 1971). In other systems studied, e.g. soyabean (O'Brien, Jarvis, Cherry and Hanson, 1968), cucumber (Johnson and Purves, 1970), increased RNA synthesis is dependent on the presence of 2,4-D. Addition of 2,4-D to the chromatin assay in vitro inhibits the polymerase reaction (for artichoke, this thesis; Hardin, O'Brien and Cherry, 1970) but if the chromatin is extracted in the presence of 2,4-D, it then shows a much elevated level of RNA polymerase activity (this thesis, and Matthyse, 1969). This has been interpreted as evidence for a factor which, after interacting with 2,4-D, enhances the chromatin-bound polymerase activity. Such a factor has been isolated from soyabean which appears to increase RNA synthesis by activating chromatin-bound polymerase (O'Brien, Hardin and Cherry, 1969). Matthyse (1969), has postulated a similar factor in peas that would function in the same way.
The results obtained by extracting chromatin from artichoke, in this thesis, can be explained by postulating a 2,4-D binding factor which interacts with the chromatin possibly by activating the endogenous polymerase activity or binding soluble activity, so resulting in an increased rate of RNA synthesis. Venis (1971) isolated a 2,4-D binding factor from pea and corn extracts which stimulated the activity of E. coli polymerase on native DNA template. This system is more removed from the in vivo situations previously described where the stimulation was by the increased activity of endogenous polymerase on chromatin template, but may be relevant since Hardin, O'Brien and Cherry (1970) showed that the factor isolated from soybean could be fractionated into a low molecular weight protein that stimulates E. coli polymerase on chromatin and a high molecular weight protein that stimulates the endogenous polymerase.

Based on this evidence for the existence of a factor intermediary in the action of 2,4-D in stimulating endogenous polymerase activity, a model has been postulated by Hardin, O'Brien and Cherry (1970) similar to that of the oestradiol system for rat uterus (Hamilton, 1968). The model suggested by Hardin, O'Brien and Cherry involves the interaction of the hormone with a receptor molecule in the cytoplasm or cell membrane. Interaction of this complex with chromatin results in a stimulation of RNA synthesis; by either, or both, changes of template availability and RNA polymerase activity.

In the artichoke system described in this thesis, stimulation of chromatin-bound RNA polymerase may be achieved by an increase of its amount, the activation of the pre-existing enzyme, or by binding of soluble polymerase to the chromatin.
Binding of the 2,4-D complex to the soluble polymerase could confer the ability to utilise the endogenous native DNA of chromatin. This would be consistent with the observation that many eucaryotic polymerases, as isolated, are unable to transcribe native DNA which is presumably the state of the DNA in vivo, and are only able to utilise denatured DNA. This has been interpreted as loss of a necessary factor for native DNA utilisation during the preparation of such enzymes. Such a factor has been isolated from rat liver (Seifart, 1970) and from calf thymus (Stein and Hausen, 1970b), which specifically stimulates synthesis on double-stranded template but not single-stranded DNA. This factor would be analogous to the bacterial sigma factor and further evidence for its existence has come from experiments in which E. coli sigma factor was injected into oocytes of Xenopus (Tocchini-Valentini and Crippa, 1970) which stimulated endogenous RNA synthesis. The level of such a factor, in its active state, could control the rate of RNA synthesis. Specific factors for polymerase I and II might control their relative activities and the existence of such factors could explain why the relative activities of polymerase I and II measured in vitro remained constant during the periods of rRNA synthesis and no rRNA synthesis in Xenopus oocyte development (Roeder, Reeder and Brown, 1970; Tocchini-Valentini and Crippa, 1970). As well as such positive control factors regulating the activity of the polymerases, negative control factors have also been postulated. Crippa (1970) has extracted a negative control factor from Xenopus oocytes which inhibits rRNA synthesis by specifically inhibiting the activity of polymerase I. This factor is only found in oocytes which are not synthesising rRNA and cannot be isolated from stage 4-6 oocytes which are synthesising rRNA.
There is therefore some experimental evidence to support interaction of a hormone and factor which then controls the rate of synthesis of RNA. However, not all the control of synthesis is restricted to transcription, since analysis of RNA synthesis in the presence and absence of 2,4-D suggests that some control is exercised at the level of post-transcriptional processing of the precursor molecule.
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