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'IN VITRO' STUDIES ON THE EFFECT OF 5-BROMODEOXYURIDINE ON CELL DIFFERENTIATION.

by

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PREFACE

The work described in this dissertation was carried out in the Department of Zoology at the University of the Witwatersrand between April 1971 and November 1973. The work is original, except where stated and no part of the thesis has been submitted for a degree at any other university.

Marlen Dyne

November 1975.

ABSTRACT

The transcription of DNA containing different proportions of BrdUrd was studied, in order to elucidate the mechanism of 5-Bromodeoxyuridine (BrdUrd) inhibition of cell differentiation. BrdUrd, an analogue of thymidine, is known to be incorporated into replicating DNA of dividing cells. Although eucaryotic cells continue dividing in the presence of low concentrations of BrdUrd their differentiation is reversably blocked. It is proposed that differentiation, but not cell division, is dependent on the presence of poly(dT) sequences in the DNA. These regions would become poly(BrdU) regions during replication in the presence of BrdUTP. Such areas may not be efficiently transcribed by RNA polymerase. However, isolated BrdUrd units may not affect RNA polymerase action.

An in vitro system was devised where the synthesis of DNA by E. coli (or M. lysodeikticus) DNA polymerase was successfully coupled to the transcription of the product DNA by E. coli RNA polymerase in the same reaction tube, i.e. DNA_I-primers $\longrightarrow$ DNA' $\longrightarrow$ RNA where DNA_I-primer was either the copolymer (dA-dT) or (dA-BrdU). The DNA product (DNA') of the first reaction was poly(dA-dT) or poly(dA-BrdU) or a related polymer containing varying proportions of thymidine and bromodeoxyuridine. For the synthesis of the latter copolymer the pyrimidine substrate dTTP was decreased in the DNA polymerase reaction mixtures and replaced by a corresponding increase of BrdUTP. The RNA product was always poly(A-U). DNA synthesis and RNA synthesis were determined by measuring the incorporation of ^{14}C and ^{3}H labelled substrate nucleotides into acid precipitable material. The results illustrated that the magnitude of RNA synthesis is inversely proportional to the BrdUrd input into the

DNA synthesis reaction and hence presumably to the RNA synthesis reaction. Experiments were carried out which demonstrated that any possible interference by the components of the DNA polymerase reaction on the RNA polymerase reaction were negligible. The ability of the synthesized DNA to prime RNA polymerase appears therefore to decrease as its BrdUrd concentration increases.

The BrdUTP required for the synthesis of DNA' was synthesized with a yield of 15.5% by direct bromination of dUTP and subsequent purification by chromatography. It was demonstrated using a calf thymus DNA polymerase assay system that BrdUTP can replace dTTP in the synthesis of DNA with an efficiency around 90%.

A brief study was made of the unprimed synthesis of poly(dA-dT) and poly(dA-BrdU) with the intention of coupling the reaction to a system of transcription. However, unprimed synthesis of these polymers appeared to be quantitatively variable and inconsistent.

It was concluded that BrdUrd does inhibit RNA synthesis in this in vitro system while DNA synthesis is unaffected. It is suggested that a possible site of action of BrdUrd inhibition of differentiation is at the level of RNA synthesis at critical sites along the genome and that these sites contain oligothymidine residues or thymidine clusters.

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I would like to thank Dr. John Ballnsky for allowing me to carry out the experimental work in the Department of Chemistry including Biochemistry.

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1. INTRODUCTION

1.1 Polynucleotide synthesis

The DNA of living cells contains the genetic information which defines the characteristics of the organism and its progeny. These characteristics are basically related to the protein type synthesized indirectly from a DNA template via its messenger RNA.

Thus, DNA $\xrightarrow{\text{Transcription}}$ mRNA $\xrightarrow{\text{Translation}}$ protein (Davidson 1972).

Replication

This process encompasses synthesis of the DNA, transcription of this DNA to a mRNA, which is then translated into protein.

In the process of replication the DNA in the cell is replicated resulting in the production of a double copy of the genetic information each of which is subsequently passed on to a progeny cell at the conclusion of mitosis. The process of transcription involves the formation of mRNA, and other RNA, on a DNA template. This mRNA in turn becomes the template on which proteins are synthesized. It is widely held that the synthesis of a specific group of proteins is characteristic of a particular state of cell differentiation.

The analogue of thymidine, 5-bromodeoxyuridine, is incorporated into the DNA of dividing cells. In the presence of bromodeoxyuridine cell differentiation has been shown to be blocked in a number of eucaryotic systems, with subsequent inhibition of synthesis of specific proteins of the differentiating system (Wilt and Anderson, 1972).

differentiation, but not cell division, is in some way
...t on the presence of small clusters of thymidine than these sequences of thymidine would become dense regions of bromodeoxyuridine after analogue substitution during DNA synthesis. Such bromodeoxyuridine rich areas of the DNA may create a "steric hindrance" for the transcribing RNA polymerase and thus derange transcription of RNA. This thesis set out to examine the plausibility of the latter hypothesis.

An in vitro system of polynucleotide synthesis is used in this work to test this hypothesis. The system uses DNA polymerase I to synthesize DNA, followed by RNA transcription of the DNA product into RNA by RNA polymerase. This DNA product of the initial reaction will contain different proportions of bromodeoxyuridine depending on the relative concentrations of precursor nucleoside 5'-triphosphates (Section 4.3). In this way the transcriptional efficiency of DNA, containing increasing amounts of Bromodeoxyuridine, can be measured.

1.1.1 DNA and RNA - their basic structure

DNA is a high molecular weight polymer of deoxyribonucleoside-5'-monophosphate units. These units consist of a deoxyribose sugar, phosphoric acid group and either a purine or pyrimidine base. The bases which most commonly occur in DNA are the pyrimidine bases cytosine and thymine and the purine bases adenine and guanine. The DNA molecule usually consists of two polynucleotide strands of complementary structure (except in some viruses, Davidson, 1972). The two strands, which are of opposite polarity, are held together by hydrogen bonds between pairs of complementary purine and pyrimidine bases. Adenine is always bonded to thymine and guanine to cytosine (Watson & Crick 1953 a and b). A diagrammatic representation of the structure of DNA is shown in Fig. 1 and the structural and H-bonding relationships of the bases is shown in Fig. 2.

In RNA, as opposed to DNA, the sugar moeity is of the ribose form and thymine is replaced by the closely related pyrimidine uracil, which similarly pairs with adenine (Davidson, 1969). However, unlike DNA, it exists as single polynucleotide strands. The structures of the ribose sugar and uracil are shown in Fig. 3.

Due to its base sequence and complementarity DNA may serve as a template, i.e. primer during the processes of replication and transcription. Thus DNA primer, as a single or double stranded DNA molecule or polydeoxynucleotide, is required in the synthesis of new strands of DNA or RNA (except in unprimed synthesis, Section 1.5). The nucleotides of the new DNA or RNA are aligned by complementary base-pairing with those in the pre-existing strands. Therefore, the nature and sequence of the nucleotides in the new strand are determined by those in the primer DNA (Smellie, 1965). Bessman (1963) and Hurwitz et al. (1961) showed that the base ratios in a variety of DNA and RNA products respectively were very similar to the base ratios of their respective primers.

1.1.2 *DNA polymerase and DNA replication*

DNA polymerase, an enzyme responsible for DNA synthesis has been isolated and purified from a variety of mammalian systems. These include ascites tumour cells, normal and regenerating rat liver, several hepatomas, calf thymus, mouse leukaemic cells, sea urchin embryos and KB human cells (Davidson 1972, and Goulian 1971). However, even the most highly purified samples of soluble mammalian DNA polymerases (Greene and Korn 1970) have not been as extensively purified as those of microorganisms.

It has been shown that the primary *in vivo* role of the most highly purified bacterial DNA polymerase, otherwise known as the Kornberg enzyme or DNA polymerase I, is not replicative synthesis but one of repair synthesis (Becker & Hurwitz, 1971). Kornberg and Gefter (1971) and Gefter et al. (1971) have demonstrated the existence of membrane

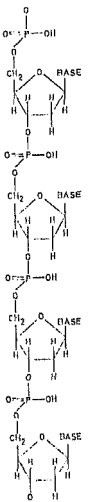
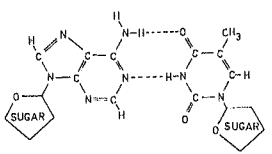


Figure 1. Part of the polynucleotide chain in DNA (Davidson 1969).

Hydrogen bonding of adenine to thymine



Hydrogen bonding of guanine to cytosine

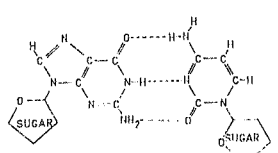
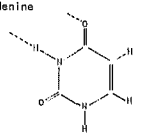
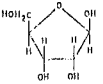


Figure 2. The pairing of adenine and thymine, and of guanine and cytosine. (Bassman 1963)

Possibility of
hydrogen bonding
to adenine



Uracil



Ribose

Figure 3. The structures of Uracil and ribose. (Watson 1965)

associated replicases, namely DNA polymerase II and III, which are more likely to be responsible for in vivo DNA synthesis. Soluble DNA polymerase I is readily available for in vitro studies of DNA replication and has been used by numerous workers.

For most of this study the easily obtainable and purified bacterial enzymes have been used. DNA polymerase has been isolated from micro-organisms since 1956 when the enzyme from Escherichia coli was first extracted by Kornberg et al. (1956). An in vitro system was devised by these workers to study the synthesis of DNA (Lehman et al. 1958). It has been shown that the incubation requirements for DNA synthesis are basically the same regardless of the source of the enzyme. These requirements which are contained in a buffered solution are outlined in the formula shown in Figure 4(a).

The DNA polymerase I used in these studies has been purified several thousand fold from Micrococcus lysodeikticus (Zimmerman 1966) and E. coli (Richardson et al. 1964). These preparations are homogeneous by criteria of sedimentation chromatography and starch gel electrophoresis. However, even the most highly purified preparations still contain exonuclease II, an activity which appears to be an intrinsic part of the molecule. As this nuclease exhibits its optimal activity at pH 9.0 and hydrolyses DNA at a slow rate in the neutral range in which polymerase activity is optimal, its activity is negligible in in vitro studies (Richardson et al. 1964). Exonuclease appears to be an essential property of all DNA synthesizing enzymes since purified Bacillus subtilis DNA polymerase shows no significant exonuclease activity in vitro (Okazaki and Kornberg 1964).

1.1.3 RNA polymerase and transcription

DNA-dependent RNA polymerase, the enzyme of transcription, has been isolated from several sources since 1960. These are rat liver nuclei (Weis and Gladstone, 1959), mouse ascites tumours (Smellie, 1965), E. coli (Hurwitz et al. 1961), and M. lysodeikticus (Nakamoto et al.1964). As in the case of DNA polymerase, the most highly purified preparations have come from bacterial sources.

The characteristics of mammalian RNA polymerase extracted and purified from rat liver nuclei appears to be essentially the same as that of the enzyme isolated from bacterial species in most of its basic properties, in in vitro synthesis. It has an absolute requirement for a DNA-primer which determines the overall base composition and sequence of the RNA, the four ribonucleoside 5'-triphosphates ATP, GTP, CTP and UTP, and a divalent cation, preferably Mn^{2+} which can only in part be replaced by Mg^{2+}, and an optimum activity at pH 7.9. Heat denatured DNA is transcribed more efficiently, suggesting that the enzyme lacks a factor responsible for efficient utilization of helical DNA (Seifart 1970). However, little is known about the subunit structure of mammalian enzyme whereas that of E. coli has been well characterised (Burgess 1971).

In this investigation E. coli RNA polymerase was used for all transcription studies. The E. coli RNA polymerase molecules as described by Burgess et al. (1969) and Burgess (1971), is a multi-subunit enzyme made up of the following polypeptide chains, one β' subunit, one β subunit, two α subunits and a sigma (σ) subunit. This holoenzyme can be separated into two functional parts; a core enzyme ($\alpha_2 \beta \beta'$), which can synthesize RNA but lacks the ability to specifically initiate synthesis, and a sigma factor (σ) which acts catalytically to allow the efficient initiation of RNA synthesis at specific sites. Poly(dA-dT) can be read in absence

of σ factor (Berg et al. 1971). In addition to the sigma factor there are other transcription factors which have a pronounced effect on the transcription reaction, but as they do not bind tightly to the core enzyme they are lost during purification. The absence of necessary factors creates difficulties in the evaluation of the mechanism of in vivo transcription as compared to the in vitro reaction.

These difficulties are principally involved in the correct in vivo *initiation and termination events. For example, using phage* λ DNA as primer in such an in vitro system together with RNA polymerase holoenzyme, results in the synthesis of heterogeneous RNA quite unlike phage specific RNA synthesized in phage infected cells. However, the addition of rho factor, which is responsible for correct chain termination, results in the synthesis only of the two phage specific RNA species found in vivo (Davidson 1972). Also in in vitro, E. coli RNA polymerase transcribes double helical DNA primer asymmetrically which appears to be characteristic of transcription in vivo (Burgess et al. 1969).

The general reaction for RNA synthesis in vitro may be represented by the formula shown in Figure 4(b). It can be seen that the genera' reaction is similar to that of DNA synthesis as outlined in Figure 4(a).

Figure 4 *In vitro* reactions of (a) DNA replication and (b) transcription

(a) DNA (primer) + n dATP, n dTTP, n dGTP, n P $\xrightarrow[\text{DNA polymerase}]{Mg^{2+}}$ DNA + 4(n)PPi

(b) DNA (primer) + n ATP, n UTP, n GTP, n CTP $\xrightarrow[\text{RNA polymerase}]{Mn^{2+}}$ RNA + 4(n)PPi

Incubation is generally at 37°C

1.2 Bromodeoxyuridine

1.2.1 The structure of bromodeoxyuridine and its incorporation into DNA

BrdUrd is an analogue of thymidine which when incorporated into the DNA permits the continuation of DNA synthesis. It can enter the DNA precursor pool of organisms and cells dividing in culture, after conversion to the nucleotide form by thymidine kinase and other kinases along the thymidylic acid pathway (Szybalski, 1962). The BrdUrd molecule incorporated into the DNA may be represented by the model shown in Fig. 5(a).

In BrdUrd the methyl group of the 5 carbon position of the pyrimidine ring is replaced by bromine. Hanawalt (1967), suggested that the ease of incorporation of BrdUrd into the DNA is probably reflected in the similarity between the van de Waals radii of the two groups as shown in Fig. 5(b). However, the greater electronegativity of the Br atom alters the electron distribution of the pyrimidine ring. This may give rise to the conversion of the pyrimidine ring from its normal keto state to the enol state which in turn results in a pairing with guanine instead of adenine (Hanawalt, 1967). This has been shown in bacteria to result in an increase in the mutation rate (Freese 1963). However, Trautner et al. (1962) demonstrated that the incorporation of guanine instead of adenine only occurred at a frequency of one per 2,000 to 25,000 nucleotides polymerized. Incorporation of the halogen into the DNA of phages, bacteria and human cells grown in vitro, results in a sensitization to radiation and light (Szybalski, 1962).

Although halogenation of DNA allows continued cell multiplication the physicochemical properties of the molecule are altered. *Substitution of bromine for the methyl group of thymine affects* the

stability of the copolymer (dA-BrdU) both in relation to pH and temperature. It also results in an increase in the density of the DNA. Inman and Baldwin (1962) showed that halogenation of the molecule results in an increased melting temperature (Tm). The difference in Tm observed between normal and BrdUrd-labelled DNA was accentuated in the case of the (dA-BrdU) copolymer as compared to the (dA-dT) copolymer. As described by Inman and Baldwin (1962), this increase in Tm may possibly be explained by a net increase in hydrogen bond strength, resulting from the inductive effect of bromine in withdrawing electrons from the pyrimidine ring. The hydrogen bond involving the 6 Keto group of BrdUrd should be weakened while the one involving the N-1 should be strengthened (Inman and Baldwin 1962). Thus a requirement for an increase in energy to unwind the DNA helix could possibly result and so interfere with the catalytic function of the polymerase enzymes.

1.2.2 The effect of bromodeoxyuridine on He La cells.

It has been suggested that the inhibitory action of BrdUrd on differentiation is a result of its incorporation into the DNA (see Section 1). Studies of BrdUrd action on the inhibition of He La cell growth affords a possible clue to the site of BrdUrd action at this level. He La cells are a tissue culture line of human carcinoma of the cervix. They are one of the first eucaryotic systems used for the study of the effects of thymidine replacement by BrdUrd (Hakala 1959, Eidinoff et al. 1959, Cheong et al. 1960, and Littlefield and Gould 1960).

When BrdUrd is incorporated into the DNA of He La cells in place of thymidine, there is little effect on the first replication cycle of the cells. The second cycle is inhibited to a degree dependent on the concentration of BrdUrd employed in the culture medium. At least one cell division must take place before cell growth is affected. It also appears that BrdUrd is toxic to cells but this is dependent on the

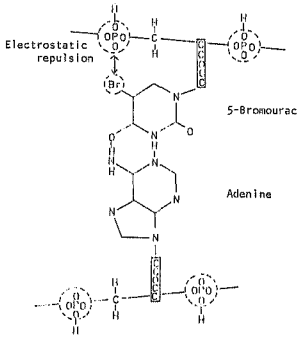
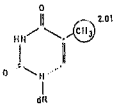
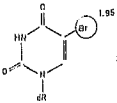


Figure 5(a) Steric relationship between 5-bromodeoxyuridine and the phosphate groups in the DNA molecule, and the adenine-5-bromouracil deoxynucleotide pair (from Szybalski, 1962).



deoxythymidine



5-bromodeoxyuridine

Figure 5(b). Chemical structure of deoxythymidine and 5-bromodeoxyuridine.

Circles represent the van der Waals radii of the atoms or groups occupying position 5.

concentration of BrdUrd used. Simon (1963) found that in the presence of 20 µg/ml BrdUrd the first division of He La cells was not delayed. Subsequent divisions were however, delayed. Only a small proportion of the cells capable of growing in the presence of BrdUrd (but containing BrdUrd in their DNA) arose. These cells grow more slowly than normal ones. At concentrations up to 80 µg/ml most of the cells can divide once and many undergo a second division. At concentrations below 20 µg/ml an increasing proportion can adapt to the analogue and divide indefinitely. Kajiwara and Mueller (1964) used 2.5, 5.0 and 10 µg/ml of BrdUrd. They found that the ratios of the generation time for the BrdUrd-treated cultures to the control cultures between the first and third days were 1.44, 1.62 and 2.40, respectively.

Tolliver and Simon (1967) demonstrated that the BrdUrd effect was reversible when BrdUrd was removed from the system. This suggests that it is not mutagenic in nature. They found that the BrdUrd-tolerant He La cells grew indefinitely but at a slower rate due to an increase in the S period of their cell cycle. When transferred to a BrdUrd free medium the cells returned to a normal growth rate. Berkowitz et al. (1968) illustrated that although cells treated with BrdUrd + FdUrd (which inhibits endogenous thymidine synthesis) can only divide once, their DNA does replicate at least twice to possibly four cycles of replication. Simon (1963) found that BrdUrd + FdUrd treated cells incorporated only slightly more BrdUrd than cells grown in BrdUrd alone. According to Tolliver et al. (1968) this slight difference in incorporation appeared to be important since it was paralleled by the inability of the cells to divide. These results suggested that there was a small fraction of sites on the DNA molecule with an absolute requirement for thymine. Under conditions where a small amount of thymidine competes with a great deal of BrdUrd for incorporation into

the DNA, Simon (1963) found the thymidine unusually effective in replacing the BrdUrd in a He La cell system.

Tolliver et al. (1968) hydrolysed DNA from BrdUrd and FdUrd treated He La cells to pyrimidine oligonucleotides, and separated the oligonucleotides chromatographically. An unexpected radioactive peak appeared between the di- and trinucleotide peaks in the pyrimidine oligonucleotide profile of DNA from cells grown for more than one generation in BrdUrd alone, but not from control He La cells. Although they do not resolve the biological significance of this peak, these authors suggest that the peak material might be associated with the control of either DNA synthesis or cell division, or with both processes. Taylor (1966) suggested that the initiation region of replicons is composed of DNA rich in dA-dT pairs in which most of the dA is in one strand and the dT is in the complementary strand. This interpretation was based on the results of studies of bromodeoxyuridine substitution in Vicia faba bean roots grown in solutions containing the analogue (Haut and Taylor 1967).

Thus there is preliminary evidence that the existence of thymidine clusters may play an important role in explaining how BrdUrd inhibits division. Furthermore if division is coupled to differentiation, then BrdUrd would in turn block differentiation. It is partly from this observation that the proposal for this investigation was formulated.

1.2.3 The effect of bromodeoxyuridine on differentiation

In their review, Wilt and Anderson (1972), cite numerous experimental observations which show that when BrdUrd is incorporated into the DNA of dividing eucaryotic cells, cell multiplication continues but the differentiation of these cells is blocked. In all these studies this inhibition was found to be reversible. When the cells were remov-

from the BrdUrd and allowed to grow recovery of differentiation took place. This is evidence that the effect of BrdUrd is not mutagenic in nature.

Although for phages and bacteria BrdUrd is a potent mutagen, the mutagenic effect is a rare occurrence. For example Freese (1959) produced 1% T_4 phage mutants in the presence of 70µg/ml of BrdUrd. As the inhibitory effect of BrdUrd on the differentiation of developing cells is virtually 100% and reversible Miura and Wilt(1971) and Mayne et al. (1971) conclude that the effect of BrdUrd is not a result of mutation. This reversibility of the BrdUrd block has been demonstrated in numerous systems such as myoblasts (Stockdale et al 1964) chrondrocytes (Abbott and Holtzer 1968a), TAT synthesis in rat hepatoma cells (Stellwagen and Tomkins 1971a) and, exocrine pancreas cells (Walther et al. 1974).

Stahl et al. (1961) showed that when BrdUrd treated bacteria were exposed to near visible light, there was an increase in the ability of BrdUrd to cause mutagenesis. This does not appear to be the case in rat hepatoma cells where Stellwagen and Tomkins (1971a) have shown that maintenance of these cells in the dark had no effect on the inhibition of cell specific proteins by BrdUrd. It has therefore been concluded that the inhibitory effect of BrdUrd is not a result of mutagenesis.

The effect of BrdUrd on cell differentiation does not appear to be related to its effect on the cells. Toxicity due to BrdUrd appears to be apparent only at high concentrations (above 20 µg/ml) of BrdUrd in the culture medium. The toxicity of BrdUrd in cultured cell lines, such as He La cells, was outlined in the previous section. With myogenic cells (Bishoff and Holtzer 1970) and chondrocytes (Lasher and Cahn 1969) there is definite growth inhibition at high concentrations

of BrdUrd. However at low concentrations (3µg/ml), where cell production is not interfered with, cell differentiation is inhibited. Thus it must be emphasized that the toxic effects observed at high concentrations of BrdUrd are not responsible for the inhibition of differentiation of these cells.

Examples are given below to illustrate in more detail the various aspects of the inhibitory action of BrdUrd on differentiation.

1. Myogenesis

As an introduction to the phenomenon of BrdUrd inhibition of differentiation the effect of BrdUrd on Myogenesis is presented in detail[1]. This was one of the first systems in which the effect of BrdUrd was well defined.

Stockdale et al. (1964) and Bischoff and Holtzer (1970) cultured presumptive myoblasts from chick muscle. These replicating cells stop dividing by the fourth or fifth day and give rise to postmitotic mononucleated myoblasts. These cells synthesize myosin and actin and fuse with other myogenic cells to form multinucleated myotubes. If myogenic cells are exposed to BrdUrd during their division stages they do not differentiate into muscle cells. Instead of fusing the cells move away from each other and it has not been possible to demonstrate the formation of contractile protein. Stockdale et al. (1964) using caesium chloride, density gradient centrifugation, demonstrated that BrdUrd is incorporated into the DNA. Furthermore, it was shown that the inhibition of differentiation was not due to an inhibition of DNA synthesis (Stockdale et al. 1964). If cells are exposed to BrdUrd after division ceases the cells are unaffected and differentiation proceeds. Thus BrdUrd can only inhibit proliferating cells from differentiating, and virtually all these cells incorporate BrdUrd into their DNA (Coleman et al. 1969). However the bromouracil need

not be incorporated into both strands of the DNA helix to cause inhibition of differentiation, as daughter cells with bromouracil in only one strand do not differentiate (Bischoff and Holtzer 1970).

More specifically, BrdUrd appears to repress protein synthesis which is specific to and characteristic of the particular type of cytodifferentiation. For example, Coleman and Coleman (1968) examined the effect of BrdUrd on myosin synthesis. In the normal untreated cells after the period of cell fusion myosin increases preferentially with respect to the increase of total protein. In BrdUrd inhibited cultures no myosin was detected.

It has been mentioned (page 17) that the inhibitory effect of BrdUrd on many differentiating systems is not permanent and can be reversed. It can also be specifically and competitively inhibited by thymidine. Cells cultured in BrdUrd and in an excess of thymidine are indistinguishable from untreated cells (Stockdale et al. 1964). 5-Fluorodeoxyuridine (FdUrd) inhibits the enzyme thymidylate synthetase and therefore inhibits DNA synthesis when endogenous thymidine is used up. This inhibition by FdUrd may be prevented by adding BrdUrd or thymidine exogenously. This is additional evidence that BrdUrd can replace thymidine in its incorporation during DNA synthesis (Bischoff and Holtzer, 1970).

In addition, Coleman et al. (1969) and Bischoff and Holtzer (1970) showed that other halogenated derivatives such as 5-chlorodeoxyuridine and 5-iododeoxyuridine could mimic the effect of BrdUrd in blocking the differention of myoblasts. These are also analogues of TdR, which are known to enter DNA specifically as thymine replacements and as such mimic the effect of BrdUrd. This lends further evidence for DNA as the possible site of BrdUrd action.

To summarise, Myogenesis is a system in which DNA synthesis and division stops before differentiation begins. (Bischoff & Holtzer 1970). From the studies of BrdUrd inhibition it appears that the cells are partially determined prior to the terminal division which precedes histological expression of differentiation.

II Erythropoiesis

The effect of BrdUrd inhibition has been studied in an embryonic erythropoietic system. Here haemoglobin (Hb) is formed while cell division continues. This is in contrast to myogenesis where cell division stops prior to differentiation. Miura and Wilt (1971) showed that the presumptive blood island tissue of the blastoderm, cultured at the primitive streak stage, failed to synthesize haemoglobin in the presence of BrdUrd. At later stages of development, however, BrdUrd treatment had no effect on haemoglobin production. It is of interest that these workers also demonstrated with the use of autoradiographic techniques that at both the BrdUrd sensitive and insensitive stages all the erythroblast precursor cells incorporate BrdUrd into their DNA.

Fabian and Wilt (1973) have demonstrated in erythropoietic cells that, during recovery from BrdUrd treatment, the previously incorporated BrdUrd in the DNA is diluted out by further replication during the recovery phase when the block on differentiation is reversed.

Weintraub et al. (1972) employed an in vivo system in which BrdUrd is introduced into the chick eggs. They showed that there is a transition in the erythropoietic lineage before which the ability to initiate synthesis of Hb is prevented by BrdUrd, and after which the continued synthesis of Hb is unaffected. This demonstrates that BrdUrd inhibition of Hb production occurs in vivo as well as in vitro.

The evidence of BrdUrd action on myogenesis and erythropoiesis is consistent with the possibility that BrdUrd interferes with an event in transcription resulting in a derangement of RNA synthesis. (See bottom pg. 24).

iii The effect of BrdUrd on cleaving eggs

In the development of Echinoderm eggs Mazia and Gontcharoff (1964) found a different type of BrdUrd effect. Incorporation of BrdUrd into the replicating sand dollar egg prevented subsequent blastula formation. Closer examination showed that the mitotic behaviour of the treated eggs was highly abnormal. Nuclear bridges formed between daughter blastoderms. Not only were abnormal blastula and lethal gastrula formed, but BrdUrd also affected the production of echinochrome pigments in the sea urchin embryo. However after the 16 cell stage of development BrdUrd has no effect on echinochrome production (Gontcharoff and Mazia 1967). This insensitivity to BrdUrd substitution after a later cell stage may be compared with the effect of BrdUrd substitution into the DNA during erythropoiesis. In both examples even though BrdUrd is incorporated into the DNA the cells become insensitive to its ability to inhibit specific protein production.

iv The effect of BrdUrd on an established differentiated state.

The studies of myogenesis and erythropoiesis described above suggest that BrdUrd inhibits the initiation of programmes resulting in the differentiation of these cells. The examples given below will illustrate that BrdUrd can reversibly suppress the expression of a differentiated state which has already been attained (Wilt and Anderson 1972).

Postmitotic chondrocytes are surrounded by a metachromatic matrix into which they deposit newly synthesized chondroitin sulphate. When postmitotic cells are liberated from their matrix and cultured as single cells they are induced to enter the mitotic cycle. The daughter cells

form multicellular colonies which deposit the matrix and synthesize chondroitin-4-sulphate. However, chondrocytes liberated from vertebral cartilage of chick embryo do not deposit matrix or synthesize chondroitin-4-sulphate when cultured in BrdUrd. (Lasher and Cahn 1969, Abbot and Holtzer 1968a and b). It should be noted that these cells have recommenced division and that BrdUrd has been incorporated into the new DNA (Anderson et al. 1970). It is likely that BrdUrd incorporation has prevented the formation of the cell specific product, chondroitin sulphate. The possibility that the incorporation of BrdUrd into DNA is only a concomitant event and not the causal event cannot be excluded. This inhibition of cartilage development by BrdUrd has also been demonstrated by Abbot et al. (1972) using organ cultures of chick embryo somites, and by Mayne et al. (1973) and Coleman et al. (1970) using mature dividing chondrocytes.

There are other examples of differentiated cells which can be prevented from synthesizing their cell specific products by BrdUrd treatment. These include fibroblast cells from chick amnion which synthesize hyaluronic acid and chondroitin sulphate (Bischoff and Holtzer 1968, Bischoff 1971 and Mayne et al. 1971), hybrid mammalian cells in culture which synthesize hyaluronic acid (Koyama and Ono 1971, and pigment producing mouse melanoma cells (Silagi and Bruce 1970 and Wrathal et al. 1973). The common feature of these examples is that for effective BrdUrd inhibition cell division must also be taking place. This observation correlates with BrdUrd inhibition of erythropoiesis and myogenesis where cell division proceeds the establishment of the differentiated state. This suggests that BrdUrd may have a similar mode of action in all these systems.

The example of pigment producing mouse melanoma cells quoted above is of particular interest because of the very low levels (1-3µg/ml) of BrdUrd required to suppress the formation of pigment. (Silagi and Bruce 1970, and Wrathal et al. 1973). At these low concentrations the rate of growth is not affected but the cells grow without pigment and

grow in flattened monolayers. This is in contrast to the normal dark clones of melanoma cells which form in normal medium. With the suppression of pigmentation these workers also found an independent suppression of tumourigenicity. These two effects are not dependent on each other as suppression of malignancy in amelanotic melanoma cells is also apparent.

v. The effect of BrdUrd on specific proteins characteristic of the differentiated state.

The inhibitory effect of BrdUrd on cell specific proteins has been partly discussed in relation to myosin synthesis and haemoglobin synthesis in section 1.2.3, points (i) and (ii) respectively. Other examples relating to the inhibitory effect of BrdUrd on cell specific protein synthesis are dealt with below.

Holthausen et al. (1969) studied the effect of BrdUrd on the level of enzymes characteristic of the extracellular polysaccharide matrix of cultured vertebral chondrocytes. There are three enzyme systems essential for the production of this matrix which is a protein-polysaccharide complex. These enzymes are UDP-glucose dehydrogenase, UDP-N-acetyl-D-glucosamine-4-epimerase and sulphokinase. The inhibition of differentiation by BrdUrd coincides with a progressive decline in the activity of these enzymes. In contrast acid phosphatase, cytochrome oxidase and hexokinase levels were the same for both BrdUrd treated cells and untreated cells. This suggests that the specific proteins of a differentiated condition are inhibited by BrdUrd in contrast to the proteins required for general cell function. Another example to illustrate this is presented below.

Stellwagen and Tomkins (1971a and b) treated an established line of rat hepatoma cultures with BrdU. Untreated rat hepatoma cells synthesize the liver specific steroid inducible enzyme, tyrosine amino-

transferase (TAT). These cells when grown in BrdUrd synthesize TAT at a reduced level of only 20% of the untreated cells. Stellwagen and Tomkins (1972a) correlated the decrease in the activity of TAT with the amount of BrdUrd incorporated into the DNA, which was dependent on the concentration of BrdUrd added to the medium.

Enzymes such as malate dehydrogenase, acid phosphatase and alanine amino transferase, which are not liver specific proteins, were unaffected. These workers confirmed that the inhibitory effect of BrdUrd is accompanied by its incorporation into the DNA. They also confirmed that the inhibitory effect of BrdUrd is reversible, and can be prevented by the addition of thymidine to the culture medium.

The interpretation of the above studies is complicated by the incomplete knowledge of the turnover rates of these enzymes and their RNA messengers. While Stellwagen and Tomkins (1971b) present some information about enzyme half-lives, more detailed study of these aspects is required for a full understanding of this aspect of BrdUrd action.

Wrathall et al. (1973) studied the effects of BrdUrd suppression of pigmentation at the level of tyrosinase activity and melanosome formation. The suppression of pigmentation can be related to a loss of tyrosinase activity and melanosome formation.

In addition, it should be noted that abnormal proteins have not been found in in vivo studies (Wilt and Anderson 1972). Immunological tests on BrdUrd treated hepatoma cultures do not produce altered proteins (Stellwagen and Tomkins 1971a and b), nor do pancreas treated cells tested by zone electrophoresis (Walther et al. 1974). This suggests that the effect of BrdUrd is not due to the synthesis of improper RNA molecules, but a block in the effective process of transcription, perhaps at the DNA location which controls whether or not a second DNA location synthesizes RNA required for the protein synthesis of a differentiated state (Britten and Davidson 1969, also Section 1.3 page 27, paragraph 3 this thesis).

vi. Is RNA synthesis affected during the inhibition of differentiation by BrdUrd?

In addition to the studies on the effect of BrdUrd on specialized proteins as described above, many investigators have studied the levels of DNA and RNA in these BrdUrd affected cells.

Stellwagen and Tomkins (1971a) found only a 10% decrease in the rates of synthesis of ribosomal, soluble and heterogeneous nuclear RNA's in rat hepatoma cells affected by BrdUrd. They failed to detect a particular BrdUrd sensitive class of RNA's from cells grown in BrdUrd by fractionating radioactively labelled RNA on polyacrylamide gels. This may have been due to the fact that the mRNA was a minor portion of some larger fraction.

Hayne et al. (1971) measured the total DNA content and the RNA:DNA and protein:DNA ratios of fibroblast cells grown in BrdUrd (5µg/ml). No significant depression of total DNA content was observed. The RNA:DNA ratio was slightly reduced, and similar but more marked depressions were observed in the protein:DNA ratios, compared with untreated cells. As their study encompassed the overall synthesis of protein and RNA in these cells it is unknown whether the reduction was due to a specific fraction. However studies are presented below which are related to specific classes of RNA.

The synthesis of mammary gland secretions affords a system in which BrdUrd inhibition of specific proteins can be studied at the level of transcription. Proliferating mammary epithelial cells can be induced to synthesize casein and α-lactalbumin. This synthesis of specific proteins is preceded by an increase in the rate of synthesis of specific classes of RNA (45S and 32S preribosomal RNA, heterogeneous DNA like RNA of the nucleus and transfer RNA). Furthermore, there is a concomitant increase in polysomes with the initiation of casein synthesis. This has been interpreted as the translation of new casein mRNA on cytoplasmic polysomes.

Although the synthesis of casein and α-lactalbumin was inhibited by BrdUrd incorporation into the DNA, the specific classes of RNA were not shown to be affected in their experimental system. However, there was a marked reduction in the polysomes and there is some decrease in the total number of ribosomes (Turkington et al. 1971).

Kotzin and Baker (1972) found that the addition of BrdUrd to a culture of developing gastrula stage sea urchin embryos caused a reduction in the rate of incorporation of (^{3}H)-uridine into new RNA. They also found that an 8-15S nuclear DNA, which was the precursor of 15-60S DNA, was prevented in the BrdUrd inhibited gastrulae from becoming part of the higher molecular weight DNA. They interpreted this as meaning that the BrdUrd in DNA prevented the processing of the 15-60S DNA from the 8-15S DNA precursors. Furthermore, DNA-RNA hybridization assays indicated that gastrula stage 20-60S nuclear DNA had greater sequence homology for (^{3}H) RNA of the same stage. This hybridization was not found in BrdUrd exposed embryos. This example supports the view that BrdUrd incorporation into DNA, does effect the synthesis of specific RNA species in the developing sea urchin embryo.

1.3 Evidence for thymidine rich areas in DNA

It was proposed earlier in this work that the inhibitory effect of BrdUrd is due to the replacement of thymidine by BrdUrd in thymidine rich areas of the DNA. Do such thymidine clusters exist?

Using the formic acid-diphenylamine reaction of Burton and Petersen (1960), Tolliver et al. (1968) demonstrated the presence of pyrimidine clusters in He La cell DNA.

The presence of repetitive DNA was found in species of higher organisms (Britten and Kohne 1968). They examined various species of

plants, protozoa, invertebrates and vertebrates. The actual sequence of these nucleotides is unknown, but it is possible that there are many homopolymer and copolymer types within the DNA. An example of the copolymer type of repeated nucleotide sequence is found in the satellite from crab DNA (Sueoka 1961), and from Yeast and Drosophila DNA (Guille and Quetier 1973). The crab satellite DNA consists of an alternating sequence of adenine and thymine units. In the same context Szybalski et al. (1966) found pyrimidine rich clusters, such as cytosine and guanine paired sequences, in the DNA from a variety of organisms.

In the DNA to RNA transcription process the dC rich sequence rather than the dG rich sequence in the DNA is the transcribed strand, and similarly with dT and dA rich sequences, dT is preferentially transcribed. This asymmetry of copying the pyrimidine rich strand has been demonstrated by Morgan (1970) using synthetic DNA polymers and E. coli DNA dependent RNA polymerase.

Assuming that thymidine rich sequences do exist, could these areas of repeated nucleotide sequence be sites of regulation for gene expression? Specific nucleotide sequences have been proposed to be sites of control and initiation in the regulation of gene expression (Britten and Davidson 1969). They and other workers have established that the genome of higher cell types contain large fractions of repetitive nucleotide sequences. Consequently they proposed a theoretical model of gene regulation in which multiple controlling elements are used for regulating the action of genes. Central to their theory is that these controlling elements are transcribed regions of the genome. They proposed that these RNA products act at the level of controlling mRNA synthesis for specific proteins.

At the initial stages of this investigation the findings of Darnell et al. (1971) appeared to be relevant to the hypothesis proposed in this

work. They demonstrated the existence of poly A sequences in mRNA. This observation initially inferred the transcription of poly dT sequences in DNA. However subsequent work (Wilt 1973) demonstrated that the poly A residues were added to the 3' end of the mRNA post transcriptionally. This precludes the participation of poly dT residues in the DNA in both initiation and synthesis of these poly A regions in mRNA. Additionally Ingram et al. (1974) demonstrated that *inhibition of erythropoiesis by BrdUrd did* not block polyadenylation of mRNA.

It can therefore be concluded that the existence of thymidine rich areas in the DNA is probable. The possibility also arises that these areas may be involved in gene regulation.

1.4 Binding of the Lac repressor of E. coli to operator analogues.

Lin and Riggs (1971) have shown that lac repressor protein in E. coli binds to poly(dA-dT). They have also shown that repressor protein binds very much more strongly to poly(dA-BrdU) than to poly(dA-dT). The dissociation constant of the repressor protein - poly(dA-dT) complex is ten times greater than that of repressor protein - poly(dA-BrdU) complex (*Lin and Riggs 1972*). *Although this is a bacterial system* the evidence presented in their work demonstrates that poly(dA-dT) residues play an important role in the control of gene expression. Possibly those sequences may also control gene expression in the differentiated state. If this is the case BrdUrd substitution of poly(dA-dT) rich areas might result in the inhibition of initiation of specific protein synthesis due to non-release of the repressor.

1.5 The in vitro synthesis of synthetic poly(dA-dT) and its analogue poly(dA-BrdU)

As the practical aim of this investigation is to study the transcription of the BrdUrd-containing DNA polymers, a discussion of the in vitro synthesis of these polymers by DNA polymerase is of importance. The synthetic polymer of (dA-dT) was used as a first approach to examine a double stranded DNA of high thymidine density. As well as being a polymer which is known to be transcribed (Chamberlin et al. 1963); TdR can be completely substituted for by BrdUrd to form poly(dA-BrdU) during replication (Inman and Baldwin 1962). Evidence for the replication of these polymers and the transcription of poly(dA-dT) to poly(A-U) in a DNA polymerase and RNA polymerase system respectively, is described below.

Schachman et al. (1960) have shown that in the absence of a DNA primer and in the presence of dATP and dTTP in an in vitro E. coli DNA polymerase system a polymer of dAMP and dTMP is formed. This synthesis takes place after a long lag period. The polymer was found to contain equal proportions of dAMP and dTMP and these were in strictly alternating sequence. Furthermore, the polymer was a double helical, hydrogen bonded macromolecule (MW2-8 x 10^6), thus of the same configuration as natural DNA. In the synthesis of poly(dA-dT) the lag period is abolished if the polymer synthesized in the unprimed reaction is subsequently used as primer. This substantiates that the polymer is poly(dA-dT).

The mechanism by which the poly(dA-dT) is synthesized initially in an unprimed system has not yet been elucidated. Setlow et al. (1972) separated and compared the enzymatic action of the two distinct enzymes of DNA polymerase I (see section 1.1.2). They found that only the intact enzyme can catalyse de novo synthesis of poly(dA-dT).

Radding and Kornberg (1962) investigated the kinetics of unprimed synthesis of poly(dA-dT). They observed that after 5% of the reaction

had occurred the size of the poly(dA-dT) molecules formed were the same as those produced at the end of the reaction. Therefore the process is not a progressive lengthening of the polymer throughout the course of synthesis. The macromolecules formed during the lag period are replicated as in the synthesis of DNA by DNA polymerase.

The polymer is synthesized within the first 20% of the lag period and the further time course of synthesis follows from the autocatalytic replication of (dA-dT) primer. Radding and Kornberg (1962) hypothesize that the polymer is started by a number of random combinations of nucleotides, catalyzed by the enzyme, and that some nucleotide product possessing a simple and highly regular structure is then favoured for further growth. Ordered polymerization continues until the molecule reaches a size suitable for priming. Upon its replication by DNA polymerase each of the daughter molecules then becomes a template for further replication in semi-conservative fashion. Wake and Baldwin (1962) confirmed that the semi-conservative system of replication applied for primed poly(dA-dT) synthesis. Thus the synthesis of poly (dA-dT) is analogous to that of in vivo DNA synthesis in that they are both semi-conservative (Meselson and Stahl 1958).

Inman and Baldwin (1962) have successfully prepared the poly(dA-BrdU) analogue of poly(dA-dT), using an unprimed or poly(dA-dT) primed DNA polymerase reaction, where dTTP is replaced by BrdUTP. The base sequence in the poly(dA-BrdU) product has also been shown to be alternating by Trautner et al. (1962). When either of the copolymers of (dA-dT) or (dA-BrdU) are used as primers, synthesis begins promptly. Poly(dA-dT) will serve as primer for poly(dA-BrdU) synthesis and vice versa, Wake and Baldwin (1962). Bessman et al. (1958) and Okazaki and Kornberg (1964) have shown that BrdUTP can substitute for dTTP in an in vitro system primed by calf thymus DNA.

Using poly(dA-dT) as primer in a system of transcription containing E. coli RNA polymerase ATP and UTP, a polymer containing AMP and UMP of alternating sequences can be synthesized, Chamberlin et al. (1963) and Chamberlin (1967).

Thus it can be concluded that the poly(dA-dT) system of replication and transcription was a satisfactory initial choice for the in vitro study of the transcription potential of polymers containing BrdUrd.

1.6 A possible mechanism by which bromodeoxyuridine inhibits cell differentiation.

The inhibition of specialized protein synthesis by BrdUrd appears to be dependent on the incorporation of the analogue into the DNA (Stellwagen and Tomkins 1971a). At least one DNA synthetic cycle is a prerequisite for the inhibitory effect of BrdUrd on differentiation. This has been demonstrated by the examples presented in section 1.2.3. This reiterates that the mode of action of BrdUrd inhibition of differentiation requires BrdUrd incorporation into DNA. A proposal pertaining to the nature of a DNA-linked mechanism of BrdUrd inhibition should also incorporate the fact that the inhibition is specific to proteins characteristic of a differentiated state (see section 1.2.3).

It has been mentioned previously (see section 1.4) that poly(dA-dT) and poly(dA-BrdU) bind to bacterial protein lac repressor molecules. In a similar fashion the protein RNA polymerase may have a greater affinity for poly(dA-BrdU) than it has for poly(dA-dT).

Further evidence indicating that RNA polymerase transcription is not as effective in the presence of BrdUrd-containing DNA may be gained from the in vivo studies of Jones and Dove (1972). They have found an inhibition of the transcription rate of λ coli phages in which thymidine is completely or partially substituted by BrdUrd. Substituted DNA was about seven times less effective than normal DNA as a primer for λ mRNA

In vivo. The authors concluded that inhibition of mRNA synthesis was due to either ineffective binding of RNA polymerase to the BrdUrd substituted DNA or to the inability of the BrdUrd substituted DNA to unwind.

Stellwagen and Tomkins (1971b) suggest that BrdUrd in DNA "inhibits the transcription of only certain genes into mRNA". Here it is proposed that these genes, which are probably rich in thymidine (possibly thymidine sequences) are involved directly or indirectly via a control loop with specific protein synthesis. These thymidine sequences may exist in the initiation regions or specific areas of the gene (see section 1.3.). Tolliver *et al*. (1968) on the basis of their studies (see section 1.2.2) suggested that thymidine rich material may be associated with the control of DNA synthesis. Similarly such regions may also play an important role in transcription. If these were specific in mRNA production related to differentiation, inhibition by BrdUrd might result. Britten and Davidson (1969) proposed that there are regulatory elements adjacent to structural genes. If these were rich in thymidine sequences their substitution by BrdUrd might result in an inhibition of their regulatory function. As proteins required for general cell function may not be affected by BrdUrd incorporation into the DNA, the initiation of their mRNA synthesis may be due to sites not rich in thymidine and consequently insensitive to BrdUrd substitution.

It is proposed that BrdUrd is incorporated into regulatory elements of the DNA which initiate the synthesis of specialised proteins characteristic of the differentiated state. These may be specifically sensitive to thymidine substitution by BrdUrd or they may be thymidine rich sites. It is this latter theory that will be tested in the *in vitro* technique, of synthesis of DNA and RNA, designed for this investigation.

1.7 Aim of this investigation

The aim of this investigation was to test the hypothesis that BrdUrd inhibition of differentiation acts specifically at the level of transcription of DNA into RNA. It was decided to approach this problem at a basic molecular level by synthesizing DNA molecules with differing amounts of BrdUrd and measuring the ability of RNA polymerase to transcribe these molecules. To this end the *in vitro* DNA polymerase and RNA polymerase systems described and evaluated in the subsequent sections of this thesis are used.

2. METHODS AND MATERIALS

2.1 Enzymes

2.1.1 DNA polymerase I

A. Micrococcus lysodeikticus. DNA polymerase I was obtained from Miles-Seravac Research Division, Miles Laboratories, Illinois, U.S.A. The activity was specified as 80 units/mg protein. This enzyme was isolated according to the method of Zimmerman (1966). It was supplied dissolved in 0.15M potassium phosphate buffer pH 6.8, 0.01M ETSH and was stored at -20°C.

B. Two preparations of Escherichia coli MRE 600 DNA polymerase I were obtained from Boehringer Mannheim, Germany. The activities were specified as 1970 and 2741 units/mg protein respectively. These preparations correspond to approximately step VII of the enzyme isolation of Jovin et al. (1969). They were supplied in 50% glycerol at a pH of about 6.0 and were stored at -20°C.

An enzyme unit is defined as the amount of enzyme required to convert 10 nmoles of isotopically labelled deoxyribonucleoside 5'-triphosphate into acid-insoluble material in the presence of poly(dA-dT) primer in 30 minutes at 37°C. Both M. lysodeikticus and E. coli DNA polymerase preparations were diluted in 0.05M Tris-HCl buffer pH 7.5, 0.01M ETSH, 1 mg/ml BSA. They were used within 15 minutes of dilution.

C. Calf thymus gland DNA polymerase from calf thymus gland was prepared using the method of Shepherd and Kalr (1966) with slight

modifications. Calf thymus gland was chopped into small pieces and suspended in 12 volumes of 0.25M sucrose, 0.001M EDTA, 0.005M ETSH. The suspension was then homogenized at low speed in a Potter-Elvehjelm homogenizer. Microscopic examination with the stain, 1% W/V crystal violet in citric acid, was used to check that cellular disruption had been achieved with minimal nuclear breakdown. The preparation was then made 0.01M with respect to potassium phosphate buffer pH 7.5 and 0.15M with respect to KCl, by the addition of 0.5M potassium phosphate buffer and solid KCl respectively. The homogenate was then centrifuged at 105,000g in a Spinco Model L. for 60 minutes. The supernatant fraction was the calf thymus DNA polymerase preparation used in this study. It was used within one hour of preparation. The protein concentration of the enzymatically-active supernatant fraction was determined by the method of Lowry et al. (1951).

2.1.2 . polymerase

Escherichia coli B RNA polymerase was purchased from Miles-Seravac Research Division. The activity was specified as 294 units/mg protein. This enzyme was prepared according to the method of Burgess (1969). The specifications were those of a purified solution of RNA polymerase containing 'Sigma' factor and essentially free of polynucleotide phosphorylase, DNA polymerase, RNAase and DNAase.

The enzyme was supplied in 50% glycerol in enzyme diluent and was stored at -20°C. The enzyme diluent was 0.01M Tris-HCl buffer pH 7.9, 0.01M $MgCl_2$, 0.01M KCl, 0.1mM dithiothreitol, 0.1mM EDTA. This solution was used to prepare the appropriate RNA polymerase concentrations. The diluted preparations were used within 15 minutes. One unit of RNA polymerase activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmole of isotopically labelled ribonucleoside 5'-monophosphate into acid-insoluble material in the

presence of calf thymus DNA primer in 10 minutes at 37°C.

2.2 DNA primers

2.2.1 Calf thymus DNA

The sodium salt of polymerized calf thymus DNA was purchased from Sigma Chemical Company, Missouri, U.S.A.

The DNA was dissolved in distilled water at a concentration of 2 mg/ml by stirring overnight at 4°C. It was denatured by heating for 10 minutes in boiling water and by rapidly cooling in ice.

2.2.2 Poly(dA-dT)

Poly(dA-dT) was obtained from the Sigma Chemical Company as the sodium salt of a high molecular weight double stranded copolymer. This copolymer consisted of alternating deoxyadenylic acid and deoxythymidylic acid residues. The copolymer was dissolved in distilled water at a concentration of 100 E_{260} units/0.5 ml and was stored at -20°C.

It was used as primer in the DNA polymerase and RNA polymerase assays with further dilution as required.

The UV absorption spectrum of Poly(dA-dT) was measured and is shown in Figure 6. The approximation that 1 mg of polydeoxynucleotide is equivalent to 20 E_{260} units was used throughout this work.

One unit of Poly(dA-dT) is defined as that amount which will yield an E_{260} of 1.0 in 1.0 ml of water (1 cm light path).

2.2.3 Poly(dA-BrdU)

Poly(dA-BrdU) was obtained, from General Biochemicals, Chagrin Falls, Ohio, U.S.A., as a double stranded polymer consisting of alternating deoxyadenylic acid and 5-Bromodeoxyuridylic acid residues in both strands. It was prepared according to the method of Inman and Baldwin (1962). The absorption maximum of this is 266.5nm and the spectrum is shown in Figure 6.

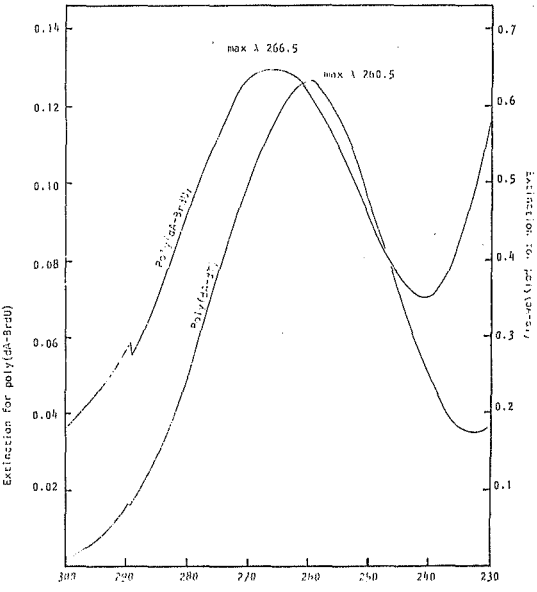


figure 6. Ultraviolet absorption spectra of poly(dA-dT) and poly(dA-BrdU)

Poly(dA-dT) was dissolved in distilled water at a concentration of 1.26 units/ml

Poly(dA-BrdU) was dissolved in 0.01M Tris-HCl pH 7.9 at a concentration of 0.18 units/ml

The polymer was supplied in 0.01M Tris-HCl buffer pH 7.9 and was stored at -4°C. It was used as required without further dilution. One unit of poly(dA-BrdU) is defined as that amount which will yield an E_{266} of 1.0 in 1.0 ml of water (1 cm light path).

2.3 Nucleotides

2.3.1 Nucleoside 5'-triphosphates

The deoxyribonucleoside 5'-triphosphates of uridine, thymine, adenine, guanine and cytosine and the ribonucleoside 5'-triphosphates of adenine and uridine were obtained from the Sigma Chemical Company.

5-Bromodeoxyuridine 5'-triphosphate was prepared initially by the author and later was purchased from Boehringer-Mannheim as the trilithium salt.

2.3.2 Isotopically labelled nucleoside 5'-triphosphates

The following labelled nucleoside 5'-triphosphates were purchased from the Radiochemical Centre, Buckinghamshire, England.

Deoxyadenosine-8-^{3}H-5'-triphosphate ammonium salt TRK 347

Deoxycytidine-5-^{3}H-5'-triphosphate ammonium salt TRK 352

Deoxythymidine-methyl-^{3}H-5'-triphosphate ammonium salt TRK 424

Adenosine-8-^{14}C-5'-triphosphate sodium salt CFA 426.

Deoxythymidine-2-^{14}C-5'-triphosphate tetralithium salt NEC 442 was obtained from New England Nuclear, Boston, Mass. U.S.A.

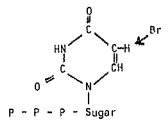
2.3.3 Preparation of 5-Bromodeoxyuridine 5'-triphosphate

5-Bromodeoxyuridine 5'-triphosphate was prepared by direct bromination of deoxyuridine triphosphate.

Markham with others (Bessman et al., 1958) had prepared BrdUTP from dCTP by bromination, followed by deamination. Markham (1971) recommended to the author the use of dUTP in order to eliminate the deamination of BrdCTP. A method was employed based on a procedure described

by Ikehara and Uesugi (1969) and recommended to the author by Kornberg and Russell (1971). This method involved the use of saturated bromine water to brominate the dUTP at pH 4.0. The product was then purified by column chromatography using DEAE sephadex (HCO_3^-) and eluting with triethylammonium bicarbonate. BrdUTP was purified by this method but a low yield was obtained. After corresponding with Grunberg-Manago and Springer (1971) their method of direct bromination of dUTP was used with slight modifications. This preparation could be completed within two days. Similar methods have been described by Chamberlin and Berg (1964) and Riley and Paul (1971).

The reaction for the direct bromination of dUTP is shown below:



dUTP → BrdUTP

Method

0.2mmole (12µl) of pure bromine was added to 0.1mmole of dUTP dissolved in 0.5 ml of formamide. This mixture was allowed to stand at 24°C for two hours. The sample was cooled to 0°C in an ice bath and then rapidly neutralized with 3 ml of 0.1M Tris at 0°C to approximately pH 7.0. Neutralization was checked with pH indicator paper. Excess bromine was then extracted from the preparation with ether until the ether layer was no longer discoloured by bromine. Four 3 ml washes with ether were required.

To ensure that complete conversion of dUTP to BrdUTP had taken place 1µl samples of the mixture were removed at various time intervals

and diluted in distilled water for spectrophotometric analyses.

All subsequent procedures were carried out at $4^{o}C$. The ether extracted preparation was then subjected to column chromatography in order to separate the various brominated and non-brominated nucleotide products of the reaction.

Dowex Bio Rad anion (Cl^{-}) exchange resin A G 1 x 4%, 200-400 mesh was used for this purpose. The dimensions of the column were 4.5 cm x 0.5 cm diameter. The resin was prepared for use by serial washes with 0.1M NaOH, water to pH 6.0, and 0.1M HCl. After the final HCl wash the resin was washed with water until the pH of the effluent was approximately 6.0. The column was packed and equilibrated with 0.01M HCl until ready for use.

The BrdUTP preparation was then applied to the column and eluted with a lithium chloride gradient consisting of the following:

Mixing vessel : 100 ml 0.01M HCl

Reservoir : 100 ml 0.01M HCl, 1M LiCl.

During elution the column was connected to the LKB Uvicord continuous flow system to monitor UV absorption. A constant flow rate of 1 ml/min was maintained using a LKB Varioperpex peristaltic pump. This was followed by a fraction collector and fractions of 10 ml were collected.

The gradient elution pattern is shown in Figure 7. Four distinct peaks were observed which were labelled a, b, c and d. Each fraction was examined between the wave-lengths of 230 to 340nm. Spectra of some of the component fractions in peaks a, b, c and d are shown in Figure 8. Bromodeoxyuridine nucleotides absorb maximally at 279nm whereas uridine nucleotides absorb maximally at 260nm. This difference in absorption maxima was taken as a criterion of conversion to the bromo species.

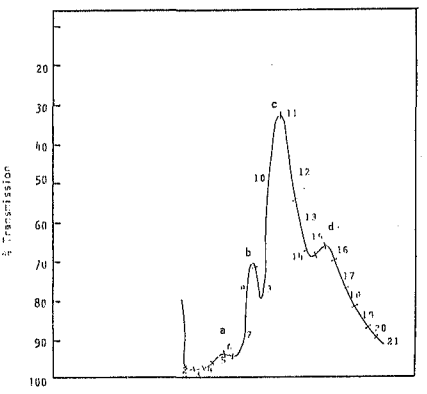


Figure 1 Dowex chromatography of the BrdUTP preparation

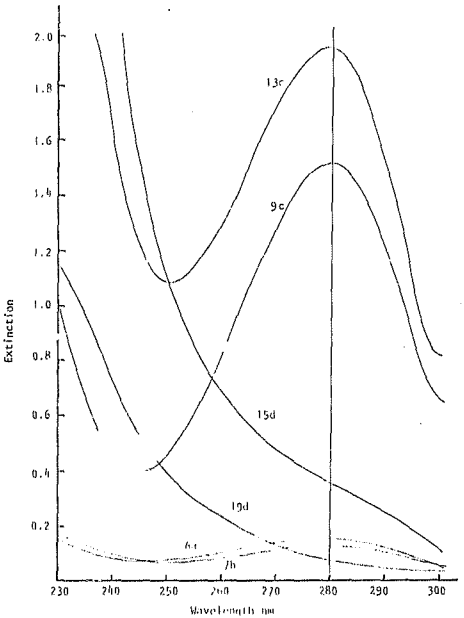


Figure 8 UV absorption spectra of some of the component fractions in peaks a, b, c, and d of Figure 7.

From Figure 8 it can be seen that fractions taken from peaks a, b and c exhibit distinct maxima at 279nm whereas peak d does not have an absorption maximum at this wavelength. It was concluded that peaks a, b and c contain brominated uridine nucleosides and peak d does not. Therefore peak a contained the BrdUMP, peak b BrdUDP and peak c BrdUTP because of the increasing magnitude of the negative charges on each nucleotide. This is a typical pattern of elution for a mixture of nucleoside mono, di and triphosphates from an anion exchange column (Smith and Khorana 1958). On this basis fractions 10, 11, 12 and 13 were considered to be pure BrdUTP. All four fractions were combined, neutralized with tributylamine and lyophylized. The LiCl was subsequently removed by washing five times with 1 ml portions of a 1:1 ethanol-acetone mixture. The BrdUTP remained undissolved during this procedure. After the final ethanol-acetone wash the preparation was washed with ether and was allowed to evaporate to dryness. The product was then dissolved in 1 ml of distilled water and 5ul of this sample was diluted in 3 ml of distilled water. The extinction of 1 ml of this solution was determined at 279.5nm and was found to be 0.22. The $E_{279.5}$ of the total BrdUTP preparation was then calculated and was found to be 132. The molar extinction coefficient (ϵ) of BrdUTP was taken as 8500 (Kornberg and Russel 1971). Therefore the molarity ($\frac{E}{\epsilon}$) of BrdUTP was calculated as 15.5umole/1 ml. Since the initial amount of dUTP used was 0.1mmole the yield of BrdUTP was 15.5%

The spectrophotometric properties of the dUTP reactant and the BrdUTP product are compared with results from other laboratories. As shown in Table I dUTP has been successfully converted to BrdUTP during the above procedure.

Table 1. Spectral properties of dUTP and BrdUTP

Spectral properties		pH 1-2				pH 12	
		1	2	3	4	2	4
λ Max	dUTP	262			262.5		
	BrdUTP	279	278	280	279	278	276
$E\frac{250}{260}$	dUTP	0.72			0.74		
	BrdUTP	0.62		0.50	0.59		
$E\frac{280}{260}$	dUTP	0.45			0.42		
	BrdUTP	1.77	1.86	1.91	1.77	1.61	1.35

Columns headed 1-4 correspond to the key below:

1. Beaman et al. (1958)
2. Kornberg and Russel (1971)
3. Riley and Paul (1971)
4. Present results

An additional check on the purity of the BrdUTP was performed using thin layer electrophoresis. Merck T.L.C. plates coated with Silica Gel 7254 were spotted with 10µg samples of dUTP and the prepared BrdUTP (in 10µl and 1µl respectively). The plates were run in 0.05M borate buffer pH 9.4 at 700 volts for 90 minutes, and finally dried in a warm oven. The nucleotides were located by absorption of ultraviolet light. Both the dUTP and BrdUTP preparation appeared as distinct spots, showing homogeneity of both molecular species. The BrdUTP preparation had moved slightly further than the dUTP. This result supports the conclusion that the dUTP has been converted to BrdUTP since halogenated

nucleotides migrate further than their non-halogenated counterparts in a borate buffer of pH 9 (Smith 1969). The above procedure was adapted from the method described by Dunn and Smith (1954). The calf thymus DNA polymerase assay system was used to demonstrate that the product BrdUTP could replace dTTP in DNA synthesis. See Section 3.2.

2.4 Additional materials

Crystallised Bovine Serum Albumin (BSA) was obtained from Armour Pharmaceutical Company, Eastbourne, England.

PPO and POPOP were obtained from Packard Instrument Company, Illinois, U.S.A.

2.5 Enzyme assays

DNA polymerase and RNA polymerase activities were determined by measuring the amount of radioactive deoxyribonucleoside or ribonucleoside 5'-triphosphate incorporated into polymer DNA or RNA respectively. These assay systems were based on a method originally developed by Bollum (1959). In this investigation all enzyme assays were performed in either duplicate or triplicate.

2.5.1 Incubation conditions

The incubation conditions varied according to the requirements of each enzyme and the requirements of specific experiments. The most frequently used incubation conditions will be described below and departure from these, for specific purposes, will be described together with the results. The reaction mixture consisted of 3 basic components; a special mixture (SM), a nucleotide mixture (NM) and an enzyme preparation. When these components were combined in the appropriate proportions the optimal conditions for the enzyme assay were attained.

The SM consisted of Tris-HCl buffer, relevant ions, sulphydryl agent and primer DNA.

The nucleotide mixture contained the appropriate nucleoside 5'-triphosphates one of which was radioactively labelled. This procedure will be described in detail for the assay of calf thymus DNA polymerase. For the microbial DNA polymerase and RNA polymerase assay the standard conditions only will be described.

A. Calf thymus DNA Polymerase

The standard assay conditions for calf thymus DNA polymerase are described in Table 2, and are based on the system of Shepherd and Keir (1966). The detailed composition of the SM and NM for 10 enzyme assays are shown in Table 3.

Table 2. Standard assay conditions for calf thymus DNA polymerase

Tris-HCl buffer pH 7.5	20mM
KCl	60mM
EDTA	0.4mM
$MgCl_2$	4mM
ETSH	5mM
Calf thymus DNA (heat denatured)	50µg
dATP, dTTP, dGTP and (^{3}H)dCTP of specific activity 2 x 10^6 c.p.m./µmole	0.2mM of each
Calf thymus enzyme fraction	153µg of protein
Total assay volume	0.25 ml
Temperature	37°C

Table 3. Composition of the SM and NM for the standard Calf thymus DNA polymerase assay

Special Mixture (SM)

Components	per assay 0.25 ml	Stock Soln.	ml of stock Soln. per 10 assays
Tris-HCl pH 7.5	5μmoles	0.2M	0.25
KCl	15μmoles	1.0M	0.15
EDTA	0.1μmoles	0.1M	0.01
$MgCl_2$	1μmole	0.5M	0.02
ETSH	1.25μmoles	0.5M	0.025
Calf Thymus DNA primer	50μg	5 mg/ml	0.30
H_2O (dist)			0.24
			1.0 Total volume

Nucleotide Mixture (NM)

Componen.,	per assay 0.25 ml	Stock Soln.	ml of Stock Soln. per 10 assays
dATP	50nmoles	5mM	0.1
dGTP	50nmoles	5mM	0.1
TTP	50nmoles	5mM	0.1
^3H dCTP	50nmoles	5mM	0.1
H_2O (dist.)			0.1
			0.5 Total vol.

Table 3 (Cont.)

The composition of the incubation mixture is described below:

	ml
H_2O	0.05
SM	0.1
NM	0.05
Enzyme extract	0.05
Total volume	0.25

The components were added in the order shown above to 10 ml conical centrifuge tubes or 3 ml test tubes. After the addition of the enzyme the tubes were capped and immediately incubated in a shaking water bath at 37°C. After incubation the reaction was terminated by freezing in a dry ice-acetone mixture. The control tubes were not incubated but directly frozen in a dry ice-acetone mixture.

B M. lysodeikticus and E. coli DNA polymerase

The standard assay conditions for M. lysodeikticus and E. coli are described in Table 4 and are based on the incubation mixture used by Zimmerman (1966). The use of Tris-HCl buffer at pH 7.9 was based on the optimum values given by Zimmerman. This pH was used instead of the usual pH of 7.4, so that the conditions could be maintained for the sequential DNA and RNA assays described in Section 2.5.1 D. The control tubes were not incubated but directly frozen in a dry ice-acetone mixture.

Table 4. Standard assay conditions for *M. lysodeikticus* and *E. coli* DNA polymerases

Tris-HCl buffer pH 7.9	66.7mM
$MgCl_2$	3.3mM
ETSH	1.0mM
Poly(dA-dT)	0.1 units
dATP, dTTP and or BrdUTP	0.3mM of each
DNA polymerase	0.02-5 units
Total volume	0.25-0.3 ml
Temperature	37°C

The isotopically labelled nucleotides used in these assays were:

(^{3}H)dATP of specific activity	6-8 x 10^5 c.p.m./μmole
(^{3}H)dTTP of specific activity	6-7 x 10^5 c.p.m./μmole and
(^{14}C)dTTP of specific activity	17-20 x 10^6 c.p.m./μmole

C E. coli RNA polymerase

The standard assay conditions for E. coli RNA polymerase are described in Table 5. These conditions were modified from those used by Lill et al. (1970). The control tubes were not incubated but directly frozen in a dry ice-acetone mixture.

Table 5. Standard assay conditions for E. coli RNA polymerase

Tris-HCl Buffer pH 7.9	50mM
$MgCl_2$	8mM
$MnCl_2$	2mM
Poly(dA-dT) or Poly(dA-BrdU)	0.2 units
UTP, (^{14}C)ATP of specific activity 2×10^6 c.p.m./μmole	0.64mM of each
RNA polymerase	0.3 units
Total assay volume	0.25 ml
Temperature	37°C

D RNA polymerase assay preceded by DNA polymerase action

This experimental technique was developed to study the ability of RNA polymerase to form Poly(A-U) on DNA templates containing varying amounts of BrdUTP. DNA polymerase action was followed by sequential RNA polymerase action in the same incubation tube. Thus after DNA polymerase action the requirements for RNA polymerase were added to the reaction mixture to study the transcription of the DNA polymerase products.

The standard DNA polymerase incubation conditions are described in Table 6. The incubation period was for 180 minutes, 10 ml conical centrifuge tubes were used to reduce evaporation losses.

Table 6. Standard assay conditions for DNA polymerase action

Tris-HCl buffer pH 7.9	80mM
$MgCl_2$	2.5mM
ETSH	1.05mM
Poly(dA-dT) or Poly(dA-BrdU)	0.1-0.2 units
dATP	0.5mM
dTTP and or BrdUTP	0.5mM total
DNA polymerase	5-5.3 units
Total assay volume	0.2 ml
Temperature	37°C

These conditions were modified slightly compared with those described in Table 4 in order to approach more closely the requirements for the subsequent RNA polymerase incubation.

After the DNA polymerase incubation 1.7μmoles $MgCl_2$, 0.6μmoles $MnCl_2$, 160μmoles UTP, 160μmoles (^{14}C)ATP and 0.413 units of RNA polymerase were added to the reaction mixture in a total volume of 0.1 ml to fulfil the requirements for RNA polymerase action. This yielded the RNA polymerase incubation conditions described in Table 7.

Because of the complexity of the procedures used in this assay the detailed composition of the DNA polymerase incubation mixture and RNA polymerase additions are described in Table 8 and Table 9 respectively.

Table 7. Standard assay conditions for RNA polymerase when preceded by DNA polymerase action

Tris-HCl buffer pH 7.9	53mM
$MgCl_2$	8.17mM
$MnCl_2$	2.08mM
ETSH	0.7mM
UTP, (^{14}C)ATP specific activity of 2×10^6 c.p.m./μmole	0.53mM
RNA polymerase	0.413 units
Total assay volume	0.3 ml
Temperature	37°C

Table 8. Composition of the SM and NM for the DNA polymerase assay

Special Mixture (SM)

Components	per assay 0.2 ml	Stock Soln.	ml of Stock Soln. per 10 assays
Tris-HCl pH 7.9	16μmoles	0.4M	0.2
$MgCl_2$	0.7μmoles	0.05M	0.4
ETSH	0.21μmoles	0.015M	0.14
Poly(dA-dT) or (dA-BrdU)	0.1-0.2 units		0.14
H_2O (dist.)			0.12
			1.00 Total

Nucleotide Mixture per assay

Components	per assay 0.2 ml	Stock Soln.	ml of Stock Soln. per assay
dATP	100nmoles	4.0mM	0.025
dTTP and/or BrdUTP	as required	5.0mM	as required
	total 100nmoles		total 0.02 ml
H_2O (dist.)			0.005
			0.05 total/assay

Composition of the completed incubation mixture

	Volume (ml)
SM	0.1
NM	0.05
DNA polymerase	0.05
	0.20 total

Table 9. Additions for RNA polymerase activity

Components	per assay (Addition)	Stock Soln.	ml of stock soln. per 10 assays
$MgCl_2$	1.7μmoles	0.34M	0.05
$MnCl_2$	0.6μmoles	0.125M	0.05
			0.1 Total volume

Nucleotide Mixture

Components	per assay	Stock Soln.	ml of stock soln. per 10 assays
UTP	160μmoles	4.0mM	0.4
(^{14}C) ATP	160μmoles	4.0mM	0.4
			0.8 Total volume

Composition of the completed incubation mixture:

	Volume (ml)
$MgCl_2$ + $MnCl_2$	0.01
Nucleotide Mixture	0.08
RNA polymerase	0.01
	0.1 ml Total

2.5.2 Analytical techniques

Polynucleotide synthesis was estimated by determining the incorporation of an isotopically labelled nucleoside 5'-monophosphate from its triphosphate form into acid-insoluble products according to the method of Ballum (1959), with slight modifications.

The frozen assay mixtures were thawed and 0.05 ml of BSA (2 mg/ml in water) were added to each tube as co-precipitant. Aliquots of 0.05 ml were taken from each tube and pipetted onto discs of Whatman (2.5 cm diameter) GF/C glass fibre paper. Duplicate discs were prepared for each assay tube. Each disc was immediately plunged into a beaker of cold 5% (W/V) TCA. This resulted in precipitation of labelled polynucleotide and protein onto the disc. There is no exchange of labelled product among discs in this system and consequently many discs may be processed together in this way. A volume of 20 ml 5% TCA was provided for each disc. After a minimum precipitation time of 5 minutes the TCA was decanted and replaced with fresh cold 5% TCA. This procedure was repeated five times. Residual TCA from the last wash was removed by successive washes in absolute alcohol and ether. The discs were then replaced individually in potassium free glass vials and vacuum desiccated to remove residual water. To each vial 10 ml of cold scintillation fluid was then added. The scintillation fluid was made up of 4g of PPO and 0.1g of POPOP per litre of toluene. The vials were then counted in a Packard Tri-Carb Liquid Scintillation Spectrometer (model 3320). Each vial was counted for three periods of 10 minutes for (^{14}C) and 3 periods of 20 minutes for (^{3}H).

The specific radioactivity of the substrate nucleoside 5'-triphosphate used in each assay was determined as follows: 0.1 ml of appropriately diluted radioactive substrate was pipetted onto two discs which were dried and counted together with the experimental samples in each assay. Also a blank disc containing no additions was occasionally subjected to the TCA wash. Such discs did not exhibit counts higher than background. This demonstrates that

there was very little absorption of unincorporated labelled nucleotide on to the discs during preparation.

Duplicate discs were taken from each assay tube and each assay was performed either in triplicate or duplicate. Therefore a maximum of 6 discs or a minimum of 4 discs were counted for each assay. These results were averaged to determine the polymer product. This average was expressed as counts/minute/assay (c.p.m./assay), and was converted to nmoles of isotopically labelled nucleoside 5'-monophosphate incorporated into acid insoluble product per assay.

3. RESULTS

3.1 Assays of DNA polymerase and RNA polymerase

The initial work was carried out to establish valid standard assay conditions for the bacterial polymerases used. These conditions were used throughout the study except where otherwise specified. It should be noted that the bacterial polymerases were initially assayed to ensure that they were of the activity and concentration specified by the manufacturers and that they had retained activity during transportation.

The calf thymus DNA polymerase was used to establish that the BrdUTP prepared in this study (see Section 2.3.3) was incorporated into acid-insoluble polydeoxynucleotide in a DNA polymerase catalyzed reaction, as described in Section 3.2. The reason for using calf thymus extract in order to check BrdUrd incorporation was due to this laboratory's experience with the assay of this enzyme.

3.1.1 DNA polymerase from calf thymus gl.

The preparation of the soluble fraction containing DNA polymerase was described in Section 2.1.1 C. The incubation conditions were as described in Section 2.5.1 A Table 2. The time course of calf thymus DNA polymerase activity is shown in Figure 9 which indicates that the assay system was linear over an incubation period of 0 to 60 minutes. A 30 and 60 minute incubation period was chosen for the experiment with this enzyme.

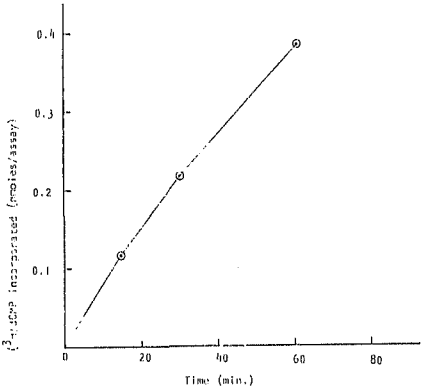


Figure 9: Time Course of DNA polymerase activity of calf thymus extract.

The components of the reaction mixture were made up as described in Section 2.5.1 A.* Tables 2 and 3.

The reaction mixture contained in 0.25 ml, 5M, 0.2mM each of dATP, dGTP, dTTP and (^{3}H)dCTP, and 153µg protein of calf thymus enzyme fraction.

The specific radioactivity of the (^{3}H)dCTP was 2.2 x 10^3 c.p.m./nmole.

Incubation was carried out at 37°C.

The results are an average of two assays each and the control tube was not incubated.

3.1.2 DNA polymerase from M. lysodeikticus

The relationship between the concentration of enzyme and the synthesis of polydeoxynucleotide

The enzyme concentrations were chosen to establish the levels at which the amount of product was proportional to enzyme concentration. As shown in Figure 10, the linearity between product formed and enzyme concentration was in the region 0.08 to 0.4 units of enzyme.

Specimen calculation

An example of the calculation of nmoles of isotopically labelled nucleotide incorporation into acid-insoluble product per assay per 30 minutes (i.e. nmoles/assay) is shown below.

In this particular assay 0.21 units of DNA polymerase was used per 0.35 ml final volume of reaction mixture. Two samples of 0.05 ml were taken for analysis and each sample was read three times for 10 minutes.

Counts per 10 min. = 6276 + 6507 + 6308 + 6176 + 6215 + 6329

= 37811

Average counts per 10 min. = 6302

Counts per min. (c.p.m.) = 630.2

c.p.m. of the control = 79.5

Thus the corrected c.p.m. = 550.7

c.p.m. per assay = 3854.9 (i.e. corrected c.p.m. x 7)

The specific radioactivity of the (^{14}C)dTTP substrate nucleotide was 19066 c.p.m./nmole

∴ nmoles of (^{14}C)dTMP incorporated per assay per 30 min

= 3854.9/19066

= 0.20

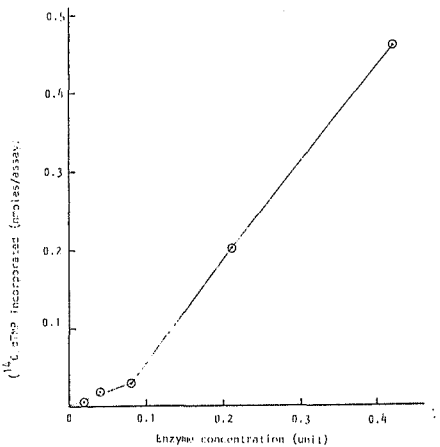


Figure 10: The relationship between the concentration of M. lysodeikticus DNA polymerase and the synthesis of polydeoxynucleotide

The components of the reaction mixture in 0.3 ml were as described in Section 2.5.1 B Table 4 and contained 5M, 0.1 units poly(dA-dT), 0.3mM each of dATP and (^{14}C)dTTP and enzyme as shown.

The specific radioactivity of (^{14}C)dTTP was 19 x 10^3 c.p.m./mmole.

Incubation was carried out at 37^{0}C for 30 minutes.

The control tube contained no enzyme.

The radioactivity incorporated in the poly(dA-dT)-primed assay was corrected to give the total nucleotide incorporation by multiplying by 2.

Thus 0.21 units of DNA polymerase activity caused the incorporation of 0.40 nmoles of total nucleotide into the acid insoluble product during 30 minutes of incubation.

According to the definition of unit activity and the specifications of Miles Laboratories 1 unit of enzyme should cause the incorporation of 10 nmoles of total nucleotide in 30 minutes.

The above calculation shows that 5.2 units were in fact required for the incorporation of 10 nmoles of total nucleotide. This discrepancy will be considered in Section 4.

These results indicate that 5 units of enzyme per assay were adequate to catalyze the formation of significant quantities of labelled product. This amount of enzyme was routinely used for the synthesis of polydeoxynucleotides in systems described later (Section 3.3).

The relationship between the concentration of DNA-primer and the activity of M. lysodeikticus DNA polymerase

To estimate the minimum concentration of primer required for maximum deoxynucleotide incorporation M. lysodeikticus DNA polymerase was assayed in the presence of increasing concentrations of poly(dA-dT)-primer. From Figure 11 it can be seen that product formation reaches a plateau between 0.1 and 0.5 units of poly(dA-dT). Therefore, 0.1-0.2 units of poly(dA-dT)-primer was chosen for all assays of this study except those of Section 3.3.5.

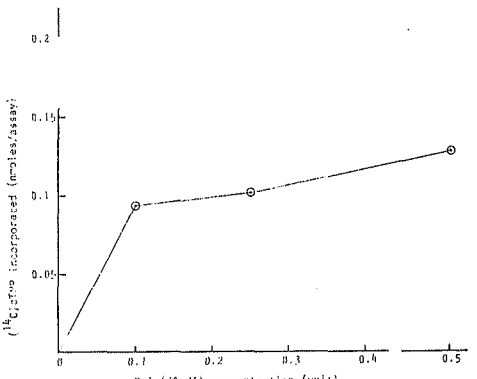


Figure 11: The relationship between the concentration of DNA-primer and the activity of M. lysodeiktikus DNA polymerase.

The components of the reaction mixture in 0.3 ml were as described in Section 2.5.1 B Table 4 and contained 5M, poly(dA-dT) as shown, 0.3mM each dATP and (^{14}C)dTTP and 0.2 units of M. lysodeiktikus DNA polymerase.

The specific radioactivity of (^{14}C)dTTP was 1×10^3 c.p.m./nmole.

Incubation was carried out at 37°C for 10 minutes.

The control tube contained no primer.

3.1.3 DNA polymerase from E. coli

The relationship between the concentration of E. coli DNA polymerase and the synthesis of polydeoxynucleotide was studied using an enzyme preparation with a specified activity of 2741 units/mg protein. As shown in Figure 12, linearity between product formed and enzyme concentration was in the region 0.02 to 0.75 units of enzyme.

The activity of this enzyme preparation more closely approached the specifications of its suppliers than did that of the M. lysodeikticus preparation. However, the levels of activity of both preparations were very similar. Consequently 5 units of enzyme were routinely used (Section 3.3).

3.1.4 RNA polymerase from E. coli

A time course of E. coli RNA polymerase activity is shown in Figure 13. An average of these results gave an incorporation of 0.89 nmole of (^{14}C)AMP incorporated in 10 minutes using poly(dA-dT)-primer, this is equivalent to approximately 742 units/mg protein. The manufacturer specified an activity of 294 units/mg protein.

As Miles Laboratories used calf thymus DNA-primer while in this case poly(dA-dT)-primer was used, the result[illegible]he directly compared. However, if this difference is t[illegible] account the higher enzyme activity obtained is within the expected magnitude when total nucleotide incorporation is calculated. As 0.3 units of enzyme per assay was found to be adequate to catalyze the formation of significant quantities of labelled product, this amount of enzyme was routinely used in RNA polymerase assays.

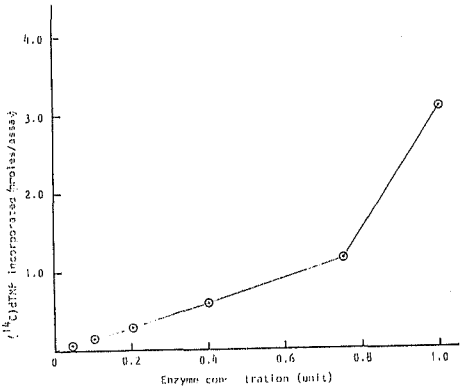


Figure 12: The relationship between the concentration of E. coli DNA polymerase and the synthesis of polydeoxynucleotide.

The components of the reaction mixture in 0.3 ml were as described in Section 2.5.1 B Table 4, and contained 5M, 0.1 units of poly(dA-dT), 0.3mM each of dATP and (^{14}C)dTTP, and enzyme as shown.

The specific radioactivity of (^{14}C)dTTP was 20 x 10^3 c.p.m./nmole.

Incubation was carried out at 37°C for 30 minutes.

The control tube contained no enzyme.

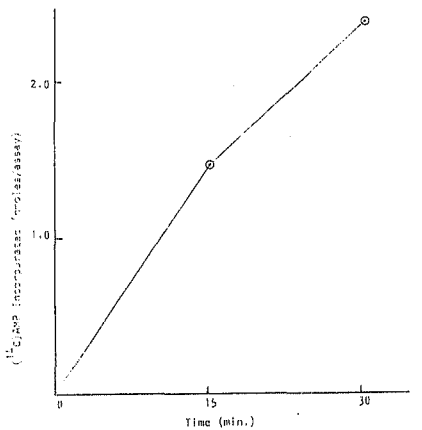


Figure 13: Time course of E. coli RNA polymerase activity.

The components of the reaction mixture were as described in Section 2.5.1 C Table 5, and contained in 0.25 ml SM, 0.2 units of poly(dA-dT), 0.6mM each of UTP and (^{14}C)ATP and 0.3 units of enzyme.

The specific radioactivity of (^{14}C)ATP was 1.3×10^3 c.p.m./nmole.

Incubation was carried out at 37°C for the indicated time intervals.

Each point on the curve is a mean of 2 assays.

The control tube was not incubated.

3.2 The incorporation of BrdUMP into DNA using calf thymus DNA polymerase and calf thymus DNA primer

Calf thymus DNA polymerase was assayed using calf thymus DNA as primer and the four deoxynucleoside 5'-triphosphate substrates, dATP, dGTP, dTTP and (^{3}H)dCTP. In some assays BrdUTP was substituted either in part or totally for dTTP, to investigate the ability of BrdUMP to be incorporated into the DNA product. The BrdUTP used, was that prepared by the author as described in Section 2.3.3. The results are shown in Table 10. It can be seen that in this calf thymus system BrdUTP can effectively substitute for dTTP. When both dTTP and BrdUTP are omitted from the reaction mixture it can be seen that the incorporation of deoxynucleotide into DNA product drops to 51-54% of the complete assay. When dTTP is totally substituted for, by BrdUTP the incorporation is 86-92% of the complete assay (i.e. 100% dTTP). In this study it has thus been assumed that within 5-10% BrdUTP and dTTP are equivalent in in vitro DNA synthesis.

Table 10. Substitution of BrdUTP for dTTP in the calf thymus DNA polymerase reaction

Relative proportions of BrdUTP and dTTP in the reaction mixture	(^{3}H)dCMP incorporated (nmoles/mg protein)		% of 100% dTTP value	
	30 min.	60 min.	30 min.	60 min.
100% dTTP	3.13	7.20	100	100
50% dTTP/50% BrdUTP	2.90	7.01	93	97
100% BrdUTP	2.69	6.59	86	92
dTTP, BrdUTP omitted	1.61	3.86	51	54

Table 10 : Continued

The components of the reaction mixture were made up as described in Section 2.5.1 A Tables 2 and 3, except where dTTP was replaced by BrdUTP as listed above.

The reaction mixture contained in 0.25 ml, SM, 50µg heat denatured calf thymus DNA, 0.2mM each of dATP, dGTP, dTTP (and or BrdUTP) and (^{3}H)dCTP and 153µg protein of calf thymus enzyme fraction. Incubation was carried out at 37°C for 30 or 60 minutes as indicated.

The specific radioactivity of the (^{3}H)dCTP was 24.7×10^3 c.p.m./nmole.

The values are a mean of two assays. The control tubes for each assay type were not incubated but frozen in a dry ice-acetone mixture.

3.3 The effect of DNA-primers containing bromodeoxyuridine on poly(A-U) synthesis

The following in vitro system was devised in order to study the ability of DNA-primers containing BrdUrd to be transcribed to poly(A-U). The DNA primers were synthesized in an in vitro DNA polymerase catalysed reaction, and subsequently transcribed by RNA polymerase. These two reactions were sequentially carried out in the same test tube. This process was devised to eliminate the procedure of first isolating the products of DNA synthesis and then subjecting them to transcription. Additionally, the high cost of the microbial enzymes used in this study necessitated the use of minimal quantities of materials and hence the development of the above system. The method used is described in detail below.

3.3.1 The synthesis and transcription of DNA in a sequential system

The following reactions were designed to synthesize DNA polymers for use as primers in an in vitro RNA polymerase system.

Poly(dA-dT), poly(dA-BrdU) and related polymers containing varying proportions of thymidine and bromodeoxyuridine were synthesized in an in vitro DNA polymerase system. The DNA polymers were then used as primers for the synthesis of poly(A-U). The ability of RNA polymerase to form poly(A-U) on primers containing varying amounts of BrdUTP was thus studied. The system used is described schematically in Figure 14.

The first part of the reaction (Figure 14(a)) represents the synthesis of a variety of DNA polymers from a poly(dA-dT)-primer in the presence of DNA polymerase, and substrates dATP, dTTP and or BrdUTP. In such a system the ratio of the dTTP : BrdUTP in the polymer synthesized is determined by the ratio of dTTP : BrdUTP in the incubation medium. The second part of the reaction (Figure 14(b)), which was continued in the same incubation tube after the necessary RNA polymerase reaction additions, consisted of the transcription of the DNA product, of the first reaction, to poly(A-U).

The reactions described above and in Figure 14 will be referred to as the sequential system or DNA_1-primer $\xrightarrow{(a)}$ DNA' $\xrightarrow{(b)}$ RNA. Additionally to distinguish the primer of reaction (a) from that of (b) the former is also defined as the initial DNA-primer.

There seemed to the author to be three possible inherent complications in the DNA' → RNA reaction of the sequential system. Consequently preliminary examination of both reactions of the sequential system individually and in sequence was undertaken in an attempt to establish acceptable assay conditions. Only then could the effect of DNA primers, with varying bromodeoxyuridine content, on poly(A-U) synthesis be studied in the sequential system. The difficulties inherent in this system are outlined below.

Figure 14. A sequential system for the synthesis of DNA polymers and their subsequent transcription to Poly(A-U)

(a) Poly(dA-dT) + x% BrdUTP + (100 - x)% dTTP + dATP

Mg^{2+} (2.5mM $MgCl_2$) ↓ DNA polymerase (180 min. at 37°C)

(b) Poly(dA-dT) or poly(dA-BrdU) or a substituted variation

Mg^{2+} + Mn^{2+} (8.2mM $MgCl_2$ + 2.1mM $MnCl_2$) ↓ RNA polymerase + UTP + (^{14}C)ATP (30 min. at 37°C)

(^{14}C)poly(A-U)

Reaction (a) is the process of DNA synthesis and reaction (b) represents the transcription of DNA-primers to poly(A-U) i.e. DNA_I-primer $\xrightarrow{(a)}$ DNA' $\xrightarrow{(b)}$ RNA. This has been defined as the sequential system when it occurs in the same incubation tube. DNA_I-primer represents the initial primer used in reaction (a) and DNA' represents the DNA product of this reaction which is also used to prime the subsequent reaction (b). RNA is the product of reaction (b) and is always poly(A-U).

x% in reaction (a) represents the amount of BrdUTP in the reaction mixture added in place of dTTP, so that the total of BrdUTP + dTTP is always 100% of the pyrimidine deoxynucleotide added to the reaction mixture.

Firstly, the initial DNA-primer of reaction (a) is always available for priming in the subsequent transcription reaction.

i.e. DNA_i-primer $\xrightarrow{(a)}$ DNA' $\xrightarrow{(b)}$ RNA

This DNA_i-primer may thus mask the ability of the DNA products (DNA') to be transcribed to poly(A-U) in the reaction (b). The effect of this *initial DNA_i-primer was minimized by synthesizing in reaction (a) a* much greater amount of DNA' product than was originally present as the initial DNA-primer. Thus the intention was to dilute out the effect of the DNA_i-primer. See relevant experiment described in Section 3.3.2(1).

An attempt was also made to use the system without the addition of an initial DNA-primer, i.e. to utilize an unprimed DNA polymerase *reaction. However,* due to inconsistancies found in the rate of synthesis of DNA in an unprimed system, this idea was abandoned. See relevant experiments described in Section 3.3.2(5) and (6).

Secondly, it seemed possible that free deoxyrucleotides not incorporated into product DNA (DNA') in reaction (a) might interfere with RNA polymerase action in reaction (b). *Thus initially conditions* were designed to minimize a possible excess of free deoxyribonucleotides in the RNA polymerase reaction. However, the results of an assay described in Section 3.3.2(4) indicated that deoxynucleotides do not compete with ribonucleotides in the RNA polymerase reaction.

Thirdly, the DNA' primer for RNA polymerase synthesis in reaction *(b) is also a substrate primer for the* DNA polymerase of reaction (a). Therefore to exclude possible competition for DNA' product between RNA polymerase and DNA polymerase during reaction (b) the activity of

DNA polymerase must be eliminated after the synthesis of product DNA'. Fortunately this problem did not arise, probably because of inactivation of DNA polymerase on addition of the extra Mg^{2+} and of the Mn^{2+} required for RNA polymerase activity. See the relevant experiments in Section 3.3.2(2).

3.3.2 Assay conditions required for the sequential DNA polymerase and RNA polymerase reactions

(1) The effect of increasing incubation times on deoxynucleotide incorporation

The synthesis of DNA in a DNA polymerase reaction primed with poly(dA-dT) was followed over a long incubation period. The aim of this experiment was to find an incubation period where the amount of product DNA formed was in great excess of the initial DNA-primer. The deoxynucleotide substrates were dATP and (^{3}H)dTTP. The incorporation of (^{3}H)dTMP into the product poly(dA-dT) was measured. The results are shown in Table 11 and Figure 15. The total amount of nucleotide incorporated into product appears to be higher than total input of nucleoside 5'-triphosphate. This will be discussed in Section 4. It can be seen that the assay system is linear for approximately six hours. From these results an incubation time of 180 minutes gave a total nucleotide incorporation of 758nmoles (per 0.9 ml assay) which was calculated to be equivalent to approximately 300µg of of poly(dA-dT) product. The initial amount of poly(dA-dT)-primer used in this incubation mixture was approximately 15µg. Therefore, there was a 20-fold increase in poly(dA-dT) product over primer in 3 hours.

An incubation period of 180 minutes was selected for the DNA polymerase reaction of the sequential system, as a 20-fold excess of poly(dA-dT)

Table 11. The rate of synthesis of poly(dA-dT)

Incubation time (min.)	(^{3}H)dTMP incorporated (nmoles/assay)
30	50
60	98
90	160
180	379
360	796
1200 (overnight)	820

The components of the reaction mixture in 0.9 ml were 60μmoles Tris-HCl buffer pH 7.9, 3μmoles $MgCl_2$ 0.9μmoles ETSH, 600 nmoles each of dATP and (^{3}H)dTTP, 0.3 units of poly(dA-dT), 16 units of M. lysodeikticus DNA polymerase.

(^{3}H)dTTP had a specific radioactivity of 695 c.p.m./nmole.

Incubation was carried out at 37°C.

At the stated time intervals two samples of 50μl were removed for analysis. The discs were air blown to dryness and kept at 4°C until all the samples had been taken. The control samples were taken before incubation was started.

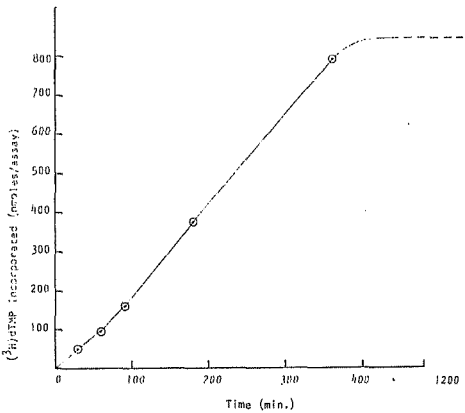


Figure 15. *The rate of synthesis of poly (dA-dT).*

For legend see Table 11.

product (i.e. DNA') over DNA-primer (i.e. DNA_i-primer) was considered to be of sufficient magnitude to minimise the effect of the DNA_i-primer in the subsequent synthesis of RNA. Thus the incorporation of ribonucleotides in the RNA polymerase reaction would be mainly due to the priming of the DNA product and not that of the initial DNA-primer.

Another feature of this combination of substrate concentration and incubation time was that there was almost total incorporation of deoxynucleoside 5'-triphosphate substrate. Consequently the amount of free deoxynucleotides present in the subsequent RNA polymerase reaction was also minimized.

(2) A preliminary study of the DNA and RNA synthetic reactions: Complete sequential reaction and isolated DNA-polymerase and isolated RNA polymerase reactions

Assays were designed to study the sequential reaction (i.e. DNA_i-primer → DNA' → RNA) and the isolated DNA polymerase reaction (i.e. DNA -primer → DNA') and isolated RNA polymerase reaction (i.e. DNA-primer → RNA).

For the sequential system (Figure 14) the incorporation of (^{14}C)AMP into RNA was measured. DNA polymerase was incubated in a buffer solution containing ETSH and Mg^{2+} for 180 minutes, with poly(dA-dT)-primer and the substrates dATP and dTTP, for the purpose of synthesizing DNA polymers (DNA'). After the completion of this 180 minute incubation the requirements for RNA synthesis were added. These were an additional Mg^{2+} and Mn^{2+} solution, (^{14}C)ATP and UTP nucleotide mixture and finally RNA polymerase. The major primer for RNA polymerase thus being the synthesized DNA (DNA'). For the isolated DNA polymerase reaction, the reaction was terminated after the 180 minute incubation and (^{3}H)dTMP incorporation into the DNA

product measured. The isolated RNA polymerase reaction mixture consisted of a buffer solution containing Mg^{2+} and Mn^{2+}, ETSH and the nucleotide mixture containing (^{14}C)ATP and UTP, Poly (dA-dT) and RNA polymerase. All components were present at the same concentration as in the sequential system. (^{14}C)AMP incorporation into RNA was thus measured.

Initially in the sequential system the reaction mixture was heated at 45°C for 60 minutes, immediately after completion of the 180 minute DNA polymerase incubation, to terminate DNA polymerase action prior to the addition of the requirements for the RNA polymerase assay. According to Richardson et al. (1964) there is a decline in DNA polymerase activity from 100% to 40% within 12 minutes at an incubation temperature of 45°C. It was found in the sequential system that addition of the Mg^{2+} and Mn^{2+} required for the RNA polymerase assay caused the formation of a fine precipitate. This precipitate was assumed to be the DNA polymerase protein. Further enzymic action of the DNA polymerase was thus curtailed. This was confirmed by the experiment described in Section 3.3.5.

The results are shown in Table 12. From these results it can be seen that the mean values of (^{14}C)AMP incorporation into RNA of 7.2 and 6.8 nmoles/assay respectively for the sequential reaction alone and for the sequential reaction with intermediary heating at 45°C for 60 minutes, are similar. Thus after completion of this experiment it was evident that probably due to the formation of the precipitate, heating of the DNA polymerase reaction mixture was no longer necessary. The incorporation of 2.9 and 3.5 nmoles/assay (assay 1) of the sequential system have been ignored as they appear to be atypically low.

The observation that the incorporation of (^{14}C)AMP for the isolated DNA_I-primer → RNA reaction is lower than that for the sequential

system indicates that the rate of synthesis is higher in the sequential system. This is possibly due either to the increase of primer concentration (i.e. DNA') or structural differences between the initial DNA-primer and DNA' product (see Section 4).

As is shown from the isolated DNA polymerase reaction the incorporation of approximately 200 nmoles deoxynucleotides is total and exhausts substrate nucleoside 5'-triphosphates supplied to the system. This is equivalent to approximately an 8-fold increase of primer (DNA') for the sequential reaction over that in the isolated DNA-primer → RNA assay. In this case the DNA_I-primer poly(dA-dT) concentration was of 0.2 units (approximately 24 nmoles). In the previous assay where a 20-fold increase was observed (Section 3.3.2) 0.1 units of initial primer was used. This discrepancy can be accounted for by the fact that both 0.1 and 0.2 units of poly(dA-dT) saturate the assay conditions used (see Figure 11).

The only other variables (besides primer concentration) in the RNA polymerase assays of the two systems is the presence of small free amounts of unincorporated deoxynucleotides and the inactivated DNA polymerase in the sequential system. However, the presence of deoxynucleotides in a complete RNA polymerase assay appears not to affect the incorporation of (^{14}C)AMP into RNA. This was illustrated in an experiment in which a full complement of deoxynucleotides was present, see this Section (4). In conclusion it appears that a valid sequential system of a DNA polymerase reaction followed by an RNA polymerase assay has been developed which obviates first having to isolate the DNA product (DNA') which primes for the RNA synthesis.

Table 12. The DNA and RNA polymerase reactions: Sequential and Isolated.

Assay Type	(^{14}C) AMP Incorporated (nmoles/assay) into RNA				
	Assay Tube 1		Assay Tube 2		Mean
Sequential : DNA_t-primer → DNA'→ RNA	2.9,	3.5	7.97,	6.45	7.21 (of assay 2 only)
Sequential : DNA_t-primer → DNA'→ RNA but DNA polymerase reaction followed by an incubation of 45°/60 min. prior to the RNA polymerase assay.	6.3,	8.2	6.8,	5.7	6.8
Sequential Control : DNA_t-primer → DNA'→ RNA	0.6,	0.58	0.53,	0.56	0.57
Isolated : DNA-primer → RNA	2.41,	2.41	2.1	1.7	2.2
Isolated Control :	0.31,	0.28	0.31,	0.32	0.31

Assay Type	(^{3}H) dTMP Incorporated (nmole/assay) into DNA				
	Assay Tube 1		Assay Tube 2		Mean
Isolated : DNA-primer → DNA	114.1,	102.0	108.9	112.9	109.5
Isolated Control :	1.26	0.98	1.17,	1.09	1.13

For legend see following page.

Legend to Table 12.

The components of the reaction mixtures were made up as described in Section 2.5.1 D Tables 6, 7, 8 and 9.

The components for the reaction mixtures containing DNA polymerase were in 0.2 ml and contained SM, 0.2 units of poly(dA-dT), and 0.5mM each of dATP and dTTP (or (^{3}H)dTTP), and 5.3 units of M. lysodeikticus DNA polymerase.

Incubation was carried out at 37°C for 180 minutes.

The components for the reaction mixtures containing RNA polymerase were in 0.3 ml and contained SM, 0.2 units of poly(dA-dT), 0.53mM each of UTP and (^{14}C)ATP, and 0.413 units of E. coli RNA polymerase.

The assay conditions for the sequential reaction were initially those of the DNA polymerase reaction.

Final RNA polymerase assay conditions were obtained on the addition of a Mg^{2+} and Mn^{2+} solution, 0.53mM each of UTP and (^{14}C)ATP and 0.413 units of E. coli RNA polymerase.

Incubation was carried out at 37°C for 30 minutes.

The specific radioactivities of (^{14}C)ATP and (^{3}H)dTTP were 1527 and 762 c.p.m./nmole respectively. Controls were not incubated.

(3) Does the precipitate formed after the DNA polymerase reaction affect the analysis of the subsequent RNA polymerase assay in the sequential system?

After addition of Mg^{2+} and Mn^{2+} required for the RNA polymerase reaction in the sequential system a precipitate formed (see this Section (2)). In order to investigate whether this precipitate affected the analysis of aliquots, taken for measurement of (^{14}C)AMP incorporation into RNA, the following experiment was performed on samples taken from experiments described in Table 19 and 14(2). After addition of BSA and mixing at the end of the incubation period two 0.05 ml samples of each assay were taken for analysis in the usual fashion. The remainder of the reaction mixture was centrifuged at 1000g for 5 minutes in a table model centrifuge at 4°C. A sediment was only observed in the samples containing DNA polymerase and the RNA polymerase additions. After centrifugation two 0.05ml supernatant samples were taken for analysis from each assay. The results are shown in Table 13. It can be seen that the results obtained from centrifuged and non-centrifuged samples were indistinguishable. Therefore it was concluded that the precipitate did not interfere with the sampling of aliquots of reaction mixture onto the discs.

(4) The effect of the poly(dA-dT)-primer and deoxynucleotides on poly(A-U) synthesis in the sequential system

These experiments were carried out in order to answer three questions:

(a) To what extent does the poly(dA-dT)-primer used in the DNA_1-primer → DNA' reaction contribute to the synthesis of RNA?

i.e. DNA_1-primer → DNA' → RNA

Table 13. A comparison of (^{14}C)AMP incorporation into the RNA product of the *sequential system, before and after* centrifugation of the incubated reaction mixture.

Relative proportions of dTTP and BrdUTP in the reaction mixture (poly(dA-dT)-primed)		(^{14}C)AMP incorporated (nmoles/assay)	
% dTTP	% BrdUTP	*Samples* taken after centrifugation	*Samples* taken before centrifugation
100	-	5.69	5.66
75	25	3.10	3.24
50	50	3.65	3.53
25	75	2.54	2.67
0	100	2.12	2.42
* 100	-	2.82	3.10
** 100	-	0.35	0.22

* No DNA polymerase (taken from Table 14, Experiment (2)).

** Control was not incubated.

All other values were taken from Table 19.

The components of the *reaction mixtures were as* described in the corresponding Tables 14(2) and 19, and the text, Section 3.3.2(3).

(b) Do deoxynucleotides present in the reaction mixture during the transcription of DNA'-primer to RNA competitively interfere with the incorporation of ribonucleotides into RNA?

(c) Can the sequential system be utilized without an initial DNA-primer? i.e. by forming DNA product (DNA') for transcription in an unprimed system (no primer → DNA' → RNA.)

In order to answer the above questions, the sequential system of DNA polymerase and RNA polymerase reactions containing all the reaction mixture components were incubated simultaneously with similar reactions which lacked either DNA polymerase or initial primer. Reactions were also performed in which BrdUTP replaced dTTP as substrate.

The results of this study are shown in Table 14 and are discussed in sequence below. The complete reaction system consisted of the poly(dA-dT)-primed DNA polymerase reaction followed by the RNA polymerase additions and synthesis of poly(A-U). (^{14}C)AMP incorporation into poly(A-U) was measured.

Assay type (i) The results of experiment (1) and (2) are of the standard reaction mixtures containing all the components of the sequential system. These values are the means of the figures quoted for each tube in Tables 17 and 19 respectively.

Assay type (ii) DNA polymerase was the only component eliminated from the sequential system. As there is no polydeoxynucleotide synthesis in the absence of DNA polymerase (Table 15), the ribonucleotide incorporated into RNA product was due only to that directed by the initial 0.1 unit of poly(dA-dT)-primer. A full complement of deoxynucleotides was thus present during poly(A-U) synthesis. It can be seen from the results that the ribonucleotide incorporation is

similar to although slightly higher than an average RNA polymerase assay in the sequential system. Compare with Table 12, where 2.2 nmoles of (^{14}C)AMP were incorporated into RNA. It can therefore be concluded that the presence of deoxynucleotides appear not to appreciably interfere with the DNA' → RNA reaction.

Assay type (iii) As poly(dA-dT) was eliminated from the DNA polymerase reaction of the sequential system, the reaction was thus unprimed. Therefore, only unprimed synthesis of poly(dA-dT) could take place. However, poly(dA-dT) was added to the subsequent reaction for DNA' → RNA. Thus poly(A-U) synthesis was due to transcription on unprimed poly(dA-dT) (DNA') and the 0.1 units of poly(dA-dT) added for the RNA polymerase assay only. The results show that for Experiment (2) there appears to be no difference in the nucleotide incorporation. However, in experiment (1) the incorporation is much higher and similar to that of assay type (i). This gave rise to the query - is the amount of unprimed synthesis constant in this system? (See page 85)

Assay type (iv) In this sequential system no poly(dA-dT) primer was added at all, thus the primer for poly(A-U) synthesis was only derived from that formed during unprimed synthesis of poly(dA-dT). The results indicate that there was sufficient unprimed polymer formed for poly(A-U) synthesis to occur.

It seems apparent from these preliminary studies of unprimed DNA synthesis in the sequential reactions, that further investigation of this phenomenon in the sequential system was necessary. These experiments are described in the following two sections.

Table 14. The effect of the initial primer on poly(A-U) synthesis

Assay Type	Assay conditions	(^{14}C)AMP incorporated (nmoles/assay)	
		Experiment (1)	Experiment (2)
(i)	a. Complete dTTP	5.79, 5.85	6.02, 5.29
	b. Control	0.45, 0.42	0.22, 0.21
(ii)	Minus DNA polymerase	2.85, 2.91	3.02, 3.19
(iii)	Minus poly(dA-dT) as initial primer in the DNA polymerase reaction mixture, but added with RNA polymerase additions	5.06, 5.05	2.83, 2.96
(iv)	Minus poly(dA-dT)	-	1.64, 1.54

For legend see following page.

Legend to Table 14

All values were subtracted from the mean of the control values shown in assay type (i) b. The control tubes were not incubated.
The results are a mean value of 2 samples each.
The components of the reaction mixtures for experiments (1) and (2) were made up as described in Section 2.5.1 D, Tables 6, 7, 8 and 9.
The DNA polymerase reaction mixture contained 0.2 ml SM, 0.1 unit of poly(dA-dT), 0.5mM each of dATP and dTTP, and 5.7 units of M. lysodeikticus DNA polymerase for experiment (1) and 5.3 units of E. coli DNA polymerase for experiment (2).
For assay types (ii) and (iii) the pyrimidine nucleotide was dTTP only.
Incubation was carried out at 37°C for 180 mins.
For the subsequent RNA polymerase reaction the following components were added to give a final volume of 0.3 ml, Mg^{2+} and Mn^{2+} solution, 0.53mM each of UTP and (^{14}C)ATP and 0.413 unit of E. coli RNA polymerase.
Incubation was carried out at 37°C for 30 minutes.
The specific radioactivity of the (^{14}C)AMP for experiment (1) and (2) were 1710 and 1878 c.p.m./nmoles respectively.

(5) Primed and unprimed synthesis of the copolymers (dA-dT) and (dA-BrdU) in a 180 minute DNA polymerase reaction

DNA polymerase assays were designed to investigate the possibility of synthesizing poly(dA-dT), poly(dA-BrdU) and substituted variations of these polymers in an unprimed system for subsequent use as primers in poly(A-U) synthesis. Deoxynucleotide incorporation in a primed system was compared with that of an unprimed system for a 180 minute incubation. The reaction for the primed synthesis of DNA was of the DNA_1-primer → DNA' type and for the unprimed synthesis DNA_1-primer was excluded. In both cases the incorporation of (^{3}H)dAMP into the DNA product was measured. The results are shown in Table 15. From these results it can be seen that the deoxynucleotide incorporation in a primed reaction is almost the same within an experiment whether the pyrimidine nucleotide is dTTP or BrdUTP. The priming ability of poly(dA-BrdU) appears to be greater than that of (dA-dT), and this observation will be discussed in Section 4. In the unprimed assays the incorporation is inconsistent within an assay type, and, between assay types containing either dTTP or BrdUTP.

(6) Time course of unprimed poly(dA-dT) and poly(dA-BrdU) synthesis over 180 minutes DNA polymerase incubation

In order to investigate the lag period and course of synthesis of the (dA-dT) and (dA-BrdU) copolymers in a DNA polymerase reaction, the synthesis of the polymers was followed over the 180 minute incubation period. In order to investigate the limits of unprimed synthesis in the system in most cases a lower concentration of DNA polymerase was employed than was used in previous experiments. The results are shown in Table 16 and

Figure 16. Four assays were performed and are denoted (a), (b), (c) and (d). It should be noted that the assay volumes were increased four-fold over those of the previous experiments to allow frequent time sampling. It can be seen that there is very little unprimed synthesis within the first 30 minutes of incubation. This lag appears to be independent of the amount of DNA polymerase added to the reaction mixture within the range of 8-20 units/assay. The lag is also independent of whether the pyrimidine nucleotide is dTTP or BrdUTP.

Radding and Kornberg (1962), have defined the lag period as the time required for 10% utilization of the substrate. In these assays it corresponds to the synthesis of 80 nmoles of poly(dA-dT) or (dA-BrdU). It is apparent that the lag period of these assays is not consistent. See Figure 16 and Table 16. Because of this variability unprimed synthesis of DNA was not used for the study of poly(A-U) synthesis in the sequential reaction system.

Table 15. Primed and unprimed synthesis of the copolymers (dA-dT) and (dA-BrdU)

Primer	Pyrimidine Nucleotide	(^{3}H)dAMP Incorporated (nmoles/assay)					
0.1 unit	100%	Experiment (1)			Experiment (2)		
		Assay tubes			Assay tubes		
		1	2	3	1	2	3
Poly(dA-dT)	dTTP	83.2	83.5		54.4	55.6	
	BrdUTP	88.5	67.5		63.4	69.0	
*	dTTP			-			0.48
Poly(dA-BrdU)	dTTP			-	75.2	73.2	
**	dTTP			-			0.64
	BrdUTP			-	96.2	85.3	
*	BrdUTP			-			0.66
No primer	dTTP	34.4	87 8		6.9	8.5	
	BrdUTP	67.4	24.2	6.6	24.24	0.24	
**	TTP			0.6			

* control no DNA polymerase

** control no incubation

The results are a mean value of two samples for each assay tube and have been subtracted from their respective control values which are each a mean of 4 samples. The components of the reaction mixtures were made up as described in Section 2.5.1 D, Table 6, and contained in 0.2 ml, 5M, 0.1 units poly (dA-dT) or (dA-BrdU), 0.5mM each of (^{3}H)dATP and dTTP (and or BrdUTP) and 5 units of E. coli DNA polymerase. The specific radioactivities of the (^{3}H)dATP for Experiment (1) and (2) were 783 and 682 c.p.m./nmole respectively. Incubation was carried out at 37^{0}C for 180 minutes.

Table 16. A comparison of primed and unprimed synthesis of (dA-dT) and (dA-BrdU) copolymers

	Time (min)	(^{3}H)dAMP incorporated (nmoles/assay) (a)	(b)	(c)	(d)
	15	0	-	-	17
	30	4	0.9	3	33
	45	-	-	-	50
	60	15	6.0	20	63
	75	-	-	-	90
	90	43	16	78	113
	105	-	-	-	135
	120	75	30	137	153
	135	92	37	166	159
	150	111	45	192	185
	165	121	55	222	195
	180	147	65	252	223
Lag period*	Time (min)	90	140	70	37

Experiment (a), (b) and (c) were unprimed

Experiment (d) was primed with 0.4 units of poly(dA-dT)

The reaction mixtures contained in 0.8 ml

64μmoles Tris-HCl buffer pH 7.9

2.8μmoles of $MgCl_2$

0.84μmoles of ETSH

400 nmoles of dTTP in (a), (c) and (d)

400 nmoles of BrdUTP in (b)

400 nmoles of (^{3}H)dATP with a specific activity of 650 c.p.m./nmole

E. coli DNA polymerase 8.3 units in (a) and (b) and 20 units in (c) and 7.9 units in (d).

0.05 ml of each assay was taken out at the indicated time intervals for analyses as described in Table II.

* Lag period defined as the time required for 10% utilization of the substrate, corresponding to an incorporation of 40 nmoles of (^{3}H)dAMP/assay.

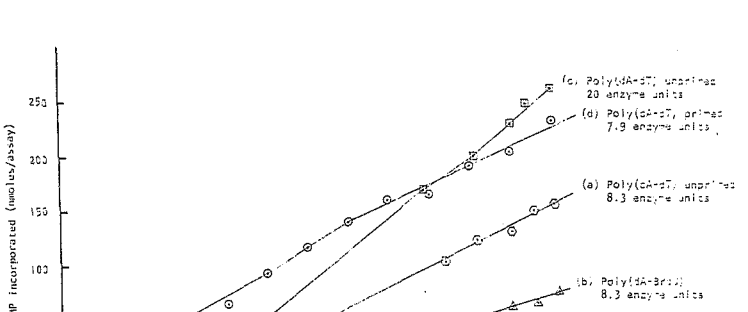


Figure 16 For legend see Table 16.

3.3.3 Poly(dA-dT) and poly(dA-BrdU) synthesis and their transcription to poly(A-U) in the reactions DNA_I-primer → DNA' → RNA

The object of this experiment was to determine the ability of poly(dA-dT) and poly(dA-BrdU) to be transcribed to poly(A-U) in an RNA polymerase reaction of the sequential system.

The reaction mixtures of the sequential system were described in Section 2.5.1 D and also in Section 3.3.2(2) and Figure 14.

The initial DNA-primer of the DNA polymerase reaction was either purchased poly(dA-dT) or poly(dA-BrdU). The pyrimidine substrate was either dTTP or BrdUTP and the purine substrate, dATP. Thus the DNA product was a polymer of alternating adenine and thymidine or bromodeoxyuridine units (Schachman et al. 1960, Inman and Baldwin, 1962).

From the results shown in Table 17 it can be seen that poly(dA-BrdU) is consistantly less able to prime RNA polymerase action as efficiently as poly(dA-dT). In later experiments the copolymers (dA-dT) and (dA-BrdU) were also transcribed in an isolated RNA polymerase assay. See Section 3.4.

3.3.4 The effect of DNA-primers containing varying amounts of bromodeoxyuridine on poly(A-U) synthesis

The aim of these experiments was to study the ability of DNA containing increasing amounts of bromodeoxyuridine to prime poly(A-U) synthesis. The design of the assays was described in Section 3.3.1 and Figure 14, and consisted of a sequential system of reactions, DNA_I-primer → DNA' → RNA. The initial DNA primer (DNA_I-primer) was either the copolymer of (dA-dT) or (dA-BrdU). The pyrimidine substrate dTTP was decreased in the DNA polymerase reaction mixtures and

Table 17. Poly(dA-dT) and poly(dA-BrdU) synthesis and their transcription to poly(A-U)

Initial Primer	Pyrimidine Nucleotide	(^{14}C)AMP incorporated (nmoles/assay)			
(0.1 units)	(100%)	Assay tube 1	Assay tube 2	Mean	Standard deviation
Poly(dA-dT)	dTTP	5.45, 6.13	6.43, 5.27	5.82	0.52
	BrdUTP	5.22, 4.74	4.41, 4.83	4.80	0.33
Poly(dA-BrdU)	dTTP	8.43 7.12	7.14, 7.78	7.63	0.67
	BrdUTP	4.81, 5.93	5.04, 5.61	5.35	0.57
Poly(dA-dT) (Control)	dTTP	0.45, 0.45	0.39, 0.44	0.44	0.00

All values were subtracted from the mean of the control values shown in the last line. The control tubes were not incubated.

The components of the reaction mixtures were made up as described in Section 2.5.1 D Tables 6, 7, 8 and 9. The DNA polymerase reaction mixture contained in 0.2 ml, SM, 0.1 unit of poly(dA-dT) or poly(dA-BrdU) (batch M_{10}), 0.5mM each of dATP and dTTP (or BrdUTP) and 5.7 units of M. lysodeikticus DNA polymerase. The BrdUTP used was that prepared by the author. Incubation was carried out at 37°C for 180 minutes. For the subsequent RNA polymerase reaction the following components were added to give a final volume of 0.3 ml; Mg^{2+} and Mn^{2+} solution, 0.53mM each of UTP and (^{14}C)ATP and 0.413 unit of E. coli RNA polymerase.

Incubation was carried out at 37°C for 30 minutes.

The specific radioactivity of the (^{14}C)ATP was 1710 c.p.m./nmole.

replaced by a corresponding increase of its analogue BrdUTP. The relative proportions of dTTP and BrdUTP are shown in the Tables 18-21. Thus in each DNA polymerase reaction the product DNA' contained dTMP and BrdUMP alternating with dAMP in proportion to the relative concentrations of substrate dTTP and BrdUTP. These polymers containing increasing amounts of BrdUMP were tested for their ability to transcribe to poly(A-U), as described in the previous experiment.

Several closely related experiments were carried out and the results individually shown in Tables 18, 19, 20 and 21 and Figures 17, 18, 19 and 20, and compared in Table 22. The reaction mixtures of the experiments were made up as described in Section 2.5.1 D. Slight variations existed in the concentrations of DNA_I-primer and DNA polymerase added to the experiments described in Tables 18-21. These variations are listed in Table 22. One important modification is shown in Table 20 where an experiment using only 2.0 units of polymerase is reported. This particular experiment was an attempt to minimize any possible effect of unprimed DNA synthesis.

From the results it can be seen that there is a relative decrease in the ability of the synthesized DNA to prime RNA polymerase as its bromodeoxyuridine concentration increases. The decrease appears to be sharpest between 0 and 25% bromination of the DNA primer. See Figures 17-20.

3.3.5 A study of the sequential system using a reduced DNA polymerase reaction period

The transcription of DNA-primers containing increasing amounts of bromodeoxyuridine was investigated as described in the previous Section 3.3.4, but with a DNA polymerase incubation period of 30 minutes, in place of the previously used 180 minute incubation period of the DNA_I-primer → DNA' reaction.

Tables 18-21. The transcription of DNA-primers containing increasing amounts of bromodeoxyuridine

Table 18

Relative proportions of dTTP and BrdUTP in the reaction mixture (poly(dA-dT)-primed)		(^{14}C)AMP incorporated (nmoles/assay)			
% dTTP	% BrdUTP	Assay 1	Assay 2	Mean	Standard Deviation
100	-	4.77, 4.69	4.96, 4.19	4.65	0.33
75	25	3.57, 3.58	3.94, 3.67	3.69	0.17
50	50	3.14, 3.07	3.78, 3.68	3.42	0.36
25	75	3.20, 2.82	3.37, 2.88	3.07	0.26
-	100	2.64, 2.44	3.3, 2.44	2.71	0.41
*100	-	0.42, 0.38	0.38, 0.34	0.38	0

Table 19

Initial Primer 0.1 units	Relative proportions of dTTP and BrdUTP in the reaction mixture		(^{14}C)AMP incorporated (nmoles/assay)			
	% dTTP	% BrdUTP	Assay 1	Assay 2	Mean	Standard Deviation
poly(dA-dT)	100	-	5.83, 6.20	5.23, 5.34	5.66	0.44
	75	25	3.28, 3.36	3.21, 3.10	3.24	0.10
	50	50	3.05, 2.94	4.47, 3.65	3.53	0.70
	25	75	2.92, 2.51	2.55, 2.68	2.67	0.20
	-	100	2.23, 2.46	2.57, 2.41	2.42	0.14
*	100	-	0.22, 0.22	0.22, 0.20	0.22	
Poly(dA-BrdU)	100	-	5.32, 5.39	5.43, 6.84	6.00	0.99
	-	100	0.68, 0.92	3.59, 3.30	2.13	4.07

* Control

For legend see Page 96.

Table 20

Relative proportions of dTTP and BrdUTP in the reaction mixture (poly(dA-dT)-primed)		(^{14}C)AMP incorporated (nmoles/assay)				
% dTTP	% BrdUTP	Assay 1	Assay 2	Assay 3	Mean	Standard Deviation
100	-	5.13, 5.11	4.94, 4.45	4.46, 4.39	4.75	0.31
75	25	4.86, 4.61	4.12, 3.72	4.22, 4.34	4.31	0.43
50	50	3.53, 4.11	3.55, 3.55	3.55, 3.16	3.55	0.31
25	75	3.11, 3.40	2.90, 2.75	3.46, 2.79	3.07	0.30
-	100	3.10, 3.08	2.89, 2.75	2.91, 3.27	3.00	0.17
*100	-	0.33, 0.33	0.30, 0.36	- -	0.33	
* -	100	0.29, 0.34	0.33, 0.31	- -		

Table 21

Relative proportions of dTTP and BrdUTP in the reaction mixture (poly(dA-BrdU)-primed)		(^{14}C)AMP incorporated (nmoles/assay)				
% dTTP	% BrdUTP	Assay 1	Assay 2	Assay 3	Mean	Standard Deviation
100	-	6.68, 7.03	4.7, 4.32	5.92, 5.68	5.7	1.06
75	25	4.82, 5.05	5.08, 4.43	- -	4.85	0.3
50	50	4.66, 4.25	4.47, 4.47	- -	4.46	0.17
25	75	3.69, 4.14	4.80, 4.25	- -	4.22	0.45
-	100	2.66, 4.45	4.06, 3.61	4.32, 4.87	4.0	0.77
*100	-	0.28, 0.27	- -	- -	0.27	
* -	100	0.29, 0.23	- -	- -		

* Control

For legend see Page 96.

Table 22. *Comparison of the mean values taken from Tables 17-21 of* the transcription of DNA-primers containing increasing amounts of bromodeoxyuridine

Relative proportions of dTTP and BrdUTP in the reaction mixture		(^{14}C)AMP incorporated (nmoles/assay) Mean values of results shown in Tables						
% dTTP	% BrdUTP	18	19	19	17	17	20	21
100	-	4.65	5.66	6.00	5.82	7.63	4.75	5.71
75	25	3.70	3.24	-		-	4.31	4.85
50	50	3.42	3.53	-		-	3.55	4.46
25	75	3.07	2.67	-	-	-	3.07	4.22
-	100	2.70	2.42	2.13	4.80	5.35	3.00	4.00
Variable components the reaction mixture								
Units of poly(dA-dT)		0.2	0.1		0.1		0.1	
Units of poly(dA-BrdU)				0.1		0.1		0.1
Units of *M. lysodeikticus* DNA polymerase		5.7			5.7	5.7		
Units of *E. coli* DNA polymerase			5.3	5.3			5.3	2.0
Source of BrdUTP		pre-pared	pre-pared	pre-pared	pre-pared	pre-pared	pur-chased	pur-chased
Specific radioactivity of the (^{14}C)ATP (c.p.m./nmole)		2060	1878	1878	1710	1710	17	1120

For legend see Page 96.

Legend to Tables 18-22 and Figures 17-20

Transcription of DNA-primers containing increasing amounts of bromodeoxyuridine.

All the values were subtracted from the mean control value shown in the last line of each table. The control tubes were not incubated. The components of the reaction mixtures were made up as described in Section 2.5.1 D Tables 6, 7, 8 and 9, except for the variations listed in Table 22.

The DNA polymerase reaction mixture contained in 0.2 ml; 5M, 0.5mM each of dATP and dTTP (and or BrdUTP), 0.2 units of poly(dA-dT) were used in Table 18, 0.1 units in Tables 17, 19, 20, 0.1 units of poly(dA-BrdU) were used in Tables 17, 19, and 21, 5.7 units of M. lysodeikticus DNA polymerase or 5.3 units of E. coli DNA polymerase except for 2.0 units of E. coli DNA polymerase in that of Table 21. Incubation was carried out at 37°C for 180 minutes. For the subsequent RNA polymerase reaction the following components were added to give a final volume of 0.3 ml; a Mg^{2+} and Mn^{2+} solution, 0.53mM each of GTP and (^{14}C)ATP and 0.413 unit of E. coli RNA polymerase. Incubation was carried out at 37°C for 30 minutes.

The specific radioactivity of the (^{14}C)ATP used for each experiment is listed in Table 22, as well as the source of the BrdUTP used.

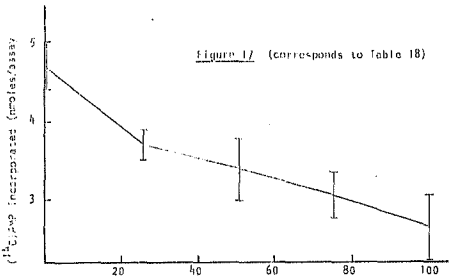


Figure 17 (corresponds to Table 18)

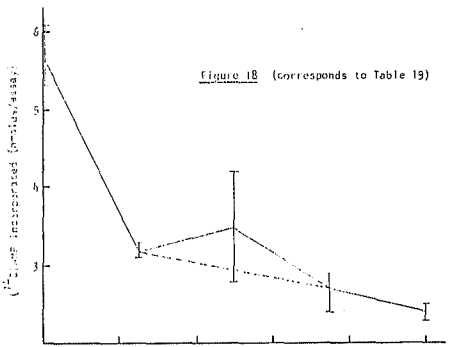


Figure 18 (corresponds to Table 19)

For legend see Page 96.

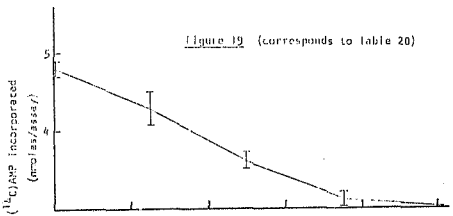


Figure 19 (corresponds to table 20)

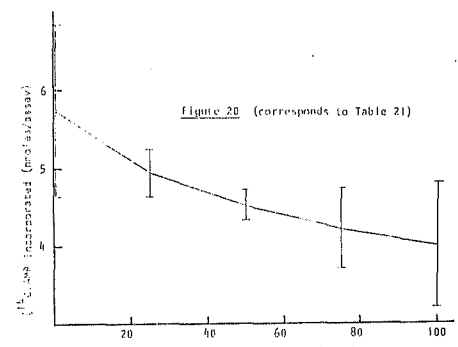


Figure 20 (corresponds to Table 21)

For legend see page 96.

A preliminary examination of the system was necessary to study the effect of various primer concentrations and incubation periods on the synthesis of the DNA polymers in the DNA_i-primer → DNA' reaction. The DNA-primer was poly(dA-dT) and the incorporation of (^{3}H)dAMP into poly(dA-dT) product was measured. The results are shown in Table 23 and Figure 21. It can be seen that there is an increase in deoxynucleotide incorporation with increasing primer concentration until 0.05 units when synthesis levels off. When 0.02 units of poly(dA-dT)-primer was used for a thirty minute incubation period 22.4 nmoles of poly(dA-dT) were synthesized. This represents an approximate 9-fold increase of product over primer, which is of similar magnitude to that obtained in experiments using the 180 minute DNA polymerase reaction period. The following experiment was carried out using these incubation conditions.

A 'double label' analysis of the sequential system,

DNA_i-primer → DNA' → RNA

In this analysis of the sequential system, the DNA synthesized (DNA') was isotopically labelled with ^{3}H and the poly(A-U) transcription product was labelled with ^{14}C. The results are shown in Tables 24a and b. The assays are similar to those described in the preceding Section 3.3.4 for the DNA polymerase reaction, using only 0.02 units of poly(dA-dT) and an incubation time of 30 minutes. The deoxynucleotide concentrations were reduced to 20 nmoles/assay to approach exhaustion of deoxynucleotide substrates after 30 minutes. The subsequent DNA' → RNA reaction was not altered. Besides the sequential DNA polymerase and RNA polymerase assays separate DNA polymerase and RNA polymerase assays were performed as controls for the above system.

Table 23. The effect of increasing primer concentrations and incubation periods on the synthesis of poly(dA-dT)

Poly(dA-dT)-primer (unit)	(^{3}H)dAMP incorporated (nmoles/assay) in	
	30 min.	60 min.
0.02	11.2	31.2
0.05	16.3	41.7
0.1	19.5	43.0
0	0.3	1.6

The components of the reaction mixtures were as described in Section 2.5.1 D Table 6 and contained in 0.2 ml, 5M, 0-0.1 unit of poly(dA-dT) as shown, 0.5mM each of dTTP and (^{3}H)dATP and 5 units of E. coli DNA polymerase.

The specific radioactivity of the (^{3}H)dATP was 440 c.p.m./nmole.

Incubation was carried out at 37°C.

The control tube was not incubated and no primer was added.

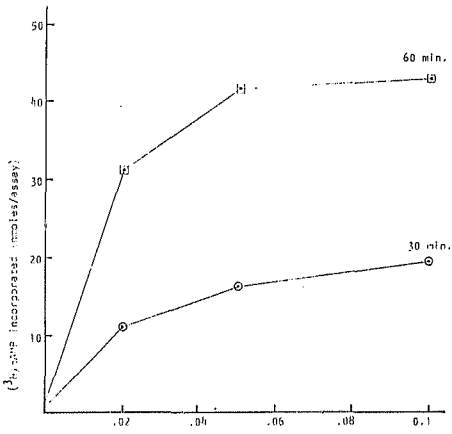


Figure 21 The effect of increasing primer concentrations and incubation periods on the synthesis of poly(dA-dT) (corresponds to Table 23).

The incorporation of deoxynucleotide in the separate DNA polymerase assay DNA_1-primer → DNA' was the same as that for the DNA polymerase reaction of the sequential system. This confirms that the action of DNA polymerase was eliminated during the RNA polymerase incubation period. The rate of synthesis of DNA polymer (DNA') is independent of the pyrimidine nucleotide whether it be dTTP, its analogue, or a mixture of both, see Table 24a.

From Table 23b it can be seen that the amount of poly(A-U) synthesized in the separate assay is substantially less than that synthesized in the sequential system. The decrease in poly(A-U) synthesis with increasing bromodeoxyuridine incorporation into the DNA' is not as marked as in the previous experiments. See Figure 22. The inconsistency in these results will be discussed in Section 4.

3.4 Transcription of poly(dA-dT) and poly(dA-BrdU)

The availability of a commercial preparation of the analogue of poly(dA-dT), poly(dA-BrdU) made it possible to measure directly the priming ability of poly(dA-BrdU) to prime RNA polymerase action. Thus poly(dA-dT) and poly(dA-BrdU) were used as primers for poly(A-U) synthesis in an isolated RNA polymerase incubation mixture. The results are shown in Table 25. The components of the reaction mixture for (a), (b) and (d) differ slightly from those of (c), (e) and (f). The former group contained the assay mixture used in the original RNA polymerase assay Section 3.1.4 while the latter contained the assay mixture adapted for use in the sequential system. However, this difference did not effect the results.

Another important difference among these experiments was the fact that 2 different preparations of poly(dA-BrdU), both purchased from General Biochemicals, were used. These are described in the legend of Table 25. It is apparent that batch M_{10} exhibits considerably lower ability to prime RNA polymerase than does poly(dA-dT) while batch M_4 exhibits a priming capacity similar to that of poly(dA-dT). These conflicting observations will be discussed in Section 4.

Table 24 (a) and (b). A double label analysis of the sequential system

Table 24 (a) The synthesis of DNA polymers

Relative proportions of dTTP and BrdUTP in the reaction mixture (poly(dA-dT)-primed		(^{3}H)dAMP incorporated (nmoles/assay)				
% dTTP	% BrdUTP	Assay 1		Assay 2		Means
100	-	11.92,	14.46	13.89,	12.80	13.27
75	25	14.03,	12.62	13.80,	12.99	13.37
50	50	13.13,	12.75	12.95,	11.82	12.67
25	75	11.87,	13.38	-	-	11.63
-	100	9.35,	13.82	12.89,	11.39	11.87
* 100	-	1.01,	0.75	0.66,	0.79	0.80
** 100	-	13.33,	10.62	11.32,	11.87	11.89

* Control

** DNA polymerase assay only

For legend see Page 107.

Table 24 (b) The synthesis of poly(A-U)

Relative proportions of dTTP and BrdUTP in the reaction mixture (poly(dA-dT)-primed		(^{14}C)AMP incorporated (nmoles/assay)					
% dTTP	% BrdUTP	Assay 1		Assay 2		Mean	Standard Deviation
100	-	2.30,	2.63	2.54,	2.37	2.47	0.14
75	25	2.40	2.29	2.47,	2.63	2.45	0.14
50	50	2.51,	2.37	2.43,	2.19	2.38	0.14
25	75	2.13,	2.15	-	-	2.15	0
-	100	1.93,	2.95	2.65,	2.33	2.46	0.43
* 100	-	0.14,	0.10	0.10,	0.12	0.12	0
** 100	-	1.20,	1.15	1.16,	1.36	1.22	0

* Control

** RNA polymerase assay only

For legend see Page 107.

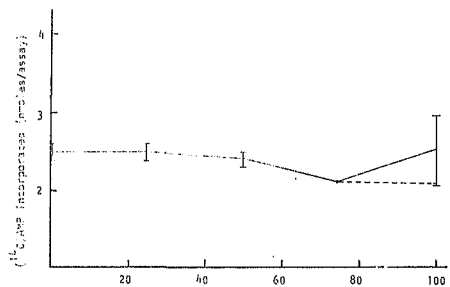


Figure 22 The synthesis of poly(A-U) from primers containing increasing amounts of BrdUrd. (corresponds to Table 24b)

For legend see page 107.

Legend to Table 24(a) and (b) and Figure 22

A double label analysis of the sequential system.

The components of the reaction mixtures were made up as described in Section 2.5.1 D Tables 6, 7, 8 and 9.

(a) The components for the isolated and sequential reaction mixtures containing DNA polymerase were in 0.2 ml and contained SM, 0.02 units of poly(dA-dT), 0.1mM each of (^{3}H)dATP and dTTP (and or BrdUTP) and 5 units of E. coli DNA polymerase.
Incubation was carried out at 37°C for 30 minutes.

(b) The components for the reaction mixtures containing RNA polymerase were in 0.3 ml and contained SM, 0.02 units of poly(dA-dT) 0.53mM each of UTP and (^{14}C)ATP and 0.48 units of E. coli RNA polymerase.
The assay conditions for the sequential reaction were initially those of the DNA polymerase reaction.
Final RNA polymerase assay conditions were obtained on the addition of a Mg^{2+} and Mn^{2+} solution, 0.53mM each of UTP and (^{14}C)ATP and 0.48 units of E. coli RNA polymerase.
Incubation was carried out at 37°C for 30 minutes.
The specific radioactivities of (^{3}H)dAMP and (^{14}C)AMP were 577 and 1.2×10^{2} c.p.m./nmole respectively.
The control tubes were not incubated. The mean value of these controls was subtracted from the results given for each assay tube.

Table 25. Comparison of the priming ability of the copolymers (dA-dT) and (dA-BrdU) in transcription

Primer	(^{14}C)AMP incorporated in 30 min. (nmoles/assay)					(^{14}C)AMP incorporated in 15 min. (nmoles/assay)	
	a+	c*	d*	e+	f*	a+	b+
Poly(dA-dT)	1.46	0.73	1.11	0.61	2.14	0.77	0.66
Poly(dA-BrdU)	0.02	0.85	1.0	0.72	2.29	0.01	0.02

\+ Results an average of 2 assays each

* Results an average of 3 assays each

The components of the reaction mixtures for (a), (b), (d) were as described in Section 2.1.5 C Table 5, and contained in 0.25 ml, SM, 0.2 units of poly(dA-dT) or poly(dA-BrdU), 0.64mM each of UTP and (^{14}C)ATP, and 0.3 units of RNA polymerase for (a) and (b) and 0.4 unit for (d).

The components of the reaction mixtures for (c), (e) and (f) were as described in Section 2.5.1 D Table 7 and contained in 0.3 ml, SM 0.2 units of poly(dA-dT) or poly(dA-BrdU), 0.53mM each of UTP and (^{14}C)ATP and 0.41 units of RNA polymerase for (c).

0.43 units for (e) and 0.5 units for (f).

As different preparations of poly(dA-BrdU) were used these are listed: (a) and (b) batch M_{10}

(c), (d), (e), (f), batch M_4

The (^{14}C)ATP had a specific activity of 1.6-1.9 x 10^3 c.p.m./nmole.

Incubation was carried out at 37°C.

4. DISCUSSION

The evidence presented in this study was the result of an attempt to elucidate the mode of inhibition of cell differentiation by BrdUrd.

On the assumption that this BrdUrd effect is a consequence of its incorporation into the DNA, it was suggested that differentiation, as opposed to cell division, is dependent on the presence of polydeoxythymidine clusters in the DNA. This suggests that polydeoxybromouridine regions which during replication are derived from polydeoxythymidine regions may not be capable of directing transcription through RNA polymerase*. DNA polymers with scattered BrdUrd units however may not appreciably effect the transcription process. As the proportion of BrdUrd in the DNA is increased it is suggested that its ability to prime RNA transcription decreases.

An *in vitro* system was designed to test this hypothesis. DNA polymerase reactions, which synthesized DNA polymers varying in BrdUrd content, were coupled to RNA polymerase transcribing systems. On subsequent incubation the amount of poly (A-U) synthesis was measured. This sequential system was described in Section 3.3 and was represented by the following reaction: DNA_1-primer $\xrightarrow{(a)}$ DNA' $\xrightarrow{(b)}$ RNA. Using poly (dA-dT) as the DNA_1-primer in reaction (a) polymers were formed containing increasing amounts of bromodeoxyuridine with correspondingly decreasing amounts of thymidine (Section 4.3). In this experimental design the fully substituted polymer was poly(dA-BrdU). These polymers were then transcribed using RNA polymerase in reaction (b).

* Poly(dT) sequences [illegible] be sites of control and initiation in the regulation of gene [illegible] ression required for the differentiated state. See page 27 and 32.

The main interpretation of the results of this investigation, is that BrdUrd incorporation into the DNA does decrease the efficiency of RNA synthesis. When poly(A-U) synthesis is directed by a poly(dA-BrdU) primer the decrease in (^{14}C)AMP incorporation into an acid insoluble product is between 20 to 60%.

In the following sections of this discussion the results, and the in vitro system of analysis used to obtain them are critically examined.

4.1 Nucleotide polymerase assay systems

The assay conditions used for both the M. lysodeikticus DNA polymerase and E. coli DNA polymerase were derived from the Tris-HCl buffer system used by Zimmerman (1966). These conditions were the same as those routinely used by Miles Laboratories for the assay of M. lysodeikticus DNA polymerase. The results in Figures 10 and 12 on the activity of these enzymes indicated a loss of 50-65% of the activity specified by the manufacturers. The assay conditions differed from those of the manufacturers only in the case of the E. coli DNA polymerase supplied by Boehringer-Mannheim Laboratories who used a phosphate buffer system. A possible explanation for these low figures was loss of activity during transport. However, the enzymes were supplied by air and the packaging usually contained some dry ice on arrival.

According to Richardson et al. (1964) the radioactivity rendered insoluble in a poly(dA-dT)-primed reaction, is proportional to the amount of E. coli enzyme added in the range 0.02 to 0.2 units. However Figure 10 shows that for M. lysodeikticus linearity between product formed and enzyme concentration was in the range of 0.08 to 0.04 units and figure 12 shows linearity in the range 0.02 to 0.75 units for E. coli enzyme.

Therefore, in effect, the enzyme concentration used for most assays was approximately 1 to 2 units and not 5 units as specified by the manufacturer (see section 3.1.2.). This however, does not affect the results and conclusions of this work as it is a comparative study. The concentration of DNA polymerase routinely used remained constant throughout except when other conditions were required for specific purposes.

The results in Figure 15 where there is no loss of product during the 400 to 1200 minute incubation period shows that the M. lysodeikticus DNA polymerase is free of DNAase activity. No similar studies were performed on the E. coli enzyme.

In Tables 11 and 12 it can be seen that the final deoxynucleotide incorporation often appears to be higher than the amount of deoxynucleotides initially available in the DNA polymerase reaction. This may be explained in terms of an error in the determination of the specific radioactivity of the labelled substrate deoxyribonucleoside 5'-triphosphate resulting in low counts. However, as this is a comparative study under constant assay conditions this problem is of little consequence.

The bacterial DNA polymerase assays were designed to determine primarily the degree of polydeoxynucleotide synthesis under defined conditions and not the activity of the enzyme per se. Thus optimal enzyme assay conditions were not of prime importance. The main aim was to produce different DNA species for use as primers in the coupled RNA synthesizing system (see Section 3.3).

The RNA polymerase enzyme assays were used to test the ability of the enzyme to transcribe the DNA products of the DNA polymerase reactions. The assay conditions for both reactions were chosen to facilitate the coupling of the RNA polymerase reaction to the preceding DNA polymerase system. It was only because of the higher ionic requirements of RNA polymerase for Mg^{2+} and Mn^{2+} that these reactions could be coupled, see Section 4.4.

4.2 The efficiency of bromodeoxyuridine substitution for thymidine during DNA replication

In order to check that the BrdUTP prepared for this study could substitute for dTTP during the synthesis of DNA, the experiment using calf thymus DNA polymerase and calf thymus DNA as primer was performed as described in Section 3.2 and Table 10.

From these results it appears that BrdUTP can replace dTTP in the synthesis of DNA with an efficiency around 90%. This is comparable with the results of other workers who used bacterial DNA polymerases with calf thymus DNA as primer. Bessman et al. (1958) found a 97% substitution using E. coli DNA polymerase. Okazaki and Kornberg (1964) observed a 95% BrdUMP replacement with B. subtilis DNA polymerase and a 93% replacement with the E. coli enzyme.

When both dTTP and BrdUTP were omitted from the reaction mixture, leaving only the other three deoxynucleotides, a 51-54% incorporation of (^{3}H)dCMP was observed (Table 10). This confirms that all four deoxynucleoside 5'-triphosphates are required for maximum enzyme activity. It also demonstrates that the BrdUTP synthesized during this work can substitute effectively for dTTP in an in vitro DNA polymerase reaction.

4.3 The synthesis of DNA polymers consisting of dAMP alternating with either dTMP or BrdUMP

Poly(dA-dT)-primed, poly(dA-BrdU)-primed and unprimed DNA polymerase reactions always yield DNA products in which dAMP alternates with dTMP(BrdUMP), see section 1.5.

Additional evidence relating to the alternating nature of these polymers was obtained from the DNA polymerase assays in which substrate concentrations were limiting, i.e. in those assays in which total substrate incorporation was achieved. For example in the last experiment described

in Table 12, where the incorporation of (^{3}H)dTMP was followed, 100% of the pyrimidine substrate was incorporated into product DNA, while in some of the experiments described in Table 15 (^{3}H)dAMP incorporation was followed, 80-100% of the purine substrate was incorporated into DNA. Thus it can be concluded from this evidence that in this system there is pproximately equal incorporation of purine and pyrimidine nucleotides into product DNA. This supports the contention that these DNA products consist of alternating sequences of dAMP and dTMP (BrdUMP).

Furthermore, from the experiments described in Table 15 it can be seen that the degree of incorporation of (^{3}H)dAMP is independent of the nature of the pyrimidine substrate, dTTP or BrdUTP.

These considerations concerning the nature of the product of the DNA polymerase reaction were substantiated in the double label experiment described in Section3, Table 24b. The magnitude of DNA synthesis is equivalent regardless of the proportions of dTTP and BrdUTP in the reaction mixture, within a reasonable margin of error.

However, this cannot be unequivocally established without performing nucleotide sequence analysis. Nevertheless, *it would seem reasonable* from the above evidence that the input of dTTP with respect to BrdUTP determines the relative proportions of *dTMP and BrdUMP in the DNA primer* for the RNA polymerase reaction.

Another aspect of *these results is that poly(dA-BrdU) appears* to be a slightly more efficient primer than poly(dA-dT), see Table 15. One *possibility is that this is due to structural variation*, in the commercially supplied polymers for example poly(dA-BrdU) may have been of lower molecular *weight and consequently may have had more free* 3' OH groups per unit amount of polymerase. DNA polymerase is well known to be stimulated *by these groups* (Davidson 1972). This is a possible explanation for the

greater priming capacity of poly(dA-BrdU). Unfortunately for reasons of quantity and cost it was not possible to structurally characterise the commercially supplied poly(dA-BrdU) and compare it with the structure of the poly(dA-dT) preparations.

It should also be noted that there is considerable variation between experiments of a similar kind, such as those described in Table 15 and 22. However, good agreement is found between duplicate assays within an experiment as seen in Table 15. This discrepancy between experiments is difficult to explain but presumably is largely due to variations which result from dealing with rather labile enzymes. Therefore despite this problem it would seem valid on the basis of the results presented to draw the conclusions made above.

4.4 RNA polymerase activity in the sequential system and in the isolated RNA synthesis system

In this section the tivity of RNA polymerase, in the sequential system DNA_1-primer →DNA' → RNA and the isolated reaction DNA → RNA, is discussed.

The results presented in Section 3.3.2 and Table 14(ii) illustrate that the presence of DNA polymerase and excess deoxynucleoside 5'-triphosphates in reaction (b) of the sequential system, do not significantly affect the activity of RNA polymerase. This observation is supported by Berg et al. (1965). They have shown that RNA synthesis primed by calf thymus DNA is unaffected by the presence of DNA polymerase and the four deoxynucleoside 5'-triphosphates.

It was only possible to couple the DNA polymerase and RNA polymerase reactions because RNA polymerase exhibits higher divalent cation requirements. According to Zimmerman (1966), the activity of DNA polymerase drops considerably with an increase in Mg^{2+} concentration to

10^{-2}M and an increase in Mn^{2+} concentration to 10^{-3}M. The divalent cation requirements of RNA polymerase are approximately 10^{-2}M for Mg^{2+} and 2×10^{-3}M for Mn^{2+}. At such high cation concentrations the activity of DNA polymerase should be negligible. This was substantiated by the double label experiment described in Section 3.3.5, Table 24a. No increase in DNA synthesis took place after the addition of the requirements for the RNA polymerase reaction and a further 30 minute incubation period, despite the presence of substrate deoxynucleoside 5'-triphosphates during that time. Furthermore, as reported in the results Section 3.3.2 a precipitate formed on the addition of the Mg^{2+} and Mn^{2+} ion requirements for the RNA polymerase reaction. This precipitate probably contained inactivated DNA polymerase.

The activity of RNA polymerase primed by poly(dA-dT) in the isolated RNA polymerase reaction was reasonably constant from experiment to experiment as shown in Tables 12, 14 (ii), 25, and Figure 13. It was frequently in the range of 1.0-3.0 nmoles (^{14}C)AMP incorporated per 30 minutes.

However, when RNA polymerase activity was followed in the sequential system there was an apparent increase in enzyme activity, for example in Tables 12, 14, 17-21 (^{14}C)AMP incorporation in the range 4.0-7.0 nmoles per 30 minutes was obtained. The isolated RNA polymerase system is primed by 0.1-0.2 units of poly(dA-dT) while the sequential system is primed by 0.1-0.2 units of poly(dA-dT) initial primer plus DNA' product synthesized during the RNA polymerase incubation. Thus the increase in RNA product in the sequential system may be attributed to the availability of a larger quantity of primer.

4.5 *The role of the initial primer in the synthesis of RNA in the sequential system*

The sequential system was developed to test the ability of the DNA' product to direct poly(A-U) synthesis. A problem inherent in this system was the possible role played by the initial DNA primer of the DNA polymerase reaction in priming the subsequent RNA polymerase reaction. In most experiments the nucleotide content of the initial primer differed from that of the DNA' product. Thus it was of critical importance to ensure that it did not significantly contribute to the synthesis of RNA.

The DNA polymerase reaction (a) was therefore designed to produce a great excess of product DNA' over initial primer. By this means the influence of the initial primer on RNA synthesis was minimized. An excess of product DNA over initial primer DNA in the range 8-20 fold was achieved by the use of long incubation periods or minimal initial DNA primer concentrations in the DNA polymerase reaction. These results are shown in Tables 11, 12 and 23.

Another approach was to attempt to completely eliminate the initial DNA primer by the use of an unprimed DNA polymerase reaction, i.e. no primer → DNA' → RNA. *It is well established that microbial DNA* polymerases are capable of synthesizing polydeoxynucleotide in the absence of DNA primer (Schachman et al. 1960). Preliminary experiments described in Table 14(iv) indicated that this might be a valid approach. However, subsequent experiments described in Table 15 demonstrated that the quantity of DNA product in unprimed reactions were variable. As shown in Table 15 there is no consistency in product synthesized even between two duplicate assays incubated for the same period.

This supports the observations of Radding and Kornberg (1962) in that the synthesis of polymers in an unprimed system is initiated by a number of enzyme catalyzed random syntheses of phosphodiester bonds. When a product able to prime polymer synthesis is formed rapid increase in reaction rate is observed.

Another feature of the unprimed reaction shown in Figure 16 and Table 4 is that poly(dA-dT) is more readily formed than poly(dA-BrdU). Thus it was concluded that unprimed DNA synthesis could not be employed in this investigation.

A further attempt to reduce the influence of the initial-primer in reaction (b) was made by using poly(dA-BrdU) as the initial DNA-primer. This aspect is discussed in the Section 4.7.

4.6 The effect of DNA-primers containing bromodeoxyuridine on poly(A-U) synthesis.

In the previous sections it was shown that the DNA polymers which are formed in reaction (a) of the sequential system and transcribed in reaction (b) consist of dTMP and or BrdUMP alternating with dAMP. It was also shown that the proportions of dTMP and BrdUMP in the DNA' product depends on the proportions of their respective deoxynucleoside 5'-triphosphates in the DNA polymerase reaction mixture. In addition it has been demonstrated that the amount of DNA' product formed is constant for all assays within an experiment.

The hypothesis underlying this work was that these DNA products would then prime RNA polymerase to a degree which was inversely proportional to their BrdUrd content. From the results in Tables 17-21 and their corresponding figures, Figure 17 to Figure 20, and table 24b (Figure 22) it is apparent that when the mean values of each table are compared there is an obvious decrease in the incorporation of (^{14}C)AMP into RNA product, as the BrdUMP content of the DNA' primer increases. The only exception to the mean values of the 50% dTTP : 50% BrdUTP assay of Table 19 and the 100% BrdUTP assay of 24b. Since these two exceptions were individual assays within 8 experiments, totalling 26 assays involving variations in BrdUMP content of the primer of transcription, it is valid to conclude that they are atypical results.

To compensate for scatter among the results of the duplicate assays of each experiment the standard deviation for each of the 4 sample discs was calculated. In Figures 17-20 and 22 each mean (^{14}C)AMP incorporation value with its standard deviation is plotted against the % BrdUTP content of the reaction mixture. This presents the results in a more graphic form. The trend to decrease in (^{14}C)AMP incorporation is again apparent.

In Table 26 the mean values of Table 22 are expressed as a % of their respective 100% dTTP value. In all cases this is the value of maximum poly(A-U) synthesis. From this table it is apparent that the % decrease is not consistant from one experiment to another. Therefore, the experiments summarized in the above table show some variation. The amount of (^{14}C)AMP incorporation varies between assays of the same type *in different experiments*. *For example* the 75% dTTP : 25% BrdUTP experiments under headings 18 and 19 show 20% and 43% reduction in (^{14}C)AMP incorporation. However, the duplicate assays within an experiment can be favourably compared as shown in Tables 17-21 and 24b. In addition similar degrees of poly(A-U) synthesis are observed whether poly(dA-dT) or poly (dA-BrdU) were used in different experiments. For example, in the 100% BrdUTP experiments under headings 19 the poly(dA-dT)-primed reaction exhibits 57% reduction in activity while the poly(dA-BrdU)-primed reaction exhibits 64% reduction in activity. This is further support for the conclusion that the initial DNA primer does not contribute significantly to the RNA synthesis reaction.

Variations between experiments cannot easily be correlated with variations in the components of the reaction mixtures from experiment to experiment. As shown in the comparative Table 26 the variations are not related to the use of M. lysodeikticus DNA polymerase or E. coli DNA polymerase. This observation might be expected as these two bacterial DNA polymerases are essentially similar in their in vitro properties (Zimmerman 1960). Furthermore, it is also not possible to relate these

Table 26 Comparison of the mean values of Table 22, of the transcription of DNA-primers containing increasing amounts of bromodeoxyuridine, expressed as a % of their respective 100% dTTP value.

Relative proportions of dTTP and BrdUTP in the reaction mixture		(^{14}C)AMP incorporated (nmoles/assay)							
		Mean values of results shown in Tables							
% dTTP	% BrdUTP	17	17	18	19	19	20	21	24b
100	-	100	100	100	100	100	100	100	100
75	25	-	-	80	57	-	91	85	99
50	50	-	-	74	?	-	75	78	96
25	75	-	-	66	47	-	65	73	87
-	100	83	70	58	43	36	63	70	?
Variable components of the DNA polymerase reaction mixture									
Units of poly(dA-dT)		0.1		0.2	0.1		0.1		0.02
Units of poly(dA-BrdU) Batch used			0.1 M10			0.1 M10		0.1 M4	
Unit of M. lysodeikticus DNA polymerase		5.7	5.7	5.7					
Unit of E. coli DNA polymerase					5.3	5.3	5.3	2.0	5.0
Source of BrdUTP		Prepared	Prepared	Prepared	Prepared	Prepared	Purchased		

variations in (^{14}C)AMP incorporation between experiments with the use of either the BrdUTP prepared by the author or the purchased BrdUTP.

Another possibility which may account for the variation in (^{14}C)AMP incorporation between experiments is the probability of variation in the amount of DNA synthesis in the initial reaction. This point is illustrated in Table 15 in the poly(dA-dT)-primed DNA polymerase reaction. Experiment (1) yielded 83 nmoles (^{3}H) dAMP incorporated per assay while experiment (2) yielded 55 nmoles per assay for *identical assay conditions*. To clarify this point completely double label experiments which follow the synthesis of both DNA and RNA in the sequential system are necessary. Most of these variations were observed using 5 units of DNA polymerase/0.2ml. Table 16 shows that unprimed synthesis is considerably reduced when 2 units of DNA polymerase/0.2ml are used. Therefore in the DNA polymerase reaction of the sequential system of Table 21 two units of enzyme were *used to minimize the possible contribution* of unprimed synthesis of DNA' product. However the variation in quantity of RNA synthesized in relation to the BrdUrd content of the DNA product, appeared to be independent whether 5 units or 2 units of DNA polymerase/0.2ml were used. This reduction in DNA polymerase concentration does not appear to affect the DNA polymerase reaction, nor does *it affect* the succeeding RNA polymerase reaction.

At this point it should be noted that all of these differences in reaction components between experiments pertain to the first reaction of the sequential system. The conditions of the RNA polymerase incubation remained constant throughout the study. However, it seems probable that the variations between experiments, which were performed on *different days, are probably due to the labile nature* of these polynucleotide synthesizing enzymes. This has been discussed previously.

Therefore, it would seem that the DNA polymerase reaction of the sequential system does exhibit variability between experiments. However, in view of the constant trend within most of the above experiments, it does seem valid to conclude that as the BrdUrd content of the DNA' product increases it becomes a less efficient primer for the RNA polymerase reaction.

4.7 Priming capacity of Poly(dA-BrdU) in replication and transcription

The ability of poly(dA-dT) and poly(dA-BrdU) to direct the synthesis of poly(A-U) by RNA polymerase was investigated at the outset of these studies. The results of the first of these assays performed indicated that poly(dA-BrdU) was incapable of directing priming for poly(A-U) synthesis. These results are shown in Table 25 columns a and b. In addition it can be seen from the experiments described in Table 15 experiment (2) that poly(dA-BrdU) is an efficient primer for DNA synthesis. It is unlikely that this incorporation of (^{3}H)dAMP is due to unprimed synthesis as the results for the latter are much lower.

Based on these results it was assumed that poly(dA-BrdU) would make an excellent initial primer in the sequential system. In such a situation using poly(dA-BrdU) as initial primer in the sequential system there could be no contribution by initial primer to the RNA synthesis reaction. There would be no doubt as to which primer was eventually responsible for the synthesis of poly(A-U). The experiments shown in Tables 17 were thus performed and repeated in Table 19. For the 100% BrdUTP containing reaction mixture primed by poly(dA-BrdU) a zero incorporation of (^{14}C)AMP was expected, however, as is apparent from these tables this did not occur. The batch (M10) of poly(dA-BrdU) used in all the experiments mentioned above was the first purchased. A second batch (M4) was then

receive transcribed in an isolated RNA polymerase reaction. As can be seen in Table 25 columns c, d, e and f there is no difference in the ability of this copolymer of (dA-BrdU) or that of (dA-dT) to prime for poly(A-U) synthesis. Batch M4 also appeared to be an efficient primer in DNA synthesis and was used for the assays of Table 21. The conflicting results in the ability of the two batches to support poly(A-U) synthesis in an RNA polymerase catalysed reaction cannot easily be explained. The assays were repeated to ensure that no experimental error had been made. Both batches of poly(dA-BrdU) were examined through the wavelength of 230 to 300 nm. For both preparations the absorption spectra were as shown in Figure 6, with an absorption maximum of 266,5 nm.

It has been suggested in section 4.8 that an RNA polymerase-(dA-BrdU)-(^{14}C)ATP-UMP complex is formed. This complex would result in a measurement of (^{14}C) AMP incorporation indistinguishable from that of (^{14}C)-poly(A-U), thus giving a false impression of poly(A-U) synthesis. However, no explanation for the differences in the priming ability of the two purchase batches of poly(dA-BrdU) can be found.

4.8 RNA polymerase and transcription

It was concluded in section 4.6 that the presence of BrdUrd in a DNA primer does appear to decrease the efficiency of the primer during transcription. This poses the question as to what the mechanism of this inhibition could be. According to Burgess 1971, Taylor 1966, Maitra et al. 1966 and Bautz 1967, transcription may be initiated at a pyrimidine rich site. With relation to this investigation this pyrimidine rich site (if thymidine) could become a BrdUrd rich site during DNA synthesis. The first stages of transcription which involve the interaction of RNA polymerase with the DNA primer might thus be modified, as a result of the presence of BrdUrd in the DNA, in line with the hypothesis presented in this thesis.

The initiation of transcription has been envisaged by Burgess (1971) to take place in six stages (see Figure 23).

The following discussion relates these six stages to the results of this investigation in an attempt to elucidate the mechanism of BrdUrd inhibition. The DNA primer under consideration would have been synthesized in this work in the presence of BrdUrd, therefore all thymidine units are considered to have been replaced by BrdUrd. It is proposed that the inhibitory effect of BrdUrd operates at the stages of strand separation and catalytic complex formation (i.e. at stages three and four).

The first stage involves the binding of free RNA polymerase to a double stranded DNA in a non-specific and readily reversible fashion. This stage would not be affected by the presence of BrdUrd. However during the next stage the enzyme locates a specific binding site, possibly pyrimidine clusters (Sheldrick and Szybalski 1967) which would therefore be made up of BrdUrd clusters. The presence of BrdUrd could then effect the third stage during which strand separation occurs. Strand separation normally occurs with the formation of a highly stable complex of RNA polymerase bound to the specific site on the single strand of DNA to be transcribed. This stage requires the s' mi. of RNA polymerase.

The nature of the effect which dA in double stranded DNA may have on strand separation is now cons .. For strand separation to take place in stage three an increase in energy may be required to split the increased strength of the dA-BrdU hydrogen bond (see section 1.2.1 and Inman and Baldwin 1962). Such isolated bonds may only result in a slowing down of strand separation and thus transcription. Clusters of the dA-BrdU bond might then be expected to result in total inhibition of strand separation. The results of this investigation using an alternating sequence of dA-BrdU bonds in the DNA primer (i.e. in which dA alternates with BrdU in the nucleotide sequence) indicate that the

Figure 23. The six stages which may occur during the initiation of transcription (Burgess 1971).

Stage 1. Free enzyme binds to double-stranded DNA in a nonspecific and readily reversible fashion. This binding is prevented by inhibitors of DNA binding such as high ionic strength, RNA, and *heparin, and does not require sigma.*

Stage 2. The enzyme locates a specific binding site, perhaps a pyrimidine cluster. This step may not require sigma.

Stage 3. Strand separation occurs with the formation of a highly stable specific complex at the initiation site. Temperatures above about 15°C are required to reach this stage, which is very stable at 37°C and very low ionic strength, and much less stable at lower temperatures or higher ionic strength. This stage may form in the presence of rifampicin and appears to require sigma.

Stage 4. A change in the enzyme occurs, resulting in the formation of the rifampicin-resistant preinitiation complex. This stage is not very stable and decays back to stage 3 in the presence of rifampicin and back to stage 1 or to free enzyme in the presence of heparin. This stage may be stabilized by sigma.

Stage 5. The 5'-terminal nucleotide triphosphate, almost always a purine, binds to the enzyme.

Stage 6. The second nucleotide triphosphate binds allowing the formation of the first phosphodiester bond in the next stage, that of RNA chain initiation.

inhibition is not total. However it can result in a 60% decrease of the normal rate of transcription (see table in section 4.6). However since this type of decrease was observed it does not eliminate the possibility that homopolymeric BrdUrd clusters could entirely prevent strand separation. In addition, due to the increased electronegativity of bromine (Hanawalt 1967) the bond between the enzyme and pyrimidine nucleotide may be so strengthened so as to retard stage four. Stage four normally consists of a change in the enzyme which may be stabilized by sigma. The inhibition of stage four may thus result in an inability of the RNA polymerase DNA complex to undergo the enzyme change indicated.

Burgess (1969) observed a positively charged binding site in the RNA polymerase molecule during purification. In addition, the site of DNA binding on RNA polymerase appears to involve positively charged regions (Burgess 1971). Either of these two effects may reduce the transcription rate or they may be cumulative. The subsequent stages resulting in translocation would then not occur.

4.9 A discussion of possible artifactual problems of the sequential system.

The formation during RNA synthesis of an Enzyme-(dA-BrdU)-(^{14}C) ATP-UMP complex:

This work has shown that increasing amounts of BrdUrd in the sequential system inhibit RNA polymerase activity. It could be argued that complete replacement of thymidine with BrdUrd might totally inhibit RNA synthesis. However Table 26 shows that the maximum inhibition of RNA synthesis observed was 74%. However, for reasons discussed below, the true levels of RNA synthesis in completely BrdUrd substituted DNA primed systems might have been lower than observed in this work.

These reasons relate to the model of Burgess (1971) which postulates the formation of an Enzyme-(dA-dT) complex during stage four of his scheme (see Figure 23). Krakow and Fronk (1969) have proposed that the initiating purine of stage five binds to the product terminus site on the RNA polymerase molecule forming an Enzyme-(dA-dT)-purine complex. The second nucleoside triphosphate then binds at the substrate site with a subsequent polymerization step followed by pyrophosphorolysis (stage 6). *Translocation then takes place as RNA polymerase moves onto the next site.*

Their studies involved the use of a system similar to that created for this investigation. Using Azotobacter RNA polymerase preparations, containing sigma factor, they studied pyrophosphate exchange during the synthesis of poly(A-U) directed by poly(dA-dT). According to their model the sigma subunit binds to the product site of the catalytic unit and is displaced as the r(A-U) synthesized moves by a series of translocation steps through the product site. Release of the sigma subunit in the poly(dA-dT)-directed system requires r(A-U) synthesis. It is proposed that under the present experimental conditions an Enzyme-(dA-BrdU)-(^{14}C)ATP-UMP complex forms. This complex, being more stable than a (dA-dT) one, retards translocation where isolated BrdU units exist and accentuates this effect where they become more concentrated in the DNA primer. If this theory is correct why do the present results, for a 100% substitution of BrdUrd in the primer, not tend towards a zero synthesis of poly(A-U)?. One possibility is that the Enzyme-(dA-BrdU)-(^{14}C)ATP-UMP complex is introducing extraneous counts in the measurements of (^{14}C)poly(A-U) synthesis. This Enzyme complex may be considered the product of BrdUrd inhibition. Due to the nature of this experimental *design this product is being measured with any* (^{14}C)poly(A-U) that may have been synthesized.

The RNA polymerase-*(dA-BrdU)*-(^{14}C)*ATP-UMP complex forms, trans*-location does not take place, and a polydeoxynucleotide complex containing (^{14}C)ATP is precipitated onto the discs in the sample being measured. Thus there would be an accumulative measurement of the (^{14}C) ATP complex and any (^{14}C)poly(A-U) product which may have formed. Thus poly(dA-BrdU) would not be able to direct the synthesis of poly(A-U). In the sequential system used in this work, fully substituted poly(dA-dT) *i.e. poly(dA-BrdU)*, still supported some synthesis of poly(A-U). In terms of the hypothesis just presented this may be accounted for by (^{14}C)ATP attached in acid precipitable form to the template enzyme complex.

Another possible explanation for the residual RNA synthesis in the totally BrdUrd substituted DNA primed system is the synthesis of homo-polymers.

This may result in an overestimation of RNA synthesis by a non-poly (A-U) precipitable product. The synthesis of (^{14}C)poly(A) or (^{14}C)-poly (A).poly(U) is unlikely under the conditions used for this study, but the possibility cannot be ignored. According to Smith <u>et al</u>. (1967) *unprimed synthesis of poly(A).poly(U) has been known to interfere with* <u>in vitro</u> systems using <u>E. coli</u> RNA polymerase. However, this is under conditions of high enzyme concentration (20 units) and a three hour lag period. Krakow (1968) has demonstrated that significant amounts of nucleotide incorporation can be measured after a 30 minute lag period using 0.6 mM each of UTP and ATP. Although this concentration of nucleotide was used in these studies they used a much higher concentration of enzyme.

Another possibility which cannot be excluded is the presence of Poly(A) polymerase and polynucleotide phosphorylase (Smith et al. 1967) in the commercial enzyme preparations used in this study.

These are possibilities which cannot be entirely excluded. However, considering the small amount of enzymes used in these assays and the relatively short incubation period for RNA polymerase, the effect of any of the above would probably be negligible.

4.10 Summary

1. It has been demonstrated that a DNA polymerase reaction can be coupled successfully with an RNA polymerase reaction in an in vitro system as follows, i.e. DNA_t-primer → DNA' → RNA (Figure 14.) The rate of synthesis of DNA is independent of the type of pyrimidine nucleotide whether it be dTTP, its analogue or a mixture of both.
2. In the above system the magnitude of RNA synthesis is inversely proportional to the BrdUrd input into the DNA synthesis reaction, and hence presumably to the BrdUrd content of the DNA template (DNA') of the RNA synthesis reaction. Therefore, there is strong evidence to indicate that BrdUrd inhibits RNA synthesis in this system, while DNA synthesis is unaffected.
3. 5-Bromodeoxyuridine 5'-triphosphate was prepared by direct bromination of deoxyuridine triphosphate. The yield was 15.5%.
4. Using a calf thymus DNA polymerase assay system it was demonstrated that BrdUTP can replace dTTP in the synthesis of DNA with an efficiency around 90%.
5. The quantity of poly(dA-dT) and poly (dA-BrdU) synthesized in an unprimed E. coli DNA polymerase assay system was found to be variable. Consistency was not found in product DNA synthesized even between duplicate assays incubated for the same period.

The main findings of this work (Points 1. and 2.) correlate well with the results of *in vivo* studies of the effect of BrdUrd on cell differentiation and cell division, (see Introduction). These *in vivo* studies have shown that differentiation is arrested at low concentrations of BrdUrd while cell division proceeds in an apparently normal fashion. Therefore, it would seem valid to conclude that a possible site of action of BrdUrd inhibition of differentiation is at the level of RNA synthesis at critical sites along the genome. It would seem possible that these sites contain oligothymidine residues or thymidine clusters. If this is the case then these sites may be involved in directing RNA synthesis into particular pathways of differentiation. When these site are substituted with BrdUrd the direction of RNA synthesis into these differentiation pathways would be blocked.

The potential of the sequential system and suggestions for future work

In the sequential system DNA polymers can be synthesized and transcribed without their previous isolation.

The sequential system is in some ways more similar to the *in vivo* synthesis of DNA and RNA synthesis in the nucleus than the separate *in vitro* systems for DNA synthesis and RNA synthesis used in most studies incorporating these reactions.

Better defined optimal enzyme assay conditions are required for the sequential system. It would also be appropriate to investigate if excess BrdUTP affects the activity of RNA polymerase. Better characterization of the DNA polymers (DNA') synthesized would be facilitated by the use of radioactively labelled BrdUTP.

Any future experiments using this sequential system should take into account the possible formation of an Enzyme-(dA-BrdU)-ATP-UMP complex. The use of (γ-^{32}P)ATP as a control for the labelling of the complex would enable a distinction to be made between the measurement of the poly(A-U) product and the product of BrdUrd inhibition, i.e. the complex. The dissociation of this complex may be facilitated by increasing the temperature to above 37^{o}C after the normal incubation period has been completed. The complex may also be digested by enzyme digestion of the protein (RNA polymerase).

A study of the transcription of a homopolymer of BrdU units should also be made before conclusive evidence in support of the hypothesis of this investigation can be obtained. Such a polymer could possibly be synthesized from a homopolymer of poly(dA).

Many kinetic experiments could be devised which might result in interesting comparisons between the two major polynucleotide synthesizing enzymes, namely DNA polymerase and RNA polymerase. For example a comparative study of the binding constants of these enzymes for BrdUrd substituted DNA templates could be undertaken.

ABBREVIATIONS

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
DNA polymerase	Deoxyribonucleoside triphosphate: DNA deoxynucleotidyl transferase
RNA polymerase	Ribonucleoside triphosphate : RNA nucleotidyl *transferase (DNA-dependent RNA polymerase)*
dA	2'-deoxyribosyladenine
dT	2'-deoxyribosylthymine (thymidine)
TdR	thymidine
BrdUrd	5 -Bromo 2'-deoxyribosyluridine
FdUrd	5 -Fluoro 2'-deoxyribosyluridine
dATP	2'-deoxyadenosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dUTP	2'-deoxyuridine 5'-triphosphate
BrdUTP	5 -Bromo 2'-deoxyuridine 5'-triphosphate
BrdUDP	5 -Bromo 2'-deoxyuridine 5'-diphosphate
BrdUMP	5 -Bromo 2'-deoxyuridine 5'-monophosphate
BrdCTP	5 -Bromo 2'-deoxycytidine 5'-triphosphate
poly(dA-dT)	Associated alternating copolymers of 2'-deoxyribosyladenine and 2'-deoxyribosylthymine poly(dA-dT).poly(dT-dA)
poly(dA-BrdU)	Associated alternating copolymers of 2'-deoxyribosyladenine and 5'-Bromo 2'-deoxyribosyluridine poly(dA-BrdU).poly(BrdU-dA)

ATP	Adenosine 5'-triphosphate
UTP	Uridine 5'-triphosphate
poly(A-U)	Associated alternating copolymers of adenine and uridine
	poly(A-U).poly(U-A)
poly(A)	Simple homopolymer of adenine
poly(U)	Simple homopolymer of uridine
poly(A).poly(U)	Associated poly(A) and poly(U)
Tris	2-amino-2-hydroxymethylpropane 1,3-diol
ETSH	2-mercaptoethanol
EDTA	Ethylenediaminetetra-acetate
BSA	Bovine serum albumin
TCA	trichloroacetic acid
PPO	2,5'diphenyloxazole
POPOP	dimethyl POPOP-1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene

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