DNA REPLICATION IN *TETRAHYMENA PYRIFORMIS*

by

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A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy at the University of Warwick, Department of Chemistry and Molecular Sciences.

July 1979

To my father

Who dreamt but was not to see

His dreams come true

To my mother

A woman of great courage

Who still thinks that I know what I am doing

For enduring so much for our sake

To Eu

And to three small people

M and N

and Kaks

Table of Contents

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I would like to thank the Association of Commonwealth Universities for financial support.

DECLARATION

Some of the work in Chapter 4 was published in my thesis submitted for the M.Sc. degree in Molecular Enzymology at the University of Warwick.

List of Figures

List of Tables

Abbreviations

AMP	Adenosine monophosphate
AraC	Cytosine arabinoside
AraCDP	Cytosine arabinoside diphosphate
AraCMP	Cytosine arabinoside monophosphate
AraCTP	Cytosine arabinoside triphosphate
AraU	Uracil arabinoside
AraUTP	Uracil arabinoside triphosphate
CDP	Cytidine diphosphate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
NEM	N-ethylmaleimide
PCMB	p-chloromercuribenzoate
PP_i	pyrophosphate
PPO	2,5-diphenyloxazole
POPOP	1,4-bis-2-(5-phenyloxazolyl)benzene
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
THU	Tetrahydrouridine
TTP	Deoxythymidine triphosphate

SUMMARY

The effect of cytosine arabinoside on DNA synthesis in Tetrahymena pyriformis has been studied. DNA synthesis was determined by measuring the incorporation of (methyl-^{3}H)thymidine into DNA. The DNA formed was analysed by sedimentation on alkaline sucrose gradients. In the presence of cytosine arabinoside labelled thymidine was incorporated into high molecular weight DNA. This indicates that in Tetrahymena, AraC inhibits the initiation steps of DNA synthesis but does not interfere with the elongation of DNA already formed.

Initial experiments indicated that Tetrahymena quickly recovered from the effect of AraC. The metabolites of AraC were analysed by ion exchange chromatography. AraC was separated from AraCMP, AraCDP and AraCTP on the anion exchanger AG1-X8. AraC and AraU were separated on Dowex 50 or AG50-X8. It was found that whereas a large proportion of cellular AraC is converted to AraU only a small fraction is converted to AraCTP, the putative active agent in inhibiting DNA replication and causing cell death. In the presence of tetrahydrouridine AraC is not deaminated, but this did not lead to increased amounts of phosphorylated products of AraC.

Two DNA polymerase fractions which differed in their sensitivity to NEM and AraCTP were prepared. The nuclear enzyme was resistant to NEM and was activated by AraCTP. The cytoplasmic enzyme was inhibited by both AraCTP and NEM. An attempt was made to fractionate Tetrahymena DNA polymerases on DEAE-cellulose. Two fractions A and B, which differed in their ability to use polyA.polydT as a template-primer were prepared. The correspondence of these to in vivo nuclear and cytoplasmic enzymes is not yet clear.

CHAPTER 1

1. DNA SYNTHESIS IN EUKARYOTES

1.1 Structure of the eukaryotic chromosome (1)

The DNA of eukaryotic cells is packaged in several chromosomes or chromatin where the DNA is closely associated with histones and other proteins. As DNA is duplicated the proper types and amounts of chromosomal proteins must be synthesized. DNA and histone synthesis are delicately controlled processes and are confined to the S phase of the cell cycle. There must be mechanisms to ensure that cellular DNA is replicated only once per cell cycle.

DNA and the nuclear proteins associated with it are called chromatin during the interphase stage of the cell and can be seen as chromosomes during the mitotic stages. Histones are the major proteins of eukaryotic chromosomes and chromatin. Most cells contain five different types of these basic proteins (3). The basic unit of chromatin, the nucleosome, is a roughly spherical body consisting of about 140 base pairs of coiled DNA (4), and an octamer comprising two molecules of each of the small histones, H2A, H2B, H3, and H4, which have a very conservative amino acid sequence (5). The precise arrangement of the four histones is not yet known. It has been suggested that the octamer forms by protein-protein interactions between individual histones involving the hydrophobic C-terminal halves of the histone molecules. The N-terminals would then be free to interact with the DNA on the outside (6).

Between nucleosomes is a stretch of DNA of between twenty to seventy base pairs. This section or linker may also be coiled. The role of the fifth histone, H1, is still unclear. Removal of this histone exposes the linker between nucleosomes to produce fibres

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Between nucleosomes is a stretch of DNA of between twenty to seventy base pairs. This section or linker may also be coiled. The role of the fifth histone, H1, is still unclear. Removal of this histone exposes the linker between nucleosomes to produce fibres

which look like a string of beads under the electron microscope.

Non-histone proteins are a heterogeneous group of acidic proteins which are thought to play structural, enzymatic and regulatory roles in chromatin. Some of these proteins may be involved in the transport of mRNA from the nucleus to the cytoplasm.

Each chromosome is divided into many replication units or replicons of varying size. For each replication unit DNA synthesis begins at an origin and proceeds semiconservatively and bidirectionally. It is not clear whether origins are defined by specific nucleotide sequences. This type of replication produces the "bubbles" which can be seen under the electron microscope.

Unlike the size of replication units which depends on the stage of development of the cell, the rate of movement of the replication fork is similar for cells which are dividing rapidly and those dividing slowly. It appears that the rate of replication depends on the number of active replicating units at any time in the S phase.

1.2 Mechanism of DNA replication in eukaryotes.

It is generally accepted that the processes by which genetic information is preserved and transmitted are essentially the same in eukaryotes and simpler organisms. Replication of DNA follows a semiconservative mechanism. Each strand of the DNA duplex is conserved and copied by base pairing with matching deoxynucleotides to yield two duplexes identical to the parental ones (7). In vitro the DNA polymerizing reaction requires a) a DNA template, b) a DNA or RNA primer with a free 3'hydroxyl end, c) the four deoxynucleotides dATP, dCTP, dGTP and dTTP and d) a metal ion such as magnesium. The reaction may be summarised as follows:

Figure 1.1

Schematic diagram showing the arrangement of DNA and histones

DNA is wound round histone octamers to form roughly spherical bodies at regular intervals. The spheres are separated by short segments of linker DNA to form a structure similar to a string of beads. The position of the histone H1 is still unclear but it is thought to be in the region of the linker DNA segment.

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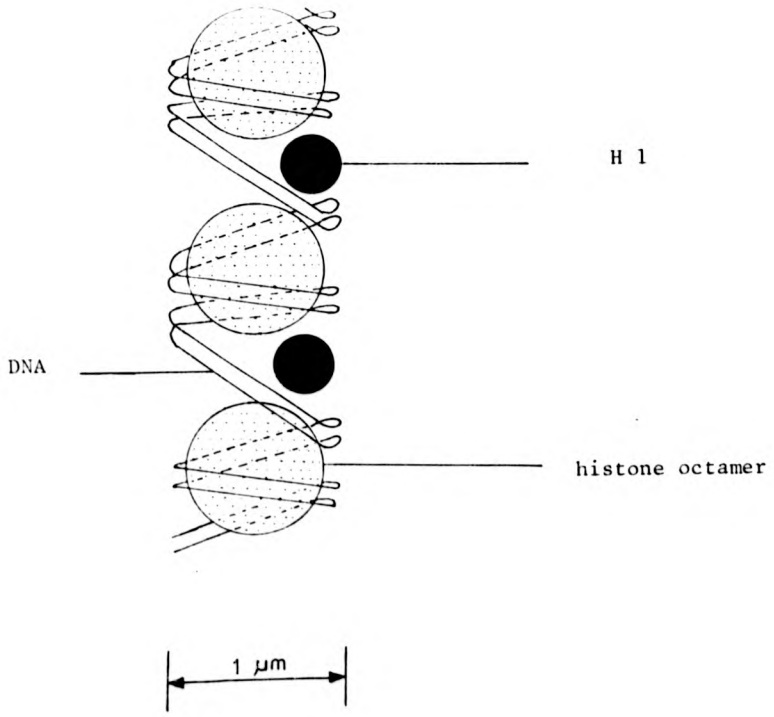
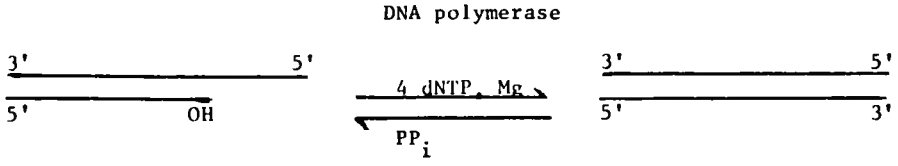
ts
The
be





Although many models have been proposed for DNA replication none is completely adequate in accounting for all the known facts concerning the replication of double-stranded DNA (8). The process of DNA replication may be conveniently considered in terms of initiation, chain growth and termination. The model described below is essentially that proposed by Huberman and Riggs (2) and expanded by Hewish (10).

1.2.1 Initiation

Although some details about the chemistry of the initiation of DNA synthesis have been elucidated little can be said about the factors which set the replication process in motion. It appears that at the beginning of DNA replication a DNA unwinding protein binds to single stranded DNA in such a way that RNA polymerase (primase) starts the synthesis of a new chain at a specific site on a single stranded DNA template (11, 12). Because of its specificity for single stranded DNA it is assumed that unwinding protein does not bind to double stranded DNA. It is proposed that the initiation site is on the relatively few sites between histone oligomers. The stretches of RNA, about ten nucleotides in length, synthesized as primers for Okazaki fragments by a specific primase, probably do not have a specific base sequence.

It has been shown that RNA polymerases are able to start new chains and that DNA polymerases can extend these chains in vivo (13) and in vitro (14). However, it has not been possible to identify RNA priming segments. This may be because of their inherently transitory

Figure 1.2

Bidirectional replication of DNA in eukaryotes

Each chromosome is thought to consist of several replication units each with its own origin. The beginning of chain replication does not necessarily occur at the same time for all the origins. Soon after initiation replication bubbles are formed. Within each bubble replication takes place bidirectionally at two forks.

(a) before replication

(b) after the initiation of DNA replication has started at two origins.

The bubble on the left started later than the one on the right.

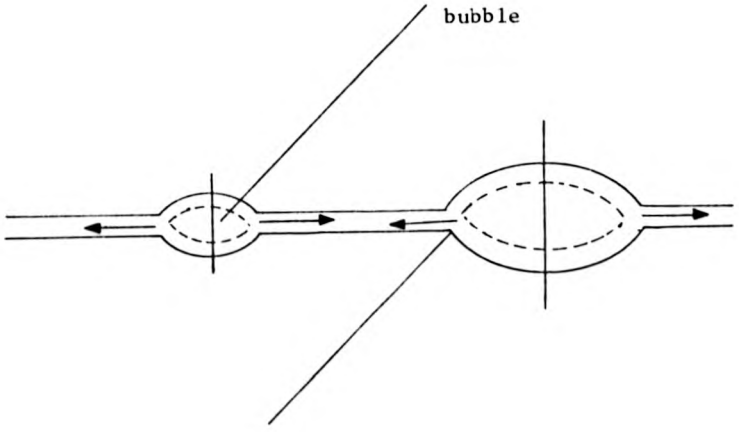
(c) the fusion of adjacent bubbles to form a single growing bubble.

———— old DNA

– – – – – – – – newly formed DNA

The direction of replication is shown by the arrows.

a



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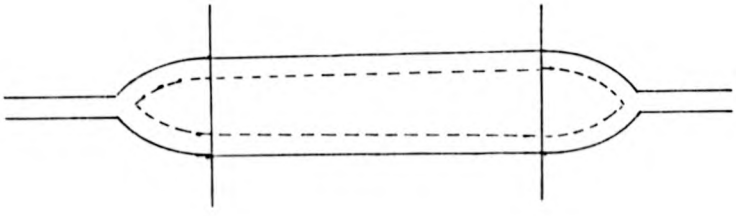


c

Figure 1.3

Initiation of DNA replication

At the beginning of DNA replication the nucleosome structure must be undone to expose sites for DNA synthesis to start. After this the following events probably take place.

(A) RNA polymerase initiates priming at an accessible site.

(B) DNA synthesis starts from the formed RNA primer.

(C) Further initiation sites are exposed.

(D) Newly formed DNA chains are joined by a ligase. Further primers are started on the displaced strand. The original initiation site is blocked to further replication.

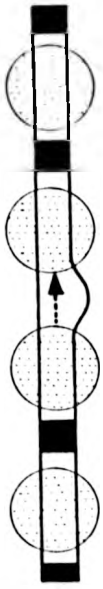
DNA

RNA

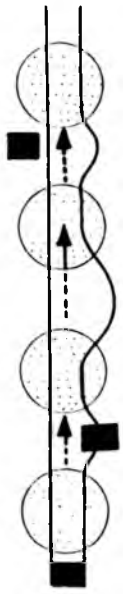
Histone complex

Blocking protein

The arrows show the direction of replication.



A



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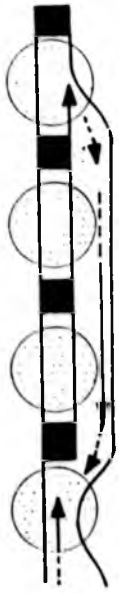

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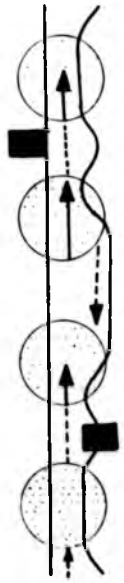

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nature.

1.2.2 Chain elongation (15)

DNA synthesis is initiated on RNA primers by a replicative DNA polymerase. It is thought that as DNA synthesis continues secondary sites for primer RNA synthesis become available on either side of the first initiation site.

Available evidence indicates that both daughter strands grow at the same time. The direction of synthesis is 3' to 5' on one strand and 5' to 3' on the other. However, no enzymic mechanism for extending DNA chains in a 3' to 5' direction has been demonstrated. There is increasing evidence that this problem is solved by synthesizing short stretches of DNA in a 5' to 3' direction (9). These segments, about ten nucleotides long, would then be joined to the growing polynucleotide by formation of phosphodiester linkages. The RNA segments have no unique base sequence but probably have a well defined size. All have pppA or ppG at their 5' end. The size of the initiator RNA may provide the signal for switching from RNA to DNA synthesis.

It seems that the synthesis of eukaryotic DNA occurs semidiscontinuously (16). Okazaki fragments are formed only on one of the daughter strands - the lagging strand. It appears that no Okazaki fragments are formed on the leading strand which replicates continuously.

The elongation reaction involves nucleophilic attack of the 3' OH primer terminus upon the alpha phosphorus of a deoxynucleotide substrate with displacement of pyrophosphate (17). The following sequence of reactions probably takes place:

a) DNA polymerase binds DNA in a complex in which the 3'-hydroxy terminus of the strand is activated. The activation may be by co-ordination to enzyme-bound zinc. There is some evidence that zinc is

involved in such a complex (18). It appears that added magnesium or manganese is not involved at this stage of the reaction.

b) In the second step the binding of the dNTP occurs by co-ordination of the alpha phosphoryl group to the enzyme bound divalent activator (Mn or Mg). Although magnesium is not essential for binding of the DNA template it is required for the binding of the nucleotide to the enzyme. Base pairing between the nucleotide to be polymerised and the template strand provides further stabilization.

c) In the next step there is a nucleophilic attack on the alpha phosphoryl group by the 3'-hydroxy group at the end of the primer strand. At the same time pyrophosphate is displaced. Zinc may facilitate the process by promoting deprotonation of the OH group in a mechanism similar to the zinc hydroxide mechanism proposed for carbonic anhydrase (19). Susceptibility of the alpha phosphoryl group to nucleophilic attack would be increased by co-ordination to Mn or Mg which withdraw electrons and neutralize the positive charge. Nucleotidyl transfer is now complete and primer translocation begins.

d) As a new primer terminus appears water ligands substitute for the phosphodiester ligand on the enzyme-bound manganese or magnesium. Zinc dissociates from the 3' oxygen atom of the previous nucleotide and co-ordinates with the new 3'-hydroxyl group.

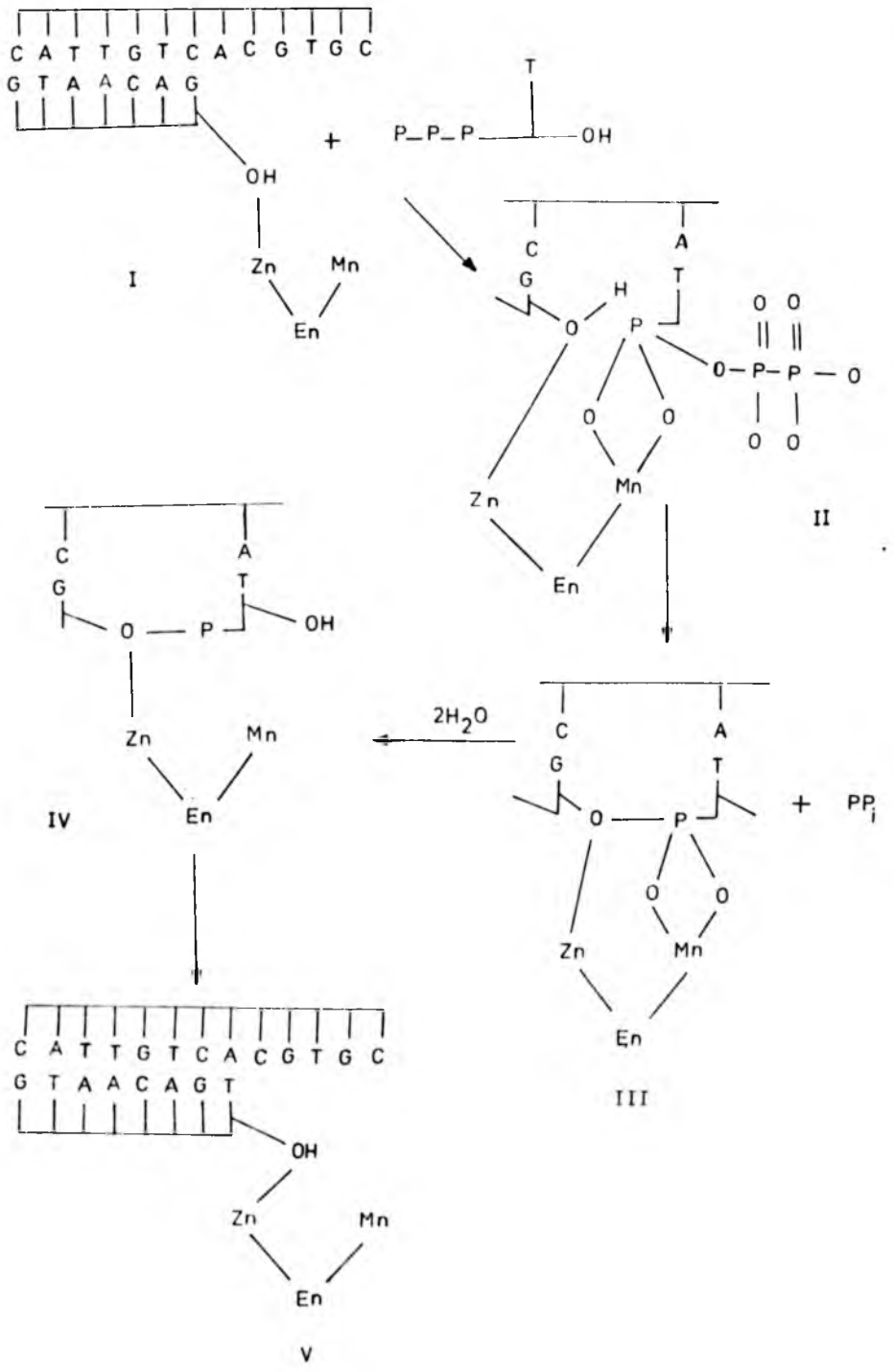
1.2.3. Termination

The final steps in DNA synthesis involve the excising of primer RNA, the joining of Okazaki fragments and restoration of the original chromatin structure. Primer RNA is removed by nucleases. The resulting gap is then filled by DNA polymerases. The short fragments are joined to form high molecular weight DNA by ligases (9). Restoration of the original chromatin structure and blocking of the initiation site prevent further replication cycles. Little is known about how these

Figure 1.4

The elongation of a DNA chain by a DNA polymerase in the presence of template

(I) The enzyme binds DNA. The 3'-hydroxyl terminus of the growing strand is activated, possibly by co-ordination to the enzyme-bound zinc.
(II) The matching deoxynucleotide binds to the enzyme/DNA complex by co-ordination of the alpha-phosphoryl group to enzyme-bound magnesium or manganese.
(III) Formation of a phosphodiester bond and the release of pyrophosphate. This marks the end of nucleotide transfer and the beginning of primer translocation.
(IV) and (V) Regeneration of a new primer terminus. The enzyme is now ready for another nucleotidyl transfer reaction.


C A T T G T C A C G T G C
G T A A C A G
OH
Zn Mn
En
I
T
P–P–P
OH
+
C A
G T
H
O P
O O
O–P–P–O
O O
Zn Mn
En
II
C A
G T
O P OH
Zn Mn
En
IV
2H2O
C A
G T
O P
O O
Zn Mn
En
III
+ PPi
C A T T G T C A C G T G C
G T A A C A G T
OH
Zn Mn
En
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events are controlled.

1.3 Inhibitors of Eukaryotic DNA Synthesis

In prokaryotes mutants have been successfully used in the unravelling of complex biochemical events. In eukaryotes this approach has met with little success. Instead the use of inhibitors has permitted the analysis of many biochemical reactions. Several inhibitors of DNA synthesis have now been discovered. The inhibitors can be classified according to their mode of action: (a) agents which bind to the enzyme, (b) template-modifying chemicals, (c) template analogs, (d) substrate analogs, and (e) a variety of chemicals whose mechanism of action is still unknown.

1.3.1 Chemical agents binding to the enzyme

1.3.1.1. Thiol blocking agents.

Thiol blocking agents inhibit those DNA polymerases which require SH groups for activity. The most commonly used are p-chloromercuribenzoate (PCMB) and N-ethylmaleimide (NEM). The extent of inhibition by NEM and PCMB has been used to distinguish the different cellular DNA polymerases. DNA polymerase alpha from calf thymus (20), HeLa cells (21) and human lymphocytes (22) are completely inhibited by concentrations of PCMB and NEM at which DNA polymerase beta from the same tissue is relatively resistant. DNA polymerase from rat liver mitochondria is not inhibited by PCMB (23). All RNA-dependent DNA polymerases from RNA tumor viruses are inhibited by thiol blocking agents (24).

As the alpha DNA polymerases are inhibited by 1 mM NEM and the beta DNA polymerases are not affected by 10 mM NEM this difference in extent of inhibition has been used to distinguish between the two types of enzyme. Some care is required, however, in differentiating

alpha and beta DNA polymerases by this method. DNA polymerases have been isolated which behave like DNA polymerase beta except that they are sensitive to NEM (25).

The requirement for SH groups may be a clue to the mechanism of catalysis by DNA polymerases and may be of help in identifying the amino acids at the catalytic site. Further it may be possible to use thiol inhibitors to distinguish between the enzymes involved in replication and those involved in repair. In eukaryotes, as in prokaryotes, it appears that repair type DNA synthesis is not inhibited by thiol blocking agents (25).

1.3.1.2. Rifamycin derivatives

In general these polycyclic compounds inhibit normal DNA dependent DNA polymerases and viral reverse transcriptases to similar degrees (26). Leukaemia DNA polymerase beta has been reported to be inhibited to a greater extent than normal lymphocyte DNA polymerase beta (27). The low selectivity of rifamycin derivatives in inhibiting nucleic acid polymerising enzymes makes them of little value as specific agents.

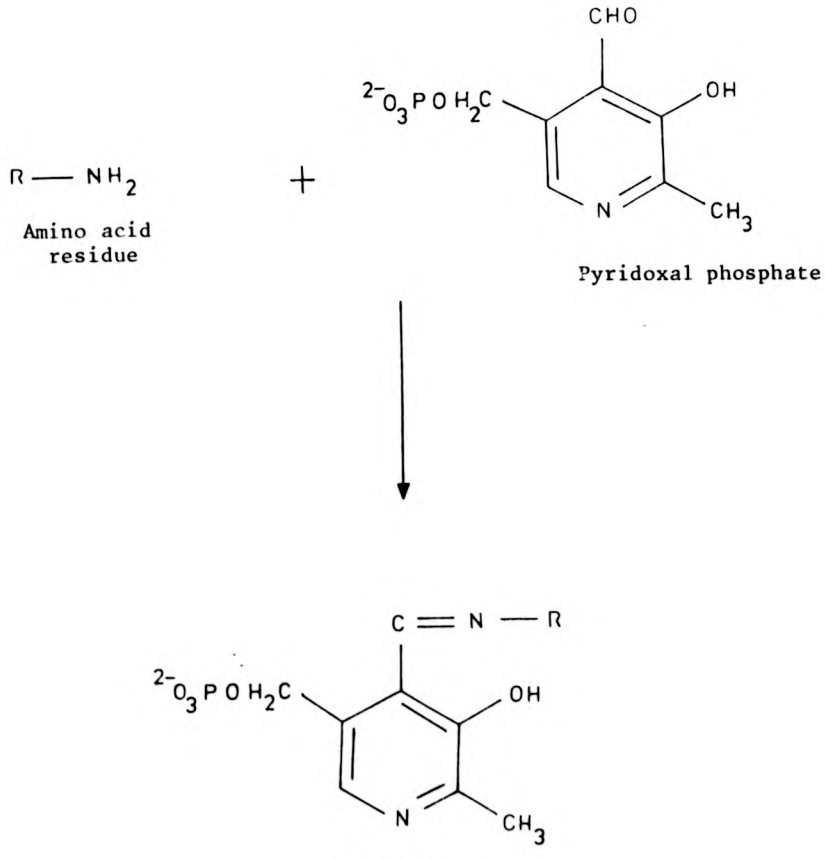
1.3.1.3. Pyridoxal phosphate

Pyridoxal phosphate inhibits DNA polymerising enzymes from a wide variety of sources (28). This probably indicates that these enzymes accept and polymerise deoxyribonucleoside triphosphates by a common mechanism. Both the aldehyde and phosphate groups are required for inhibitory action. Salvo and co-workers (29) demonstrated that both lysine and arginine were at the catalytic site of E. coli DNA polymerase. This together with the fact that pyridoxal phosphate can form Schiff bases with lysine or arginine indicates that lysine and/or arginine is involved in the inhibitory action of pyridoxal phosphate. The phosphate moiety may be required for proper orientation of the inhibitor with respect to the substrate binding site before Schiff

Figure 1.5

Formation of a Schiff base from pyridoxal phosphate and an amino acid

When pyridoxal phosphate reacts with an amino acid such as arginine or lysine an imine or Schiff base is formed. This results from the reaction of the aldehyde function of pyridoxal phosphate with the side-chain amino group of lysine or arginine.


CHO
$^{2-}O_3POH_2C$
OH
N
CH_3
R — NH_2
+
Amino acid residue
Pyridoxal phosphate
C = N — R
$^{2-}O_3POH_2C$
OH
N
CH_3
Schiff base

base formation between the amino acid residues and the aldehyde group of the inhibitor.

1.3.2 Substrate Analogs

Several deoxynucleotide triphosphate analogs have been shown to compete with normal substrates and to be incorporated into DNA in vitro. Others competitively bind to the active site of the enzymes of DNA synthesis or prevent the synthesis of nucleotide precursors.

Many purines and pyrimidines inhibit nucleic acid biosynthesis after conversion to the corresponding nucleoside-5'-phosphates (30). In addition to the polymerases various enzymes which use the normal nucleotide may be inhibited. 6-mercaptopurine, for example, is converted to 6-thioinosinic acid which inhibits enzymes that effect the interconversion of purine nucleotides. 5-fluorouracil, after conversion to fluoro-2'-deoxyuridylate, inhibits thymidylate synthetase. 6-azauridine, formed from 6-azauracil, inhibits the decarboxylation of orotidylic acid. This important reaction in the de novo synthesis of pyrimidine nucleotides is also inhibited by 5-hydroxyuridylic acid, a metabolic product of 5-hydroxyuridine. 9-beta-D-arabinofuranosyl-adenine after conversion to the triphosphate inhibits DNA polymerases. The inhibition of DNA synthesis by cytosine arabinoside is discussed in detail in a later chapter.

Some inhibitors may follow the entire reaction sequence of the normal metabolite and become finally incorporated into DNA. Abnormal nucleotides in DNA may cause genetic mutation, inhibit further growth of DNA chains or result in erroneous transcription. Cell death may result in all cases. The halogen derivatives of uracil and uridine 5-bromouracil, 5-bromo-2'-deoxyuridine, 5-iododeoxyuridine and 5'-trifluorouridine are incorporated into DNA instead of thymidine and interfere with cell replication.

1.3.3. Template Analogs

Single stranded homopolymers without primers have been reported to inhibit leukaemia virus DNA polymerases (31). Single stranded polyribonucleotides are bound but not used as templates by the polymerases. Early reports suggested that inhibition by single stranded polyribonucleotides might be used to distinguish the reverse transcriptases of RNA tumor viruses from cellular DNA polymerases. However, it has been shown that some cellular DNA polymerases are inhibited to the same extent as viral enzymes (32).

1.3.4. Template-modifying Agents

1.3.4.1. Intercalating Agents

A variety of agents act as inhibitors by binding to the template-primer. This group includes many antibiotics (actinomycin D, ethidium bromide, distamycin, neomycin, and acridine dyes, anthracyclines, kanchanomycins and 8-aminoquinolines). Many of these intercalate into the DNA double helix and do not interact covalently with DNA. Some of these may be transformed within the cell to produce an active agent which binds to DNA.

Intercalating drugs affect DNA polymerisation by increasing the energy required to separate the two strands. Nucleic acids which are complexed with acridines are less sensitive to the action of nucleases than in the free state. Intercalating dyes inhibit DNA repair by disturbing the activity of both DNA polymerases and deoxyribonucleases.

The acridines, of which proflavine is one of the simplest members, are planar molecules with three conjugated rings. These molecules intercalate with nucleic acids. The intercalation process requires planarity and certain size of the ligand and a base paired helical secondary structure of the nucleic acid. Proflavine shows two modes of binding (33). In addition to the strong intercalation

which occurs at low drug concentrations there is a weaker binding which is presumed to be to the outside of the helix.

In vitro proflavine inhibits DNA dependent DNA polymerase. The inhibition is due to the acridine binding to the DNA primer. Inhibition produced by a given concentration of drug depends on the amount of DNA primer present in the incubation mixture (34).

Actinomycin D inhibits RNA synthesis at low concentration and DNA synthesis at higher concentrations. Actinomycin D at concentrations which inhibit RNA polymerase does not affect the binding of this enzyme to the DNA template (35). As in the case of acridines the action of actinomycin D can be related to the stabilizing effect it has on the double helical structure of DNA. Deoxyguanosine residues are essential for the formation of actinomycin D-DNA complexes (36).

Ethidium bromide appears to selectively inhibit mitochondrial DNA synthesis in eukaryotic cells (37). It is possible that this inhibition of DNA synthesis is due to the inhibition of mitochondrial DNA polymerase. Isolated rat liver mitochondrial DNA polymerase has been shown to be inhibited by ethidium bromide under conditions in which the nuclear enzyme is resistant to the drug (38).

Intercalation is involved in the mechanism of action of the anti-malarial drugs chloroquine and quinacrine and anthracycline antibiotics. Chloroquine and quinacrine inhibit the in vitro activity of DNA polymerases.

1.3.4.2. Alkylating Agents

Alkylating agents generally act by replacing a hydrogen atom in a molecule by an alkyl group (39). Frequently alkylation is effected by the addition of an alkyl radical to a negatively charged species. Molecules that are reactive to alkylating agents contain nucleophilic centres involving oxygen, nitrogen and sulphur. At physiological pH

Figure 1.6

Crosslinking between guanine residues on different chains of DNA

In solution the drug forms a reactive cyclic intermediate which reacts with the 7 nitrogen of a guanine residue in DNA to form a covalent bond. The second arm can then cyclize and react with nucleophilic groups such as a second guanine residue in an opposite DNA strand or in the same strand. Reactions between DNA and RNA and DNA and protein can also occur.

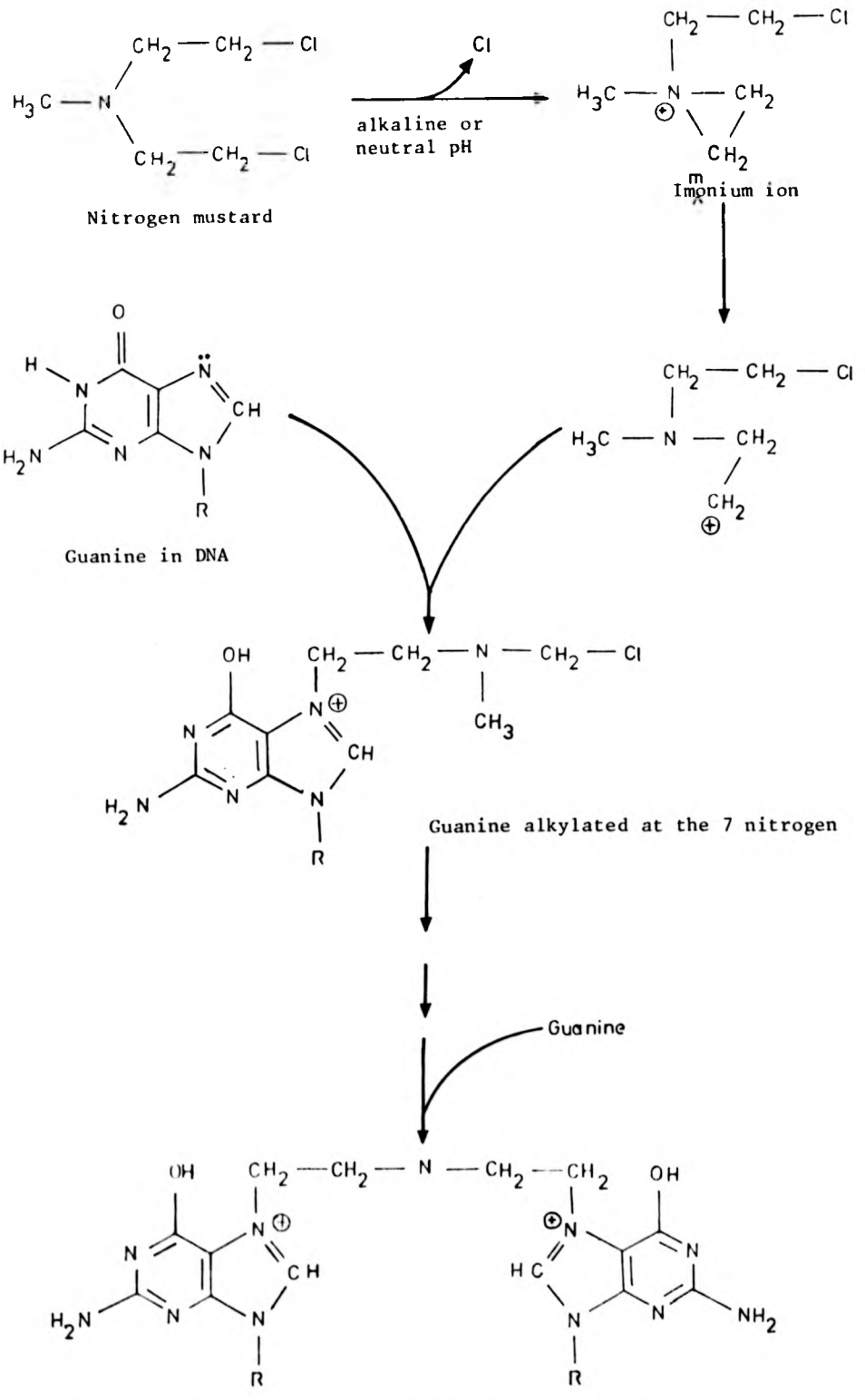
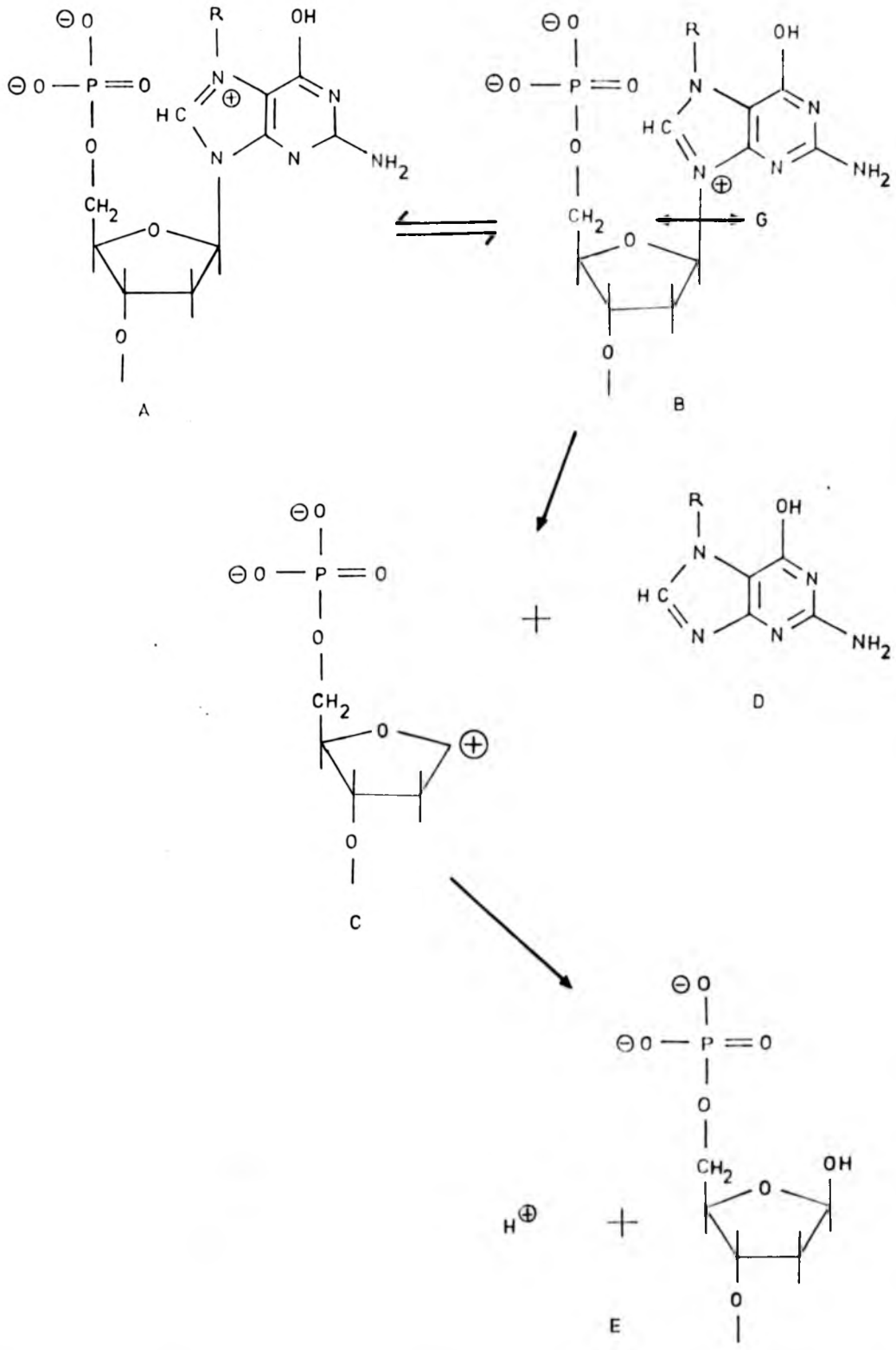

alkaline or
neutral pH
Nitrogen mustard
Imonium ion
Guanine in DNA
Guanine alkylated at the 7 nitrogen
Guanine
Cross-linkage of guanine in different DNA strands

Figure 1.7

Depurination of DNA after alkylation

In a DNA containing N_7-alkylated guanine an equilibrium is set up between this quaternary compound (A) and one with the positive charge on the N_9 (B). These compounds are unstable and alkylated guanine (D) is released by cleavage of the bond at G. The remaining part (C) rearranges and produces a depurinated patch in the DNA strand (E). DNA molecules from which guanine bases have been removed are less stable than normal DNA. They tend to break down in the area of purine deletion by hydrolysis of the phosphate bond.


A
B
C
D
E
G

the range of groups susceptible to alkylation includes the amino and carboxyl groups of amino acids and proteins, the thiol groups of cysteine and cysteine residues of protein, imino groups of histidine, the primary and secondary hydroxyl groups in nucleotides, the nitrogen atoms in purines and the O^6 atom in guanine. In general cytotoxic alkylating agents and tumor inhibitors are at least bifunctional.

There are several regions in a nucleic acid molecule which can be attacked by alkylating agents. An important product of alkylation is N_7-alkylated guanine. Minor products include N-alkylated adenine (N_3-adenine) and cytosine. O_6-alkylation of guanine also occurs and may be involved in the mutagenicity of certain alkylating agents.

The alkylation of guanine in position 7 results in the loss of the base from the main DNA chain as shown on the opposite page. The DNA molecules from which guanine residues have been removed are less stable than normal DNA and break down in the depurinated region by hydrolysis of the phosphate bond.

Bifunctional alkylating agents act by crosslinking adjacent nucleophilic centres. The cross-linked residues may be on the same chain or on different ones. If DNA treated with bifunctional alkylating agents is acid hydrolysed diguanyl derivatives of the type shown on the opposite page are found to be most abundant in the hydrolysate. The mechanism by which a nitrogen mustard becomes covalently bonded to the 7-nitrogens of two guanines is shown on the facing page.

Under neutral or alkaline conditions one of the chloroethyl side chains undergoes cyclisation, releasing chloride ion and forming a highly reactive immonium ion intermediate. Cleavage of the strained ring yields a carbonium ion which reacts with water or with nucleophilic groups like amino, carboxyl, phosphate or sulphydryl groups

of proteins and nucleic acids. Depurination in adjacent regions of DNA leads to unstable sections on both strands. This would probably lead to strand cleavage.

Although the scheme described above is the generally accepted view on the mechanism of action of alkylating agents it is not possible, at present, to state unequivocally that DNA is the only target of importance for these chemicals. Other mechanisms have been suggested for the mechanism of action of individual drugs (40).

1.3.5. Other Inhibitors of DNA Synthesis

1.3.5.1. Zinc Chelators

In DNA polymerases from a few sources it has been shown that enzyme-bound zinc is necessary for activity. This together with the observation that chelating agents inhibit DNA has led to the belief that all DNA polymerases are zinc metalloenzymes (18).

The presence of zinc in eukaryotic DNA polymerases has not been reported. None of the enzymes show a requirement for added zinc for catalytic activity. This may be due to the presence of tightly bound zinc associated with the polymerase molecule. The presence of zinc in many eukaryotic DNA polymerases is implied by inhibition studies which use chelators such as o-phenanthroline which have great affinity for zinc. The observed inhibition cannot be due to the interaction of o-phenanthroline with magnesium as this ion is added in excess. Although o-phenanthroline has a high affinity for zinc it also binds to other ions like copper and iron.

1.3.5.2. Metal Ions

All known DNA polymerases require an added divalent metal ion for activity. Several divalent cations can substitute for magnesium or manganese during catalysis (39). With activated calf thymus DNA as template-primer, sea urchin nuclear DNA polymerase can effectively

use cobalt, nickel, manganese or zinc in place of magnesium. Substitution of barium, calcium or strontium for magnesium does not lead to detectable incorporation of deoxyribonucleotides.

Some monovalent and divalent metal ions can inhibit DNA synthesis. Although information is available about the inhibition of prokaryotic DNA polymerases (7) little is known about the inhibition of mammalian DNA polymerases by metal ions. Bollum (20) has described the inhibition of DNA polymerase alpha by potassium, sodium, lithium, and ammonium chlorides. Inhibition of DNA polymerases from human KB cells (41), HeLa cells (42), rabbit (43) and mouse testis (44) by sodium and potassium has been studied.

1.3.5.3. Agents with unknown modes of action

Nitrosoureas inhibit DNA synthesis in cultured cells and in cell-free extracts. The mechanism of action is unclear but may involve alkylation of the DNA template or the formation or carbamyl derivatives of the enzyme. A number of nitrosourea derivatives have been shown to be specific inhibitors of purified DNA polymerase alpha from rat liver and hepatomas (45). Pre-incubation of the enzyme with nitrosoureas increases the extent of inhibition. Isocyanates, breakdown products of nitrosoureas inhibit DNA polymerase alpha to a greater extent than the parent compounds.

Bleomycin, a glycopeptide antibiotic inhibits bacterial and mammalian cell growth and has antitumor activity. It inhibits DNA synthesis by viral reverse transcriptase, DNA polymerases alpha and beta (46) but DNA polymerase gamma and terminal deoxynucleotidyl transferase are unaffected.

1.4 Enzymes of DNA Synthesis

1.4.1 Eukaryotic DNA Polymerases

DNA polymerases catalyze the formation of a phosphodiester bond between the 5'-phosphate group of the incoming deoxyribonucleotide and the 3'-hydroxyl end of the primer. It is now accepted that DNA from a variety of eukaryotic cells existsin multiple forms. The multiplicity of DNA polymerases raises the question of the biological function of these enzymes. Four main eukaryotic DNA polymerases can be distinguished (47, 48, 49, 50, 51, 52, 53, 54). A common nomenclature has been devised for these enzymes (55).

There is general agreement that the high molecular weight (6-8S) DNA polymerase, usually obtained from the cytoplasmic fraction of cells is DNA polymerase alpha. The low molecular weight enzyme (3-4.5S) usually isolated from nuclei is designated DNA polymerase beta. A third polymerase, DNA polymerase gamma found in both cytoplasm and nuclei shows preference for replicating oligonucleotide-primed homopolymeric ribonucleotides such as $(dT)_{15}(A)_n$. A fourth DNA polymerase activity has been found associated with the mitochondria of several mammalian cells (56).

All eukaryotic DNA polymerase use activated DNA as a template-primer. Unlike bacterial DNA polymerases they lack associated nuclease activity. The enzymes differ in size, structure, template-primer specificity and in the requirement of thiol groups for activity.

1.4.1.1. DNA Polymerase alpha

DNA polymerase alpha was the first eukaryotic DNA polymerase to be identified (57). It has been prepared from calf thymus (57), HeLa cells (58), rat tissues (59), human KB cells (60), avian cells (61), baby hamster kidney cells (62) and mouse L cells (63).

The sedimentation properties of the purified enzyme are strongly

influenced by the ionic strength. In the absence of sodium chloride DNA polymerase alpha from mouse L cells and calf thymus sediments at 7S. This corresponds to a molecular weight of 130 000. In the presence of 0.5M sodium chloride, however, the enzyme sediments at 5S, corresponding to a molecular weight of 70 000.

DNA polymerase alpha is the predominant DNA polymerase activity of growing cells where it often represents 80 - 90% of the total cell DNA polymerase content. As with all known DNA polymerases, DNA polymerase alpha requires 3'-OH priming groups as initiators. It copies gapped duplex DNA (activated DNA prepared by the action of DNAse I on duplex DNA) at a high rate. DNA polymerase alpha can use synthetic DNA homopolymer templates well with both DNA and RNA primers. It is unable to copy RNA templates. Reactions with synthetic template-primers proceed best with Mn as the divalent cation but those with natural DNA templates proceed best with Mg.

The de novo synthesis, in vitro, of polyd(A-T) by calf thymus DNA polymerase alpha has been reported (64). This synthesis, which occurs after a long lag period, occurs both with and without the addition of DNA and unwinding protein. The synthesis of polyd(A-T) was shown to be autocatalytic and exponential. Earlier the same group had reported the replication of single-stranded DNA by DNA polymerase alpha. The product was shown to be double-stranded DNA with a hairpin structure.

Calf thymus DNA polymerase alpha can carry out pyrophosphate exchange and pyrophosphorolysis reactions but no associated nuclease activity has been demonstrated (12). The pH optimum of the enzyme ranges from 7-8 depending on the buffer used. It is particularly sensitive to high ionic strength. DNA polymerase alpha is sensitive to NEM (8), high concentration of actinomycin D, and AraCTP (65).

Although most easily detected in the cytoplasm, DNA polymerase alpha has also been found in the nucleus. The sensitivity of the enzyme to promotors of cell proliferation and to inhibitors of mitosis and replication shows that the enzyme may be important in replicating DNA under physiological conditions.

DNA polymerase alpha is generally considered to be the true DNA replicative enzyme. Further work on this enzyme has been hampered by the difficulty encountered in its purification. It has not been possible to purify the enzyme to homogeneity. Heterogeneity may reflect proteolytic degradation of the enzyme during purification to yield smaller molecules which retain polymerase activity. On the other hand it is possible that the heterogeneity of the enzyme is a reflection of its biological role. The fluctuation which occurs in its activity during the growth of a cell are correlated with rate of DNA replication and may also be associated with molecular weight changes.

Although there is general agreement that DNA polymerase alpha probably consists of subunits, the number, size and structural relationship of these units is yet to be worked out (52).

In view of the observation that DNA synthesis in both prokaryotes and eukaryotes begins by the coupling of a deoxyribonucleotide to the 3'-OH group of RNA, the ability of polymerase alpha to use RNA primers is significant. Although other DNA polymerases can use RNA primers with synthetic DNA templates, only DNA polymerase alpha can synthesize DNA covalently bonded to natural RNA primers (66). This would indicate that the enzyme can initiate DNA synthesis in vivo and would make it a strong candidate as the replicative enzyme.

1.4.1.2. DNA Polymerase beta

The low molecular weight DNA polymerase beta comprises 5-15% of the total DNA polymerase of dividing cells but up to 50% of the

activity in non-dividing cells. Although the enzyme is usually found in the nucleus it is also reported to be present in the cytoplasm (67). The enzyme has been isolated from HeLa cells (57), rat liver (68) and PHA-stimulated human lymphocytes (69). Because of its size, location, charge, and relative abundance in non-proliferating tissue this DNA polymerase has been the easiest to purify. In calf thymus (70) and human KB cells (71) the enzyme has been purified to homogeneity.

DNA polymerase beta from calf thymus and human KB cells is a single polypeptide chain with a molecular weight of 43 000 - 45 000. The molecular weight of the enzyme isolated from other sources ranges from 35 000 - 45 000 (70). Although sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis shows no evidence of subunit structure under certain conditions of low ionic strength the enzyme forms aggregates with a molecular weight of 250 000 (71). In mouse cells the aggregated low molecular weight enzyme has sometimes been mistaken for DNA polymerase alpha (72).

With activated DNA as template-primer, DNA polymerase alpha requires all the four deoxynucleotides for maximal activity. However, some incorporation with only one, two or three of the deoxyribonucleotide substrates and activated template has been reported (73). DNA polymerase beta may, therefore, have been confused with terminal deoxynucleotidyl transferase, an enzyme which catalyses the polymerization of deoxynucleotides on the 3'-hydroxy ends of oligo and polydeoxyribonucleotides in the absence of template. The purified enzyme, however, does not show terminal deoxynucleotidyl transferase activity when assayed under conditions which are optimal for transferase activity.

Purified DNA polymerase beta has no associated nuclease activity nor does it carry out pyrophosphate exchange (60). The enzyme functions

optimally at alkaline pH and has an alkaline isoelectric point. The DNA polymerase beta purified from calf thymus chromatin is a basic protein. Although DNA polymerase beta is inhibited by p-chloromercuribenzoate it is less sensitive to NEM than DNA polymerase alpha (48). The enzyme is not inhibited by 20% ethanol or 25% acetone and is stable in 5M urea (74).

Mosbaugh and others (75) have reported the association of highly purified DNA polymerase beta from Novikoff hepatoma cells with another protein, Novikoff factor IV. This protein stimulates the enzyme fourfold and converts the 3.3S polymerase to the 4.1S form. Other proteins could be involved in the conversion to the observed 5.8 and 7.3S forms.

1.4.1.3. DNA Polymerase gamma

The separation of DNA polymerase gamma and its differentiation from alpha and beta DNA polymerases in HeLa cells was first described by Fridlender and co-workers (76). The enzyme has been reported in a variety of sources including human lymphocytes, HeLa cells, rat liver and chick embryo (77).

DNA polymerase gamma requires thiol groups for activity. It can use either magnesium or manganese as the metal ion. In HeLa cells the enzyme shows K_m values for deoxynucleotides that are an order of magnitude lower than the K_m values for DNA polymerase alpha and beta. It has been suggested that gamma DNA polymerase may use deoxynucleotide triphosphates during the S phase when the deoxynucleotide pool is relatively low (51).

DNA polymerase gamma has a pH optimum of between 7 and 8. It prefers manganese when copying its preferred template $(dT)_{15}(A)_n$. The unique property of DNA polymerase gamma is its ability to copy synthetic ribohomopolymers such as $(dT)_{15}(A)_n$ at a higher rate than

deoxyribohomopolymer templates such as $(dT)_{15}(dA)_n$ or activated DNA. Unlike reverse transcriptases, DNA polymerase gamma does not use poly $(C)dG_{15}$ or natural RNA to an appreciable extent (78).

In HeLa cells DNA polymerase gamma is found in both the cytoplasmic and the nuclear fractions. The cytoplasmic enzyme can be separated into two forms on hydroxyapatite columns. The two forms, both of molecular weight 100 000 daltons, differ in their ability to copy ribohomopolymers such as poly(I), poly(C), or poly(U) (79). Form I copies poly(A) templates well but copies poly(C), poly(I) and poly(U) very poorly. Form II can copy all of the homopolymers well. The two forms also show different inhibition patterns in the presence of salt or ethanol (80).

N-ethylmaleimide (1 mM) inhibits nearly half the activity of DNA polymerase gamma but p-chloromercuribenzoate (1 mM) inhibits the activity completely. The purified enzyme has been reported to be unable to catalyse pyrophosphate exchange (81).

DNA polymerase gamma comprises only one percent of the total cell DNA polymerase activity. Its function may, therefore, be a specialised one. On the other hand it is possible that the enzyme occurs in high concentration in certain cells or during certain phases of development.

1.4.1.4. Mitochondrial DNA polymerase

Mitochondrial DNA polymerase, another minor polymerase which is found in mitochondria, is active in high salt and can copy mitochondrial DNA (82). Some workers have reported differences between mitochondrial and gamma DNA polymerases from the same source in their sensitivity to inhibitors and their efficiency in using template-primers (83). In contrast others do not find any differences in the sedimentation or catalytic properties of these enzymes (84). The responses of these

enzymes as to inhibitors was found to be identical.

It has not been easy to demonstrate that mitochondrial DNA polymerase is indeed associated with that organelle. In any case mitochondrial DNA polymerase comprises only a small percentage of the total cell DNA polymerase activity. While it would be important for mitochondrial function it is not expected to play a critical role in the biology of the cell as a whole. If mitochondrial DNA polymerase and DNA polymerase gamma are indeed one enzyme, then the presence of this enzyme in the cytoplasm is baffling.

1.4.1.5. Terminal deoxynucleotidyl Transferase

Terminal deoxyribonucleotidyl transferase is not a normal DNA polymerase in that it does not require a template to direct DNA synthesis. Although low levels of the enzyme have been reported in bone marrow (85) it is almost exclusively found in the thymus (86).

The enzyme has a molecular weight of about 32 000 and comprises two subunits of 8 000 and 26 000 daltons. Two forms of terminal deoxyribonucleotidyl transferase have been described. Both have the same molecular weight and primer and substrate preferences (87). They differ in their response to cortisone treatment. Form I is more resistant than form II. Also whereas the levels of form I terminal transferase are similar for all ages and strains of mice the levels of form II vary with both strain and age (52).

Cell lines derived from cells of patients with acute lymphoblastic leukaemia have high levels of terminal deoxynucleotidyl transferase. The enzyme could, therefore, be used as a biochemical marker for certain types of leukaemia. Although terminal transferase from leukaemia cells has properties similar to the calf thymus enzyme differences have been reported. The enzymes show different divalent cation preferences when polymerising dG with $(dA)_{50}$ as initiator (88).

1.4.1.6. Location of DNA Polymerase in Cells

Except for mitochondrial DNA polymerase and polymerases of viral origin, DNA polymerases would be expected to occur in the nucleus where DNA synthesis takes place. It was surprising to find that, using standard procedures of enzyme fractionation, a fraction of the total polymerase activity was always recovered in the cytoplasmic fraction. The presence of DNA polymerase in the cytoplasmic fraction could be an artifact of the fractionation procedure (48).

The finding that when nonqueous media are used most of the cell polymerase activity is recovered from the nucleus (69), led to the conclusion that both DNA polymerase alpha and beta are nuclear. This conclusion has been challenged (89) and the question whether DNA polymerase alpha is a nuclear enzyme or not is still unresolved.

Although it has been suggested that DNA replication occurs at the cell membrane there is no agreement as to whether this is the case or whether any of the enzymes of DNA synthesis are located there.

1.4.2. Other Enzymes of DNA Synthesis

Although DNA polymerases have been the most thoroughly studied of the enzymes involved in DNA replication it is becoming clear that the process of DNA synthesis requires the interaction of several proteins. The action of most of these proteins is poorly understood. The presence of some of these enzymes is predicted from the proposed mechanism of DNA synthesis whose general outline is now widely accepted.

1.4.2.1. Ligases

The mechanism of DNA synthesis discussed earlier on in the chapter involves the formation of short pieces of DNA which are joined together in the later stages of chromosome replication. DNA ligases catalyze the formation of phosphodiester bonds between adjacent 5'-

Figure 1.8 Eukaryotic DNA polymerases

Property	Alpha	Beta	Gamma
1. Cellular Location	cytoplasm and nucleus	nucleus	cytoplasm and nucleus
2. Molecular weight (Daltons)	$1.1 - 2.2 \times 10^5$	4.5×10^4	$1.1 - 3.3 \times 10^5$
3. Sedimentation coefficient	6 - 8S	3.3 - 3.5S	6.1 - 6.3S
4. Homogeneity	heterogeneous	homogeneous	heterogeneous
5. Preferred template-primer	activated DNA	poly $dA.dT_{15}$	poly $A.dT_{15}$
6. Ability to copy ribopolymer template	UNABLE	ABLE	ABLE
7. Ability to extend oligo-ribonucleotide primer	ABLE	UNABLE	UNABLE
8. Sensitivity to NEM	very sensitive	resistant	moderately sensitive
9. Sensitivity to Ethidium Bromide	sensitive	resistant	
10. Exonuclease activity	NONE	NONE	NOT KNOWN
11. Elution from DEAE-cellulose	moderate salt (<0.3M)	low salt (<0.05M)	moderate salt (0.3M)
12. Elution from phosphocellulose	moderate salt (about 0.3M)	high salt (>0.45)	moderate salt (about 0.3M)

phosphoryl and 3'-hydroxyl ends in double-stranded DNA. These enzymes are required both for the replication and repair of DNA. In vivo ligases probably act in concert with the DNA polymerases.

DNA ligases have been detected, isolated and characterised from several vertebrate species (90). Whereas bacteria have only one DNA ligase mammalian cells contain at least two ligases (91). DNA ligase I, the larger and more abundant of these enzymes, can occur in both a dimeric and monomeric form.

The mechanism of reaction of mammalian ligases is similar to that of the DNA ligase in E.coli (92). In the presence of cofactor the enzyme forms a complex with AMP. The adenylate moiety of the complex is then discharged in a reaction that required magnesium ions.

Although DNA ligases have not received as much attention as the polymerases these enzymes are likely to play a key role in DNA synthesis.

1.4.2.2. Helix-destabilising Proteins

Proteins which bind to single-stranded DNA in preference to double-helical DNA have been isolated from mammalian systems (93). These proteins markedly depress the helix-melting temperature of DNA. Unwinding protein from calf thymus (94) was found to stimulate DNA polymerase alpha. It was suggested that unwinding proteins caused this stimulation by inhibition of the formation of hairpin helices. The biological role of unwinding protein is still unclear.

1.4.3. DNA Polymerases of the Lower Eukaryotes

Unlike the genetic approach used with bacterial systems, it has not generally been possible to use mutants in investigating various aspects of eukaryotic DNA synthesis. However, with the haploid stage of the smut fungus Ustilago maydis it has been possible to develop a mutant, Pol 1, deficient in DNA replication (95). This mutant was

temperature sensitive for both cell growth and DNA synthesis. Pol-1 contained only 10-25% of the wild type DNA polymerase activity when cells are incubated at the restrictive temperature of 32°C. When DNA polymerase was purified from the strain Pol-1 grown at the permissive temperature of 22°C, the enzyme was found to be heat-labile.

The wild type enzyme was found to have a molecular weight of 180 - 200 000. The DNA polymerase operated optimally at pH 7.5 and 120 mM KCl. The activity was inhibited by N-ethylmaleimide and ethanol. A 3' to 5' exonuclease activity was always associated with the polymerase activity. The major Ustilago maydis DNA polymerase was inhibited by the zinc complexing agent o-phenanthroline. This was taken to mean that zinc was probably a component of the enzyme (96).

In addition to mitochondrial DNA polymerase two DNA polymerases, A and B have been characterized from Euglena (97). Both enzymes have an optimum pH of 7.2, and function best in 25 mM potassium and 2 mM magnesium. Both enzymes preferred manganese to magnesium and were inhibited by NEM.

The enzymes differed in that only the B form was able to use an oligo(dT)-initiated poly(A) template and possessed exonuclease activity which could hydrolyse single-stranded DNA and the RNA of the DNA.RNA hybrid.

A multiplicity of DNA polymerases has been observed in Paramecium (98), Chlamydomonas (99) and yeast (100). The DNA polymerases of Paramecium are similar to those of Euglena. Yeast contained two forms of DNA polymerases. Yeast enzyme I was found to resemble DNA polymerase alpha but yeast enzyme II was similar to prokaryotic DNA polymerase II and III. The two enzymes could be separated by chromatography on DEAE-cellulose, hydroxyapatite or DNA-cellulose (101). Both enzymes had a molecular weight of about 100 000. Enzyme I had no associated

nuclease activity but carried out pyrophosphate exchange and pyrophosphorolysis reactions. Enzyme II carried out pyrophosphate exchange and phosphorolysis and had an associated exonuclease activity. Whereas enzyme I could not use mismatched templates, enzyme II could excise mismatched 3'- nucleotides from suitable templates. Both enzymes were sensitive to NEM.

Only one DNA polymerase has been described in some primitive eukaryotes like Tetrahymena (102), Dictyostelium discoideum (103) and Chlorella (104). DNA polymerase from Tetrahymena pyriformis was found to have a molecular weight of 80 000. In solutions of low ionic strength the enzyme formed aggregates. With manganese as the metal ion the enzyme was able to copy RNA templates and extend oligodeoxynucleotide primers. Throughout all the stages of purification the enzyme contained associated DNAse activity. In this thesis, I report evidence for the multiplicity of the DNA polymerase activity of Tetrahymena.

The enzyme from Dictyostelium was found in all stages of growth and development including spores. Unlike DNA polymerase alpha of the higher eukaryotes the level of the polymerase in Dictyostelium did not vary widely with the rate of cell growth. The enzyme was completely inactivated by NEM.

The absence of an enzyme with any resemblance to DNA polymerase beta from the primitive eukaryotes probably indicates that this enzyme evolved later than DNA polymerase alpha. It has been suggested that this more recent appearance was possibly to meet some unique function in the more complex eukaryotes (103). This new function might be a special type of DNA replication - possibly repair. Until the roles of the different eukaryotic polymerases are properly worked out these ideas will remain conjectural.

1.4.4. Control of DNA Synthesis

The factors which control the initiation, maintenance and termination of DNA synthesis are still poorly understood. DNA replication and histone synthesis are closely coupled and are probably influenced by the same control mechanisms.

A possible clue to the control of DNA initiation is the observation that the number of active origins for DNA synthesis depends on the physiological state of the cell (1). There may be different initiator proteins which interact with different sets of origins which are defined by specific base sequences. Another possibility is that a single set of initiator proteins interacts with varying affinity with different sets of origins. It is also possible that origins are defined by the secondary structure of DNA and not simply by nucleotide sequence.

Many hormones, mitogens and mitotic inhibitors that control cell proliferation act by regulating the progression of cells from the G_1 to the S phase of the cell cycle and are presumed to affect the initiation of DNA synthesis. The effect of these substances on DNA synthesis is not direct (105). Many agents which cause cells to progress from G_1 to S first lead to increased RNA (rRNA) synthesis and chemical modification (acetylation and phosphorylation) of chromosomal proteins. These events occur before the initiation of DNA synthesis.

Work with various cell types shows that a given cell type traverses the S, G_2, and M phases at a constant time during every cycle. In contrast the length of the G_1 phase varies between individuals in the same population of cells. The current view is that the G_1 phase is comprised of a fixed part, B, and a variable one, the G_o or A part (106). It is the variability of the G_o phase which is responsible for the

Figure 1.9

Circular and linear diagrams of the eukaryotic cell cycle

All cells pass through a sequence of stages which are referred to as the cell cycle. Immediately after mitosis (M) daughter cells enter the G_1 phase. Some differentiated cells, such as the enucleated erythrocytes of frogs and birds and the neurones of all tetrapods, remain permanently in the G_1 phase. In tissues whose cells divide the G_1 phase is followed by the S period during which DNA synthesis occurs. After DNA synthesis a G_2 period may intervene prior to mitosis and cell division. The length of the G_1 and G_2 periods vary markedly between species. In some cell lines the G_1 phase is virtually absent. For a particular strain of cells the G_2 and M periods appear to be constant. The approximate durations for each phase of the cycle are one hour for the M phase, 1 - 8 hours for the G_1 phase, 6 - 8 hours for the S phase and 2 - 4 hours for the G_2 phase. Some cells continuously move around the cycle but others leave the cycle temporarily to enter the G_0 period. Cells in the G_0 phase can be induced to synthesize DNA and divide again. Some cells leave the cell cycle definitely and become destined to die without dividing again.

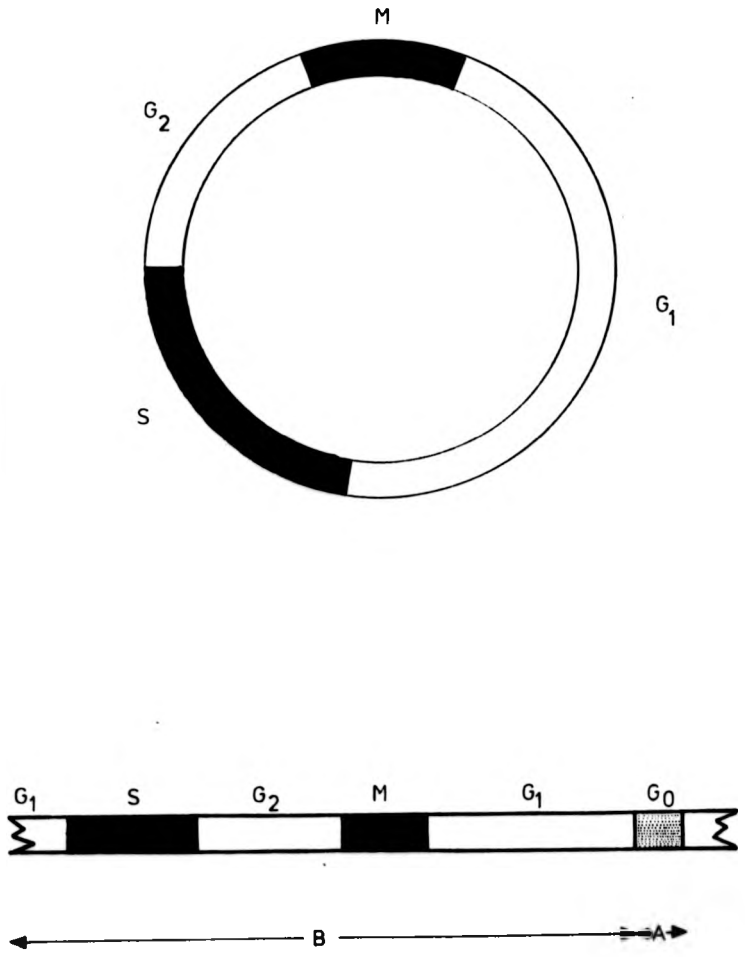


Figure 1.9. Diagram of the eukaryotic cell cycle

variability of the G_1 phase and for asynchrony of cell division in cell populations. It is not yet known whether the G_0 phase is in the early or late part of the G_1 phase. Details about the events which trigger the transition from A to B also remain unknown.

There is evidence that the cell's ability to initiate DNA synthesis is under positive control. In vitro studies with nuclei isolated from cells at different stages of the cell cycle show that differences in the rate of DNA synthesis between active and inactive cells are retained in nuclei isolated from these cells (107, 108). Nuclei from cells synthesising DNA rapidly incorporate precursors into DNA more rapidly than nuclei from inactive cells. Attempts to isolate protein factors which stimulate G_1 nuclei to synthesise DNA have not produced conclusive proof of the existence of these proteins (109).

2. Aims of the Project

In this project Tetrahymena pyriformis was used as the experimental organism. There are a number of advantages in working with this organism. Tetrahymena was the first protozoan to have its growth requirements fully characterized (110). Its nutritional requirements are similar to those of higher eukaryotes and the cells can be induced to grow in a synchronous fashion by a variety of simple techniques (111). The short generation time of about three hours and the asexual mode of replication of the amicronucleate strain used made Tetrahymena a convenient organism to work with.

Because of their central role in DNA cell replication, DNA polymerases are currently being studied as possible target enzymes in cancer chemotherapy. At present it seems that there are at least two DNA polymerases in the nuclei of higher eukaryotes (52, 53). The first, DNA polymerase alpha, is thought to be involved in DNA

replication. The second, DNA polymerase beta, is thought to be a DNA repair enzyme. No enzyme with properties similar to those of DNA polymerase beta has been detected in the lower eukaryotes (112). A primary aim of the project was to determine how many types of DNA polymerase could be detected in Tetrahymena and to determine their function. A secondary aim was to study the location of DNA polymerases in Tetrahymena cells. I hoped to develop a method of preparing nuclei which would not allow DNA polymerases to escape into cytoplasmic fractions.

A further primary aim of the project was to study the effect of AraC and AraCTP on DNA replication in Tetrahymena. Specifically the effect of AraC on the formation of intermediates in DNA replication was to be investigated. I also wanted to study the effect of AraCTP on the DNA polymerases of Tetrahymena. A clear understanding of the effect of AraC on DNA synthesis should contribute towards the more rational use of this drug in the treatment of cancer.

CHAPTER 2

2.1 THE EFFECT OF CYTOSINE ARABINOSIDE ON DNA SYNTHESIS

Cytosine arabinoside (AraC), a synthetic pyrimidine nucleoside, differs in its sugar moiety from the normal metabolites cytidine and deoxycytidine (Figure 2.1). The chemical properties of AraC have been reviewed (113) and recently the conformation of arabinosyl nucleosides has been studied (114). Soon after its discovery (115, 116) AraC was found to have antileukaemic (117) and antiviral properties (118). AraC is now one of the most effective drugs used to treat acute myelogenous leukaemia and other malignancies. The therapeutic value of AraC appears to derive from its ability to inhibit DNA synthesis (113) or from synthesis of altered DNA (119) resulting in cell death. It is unclear why AraC exhibits nonspecific toxicity to most eukaryotic cells but selectively kills fast replicating cancer cells in human and experimental animals.

Although AraC has continued to be used for treatment of some leukaemias in adults and children remission rates have been low. Improvement may be achieved by changing dose schedules to allow recovery of normal bone marrow and minimise immunosuppression. Effectiveness may also be improved by combination with certain alkylating agents and thiopurines.

2.1.1 Toxicity of AraC in Cells

Before AraC was used to treat cancer patients its toxicity in rodents, dogs and monkeys was tested (122). The main toxic effect was exerted against bone marrow. A similar effect was observed in humans (123). Human marrow cells developed marked chromosomal changes.

In bacteria large concentrations of AraC (1 mg/ml) are required for lethality (125, 126). Even strains which lack cytidine deaminase

Figure 2.1

Cytosine arabinoside and related natural nucleosides

Cytosine arabinoside differs from cytidine in the orientation of the hydroxyl group on the 2-carbon of the ribofuranose ring. Deoxycytidine does not have a hydroxyl group on this carbon.

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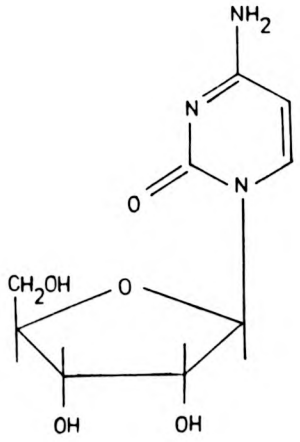
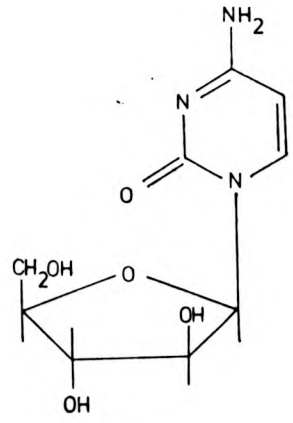
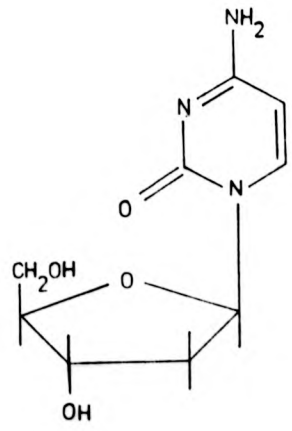


Cytidine



Cytosine arabinoside



Deoxycytidine

are not easily killed by AraC. In contrast to mammalian DNA polymerases E. coli DNA polymerase I is insensitive to AraCTP (127, 128).

Kim and Eidinoff found that in HeLa cells there was a lag in the lethal effect of AraC (132). They concluded that cells were accumulating prior to or at the beginning of the S phase of the cell cycle. When the drug was removed most of the cells entered mitosis synchronously. The synchronized cell populations were then used to study the stage at which cell death occurred in the presence of AraC. They found that the stage of cell death corresponded closely to the S phase. It has been suggested that the effectiveness of AraC in killing cells would be improved by using treatment schedules that maximize the proportion of cells passing through the S phase.

AraC does not prevent cells in G_2 from progressing to mitosis (133). The passage of cells from S to G_2 is affected to a greater extent than passage from G_1 to S (134). The greatest inhibition occurs during the last half of the S phase. This time corresponds to the period of maximal damage under the influence of AraC. It appears that the second half of the S phase is the most sensitive for cell kill which occurs when there are five or more chromatid breaks per cell. Introduction of only one or two breaks does not lead to detectable cell death (135).

In human cells AraC is thought to cause chromatid breakage in the G_1, G_2 and mitotic stages (136, 160). In these stages AraC is not an effective killing agent. At present the predominant view is that AraC kills cells by affecting semi-conservative DNA synthesis occurring during the S phase.

In fetal mice and rats AraC is teratogenic. AraC will transform hamster and rat cells in culture to the malignant state (138). The

transformations occur more easily with cells treated with the drug in the S phase than with G_1 arrested cells. The transforming and inhibitory effects of AraC in tissue culture and in intact animals can be prevented by deoxycytidine (120, 121, 137).

It appears that AraCTP is the major toxic agent formed from AraC. A subline of L1210 leukaemia which is resistant to AraC is unable to metabolise AraC or deoxycytidine (138). Development and maintenance of lethal intracellular concentrations of AraCTP depend on such factors as the rate of uptake of AraC, the rate of deamination or other degradation, the rate of phosphorylation and the turnover of AraCTP. As the external concentration of AraC decreases, the turnover of AraCTP in the cell will decrease the intracellular concentration of this toxic product and allow survival and multiplication of temporarily inhibited cells.

2.1.2. Metabolism of cytosine arabinoside (Figure 2.2)

2.1.2.1. Transport

Nucleosides such as AraC will pass across the cell membrane and will accumulate in cells in the form of their 5'- phosphates. Deaminated nucleosides do not accumulate but are rapidly excreted (138). In L1210 cells, purine and pyrimidine nucleosides inhibit the entrance of AraC but only pyrimidines inhibited the rapid exit of AraC (161).

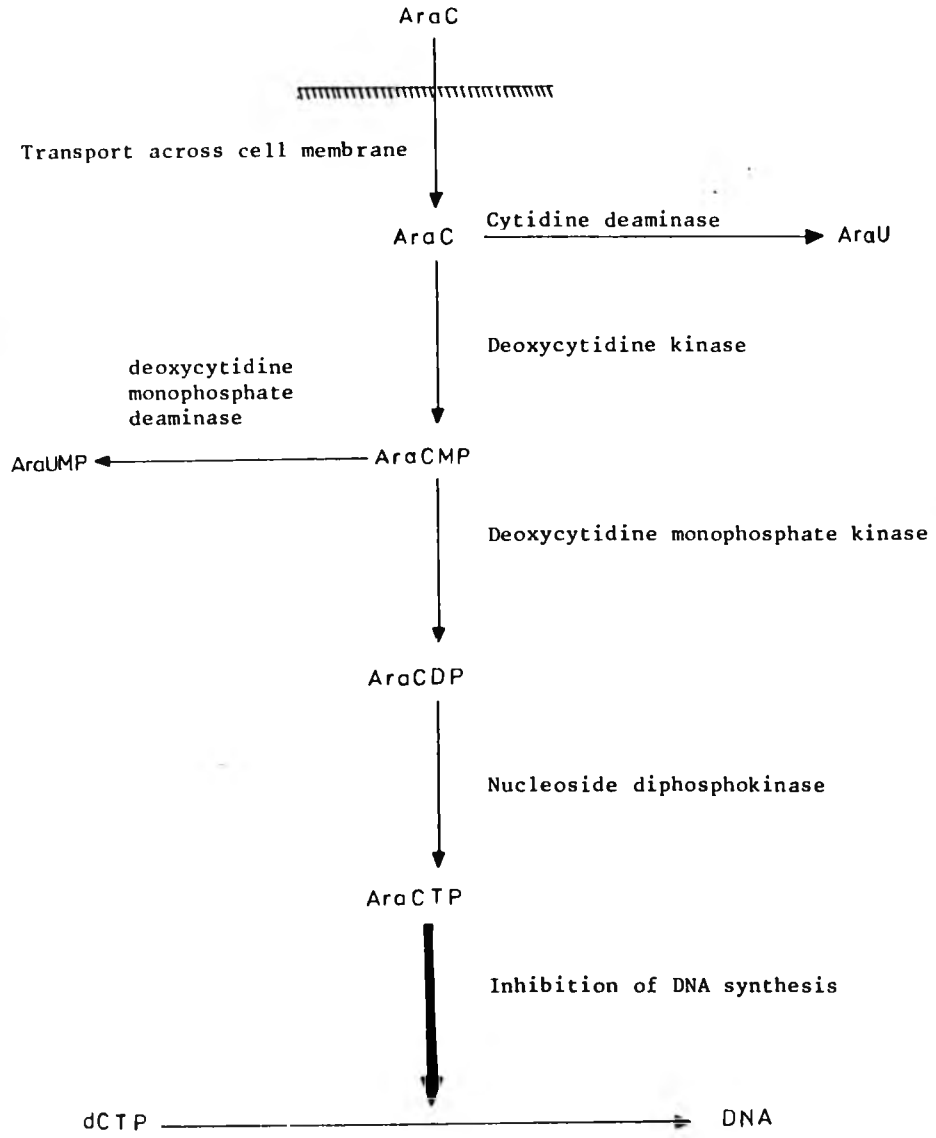
2.1.2.2. Deamination

Early studies showed that under the usual conditions of administration to humans and various experimental animals, AraC is rapidly converted by cytidine deaminase to AraU. AraU may be excreted as such in urine or it may be cleaved to uracil. About 5% of AraC is excreted unchanged. In rats which have a low deaminase activity, about 70% of the drug was excreted unchanged (162).

Figure 2.2

Diagram showing the metabolism of cytosine arabinoside in cells

After passing across the cell membrane AraC may be converted to AraCMP by deoxycytidine kinase. AraC may also be deaminated by cytidine deaminase to produce nontoxic AraU. AraCMP is further phosphorylated by deoxycytidine monophosphate kinase to yield AraCDP. A non-specific nucleoside diphosphokinase then phosphorylates AraCDP to produce AraCTP. The current view is that it is the AraCTP, an analogue of dCTP, which then interferes with DNA synthesis.


AraC
Transport across cell membrane
AraC
Cytidine deaminase
AraU
Deoxycytidine kinase
deoxycytidine
monophosphate
deaminase
AraUMP
AraCMP
Deoxycytidine monophosphate kinase
AraCDP
Nucleoside diphosphokinase
AraCTP
Inhibition of DNA synthesis
dCTP
DNA

In HeLa cell cultures, exposure to AraC causes a fourfold increase in cytidine deaminase activity even in the presence of the inhibitor of protein synthesis, cycloheximide (147). Cytidine deaminase may, therefore, be involved in resistance to AraC by certain cells (148).

During a systematic search for inhibitors of cytidine deaminase a potent inhibitor, tetrahydrouridine (THU) was discovered (149, 124). Tetrahydrouridine inhibits human liver cytidine deaminase with a K_i of 1.3×10^{-7} M. At high concentrations (10^{-4}) THU inhibited AraC (150) phosphorylation by a human liver preparation.

2.1.2.3. Phosphorylation

AraC is converted to AraCTP by three successive enzymic reactions (163). Initially AraC is converted to AraCMP by deoxycytidine kinase. Further phosphorylation by deoxycytidine monophosphate kinase yields AraCDP. Finally a nucleoside diphosphokinase converts AraCDP to AraCTP. In mice injected with L1210 ascites cells, the tissue level of AraCTP appears to determine cellular toxicity and chemotherapeutic efficacy of AraC (140). It is probably significant that the leukaemic cells of AraC-treated cells have AraCTP concentrations ten times higher than normal cells. AraUMP was detected in the monophosphate fraction from leukaemic mice (140). This may have been produced from the phosphorylation of AraU or the deamination of AraCMP (164).

2.1.3 Resistance of cells to AraC

Leukaemic cells from patients treated with AraC often develop resistance to the drug if they survive the first dose of the drug. There are at least three different hypotheses to explain the appearance of resistant cells. The development of resistance to AraC may be caused by a decrease in the kinase capable of phosphorylating AraC

(141, 142, 143). In some resistant cell lines it was found that there remained in the mitochondria a kinase capable of phosphorylating deoxycytidine but not AraC (165). Some drug resistant cells not only lack an AraC kinase but also have increased levels of ribonucleotide reductase or an insensitivity of this enzyme to dATP (144). This could lead to increased levels of protective dCTP (145). The resistance of some cells to AraC could also be associated with their ability to deaminate this drug to the nontoxic derivative AraU.

2.1.4 The mode of action of AraC

AraC at high concentrations can potentially interfere with many different biochemical processes. At low concentrations it inhibits DNA synthesis fairly specifically. A number of proposals have been put forward to explain the action of AraC in inhibiting DNA synthesis and causing cell death. Some of these are discussed below.

2.1.4.1. Inhibition of cytidine reductase

After it was observed that deoxycytidine prevented toxicity by AraC it was suggested that AraC or one of its metabolites exerted its effect by inhibiting ribonucleotide reductase (120). This enzyme converts CDP to dCDP. However, neither AraCDP nor AraCTP are inhibitors of mammalian ribonucleotide reductase (151). Also in L cells inhibited by AraC, normal levels of dCTP accumulate (152). It, therefore, appears that neither AraC nor any of its derivatives cause cell toxicity by inhibiting ribonucleotide reductase.

2.1.4.2. Inhibition of DNA polymerases

It has been suggested that AraC caused cell death by inhibiting DNA polymerases and thus inhibiting DNA (153). It is proposed that AraC, after conversion to AraCTP, competes with dCTP as a substrate for DNA polymerases. AraCTP is a powerful inhibitor of DNA polymerases

in both normal and tumour cells (152, 128, 154). The observation that AraC does not interfere with the synthesis of dCTP and dTTP from exogenously supplied deoxynucleosides, but inhibits their incorporation into DNA (152) is in agreement with the suggestion above.

Although AraCTP competitively inhibits dCTP utilization by the polymerases it does not inhibit polyd(A-T) synthesis by tumor DNA polymerase (155). In general the inhibition of cellular and viral DNA polymerases by AraCTP is dependent on the template used. DNA polymerase alpha is more sensitive to AraCTP than DNA polymerase beta (162, 167).

2.1.4.3. Incorporation into nucleic acids

Finally it is possible that AraC kills cells by being incorporated into DNA. If AraCTP is incorporated into DNA chains it could occur within polynucleotide chains or only in terminal positions where it would block extension of the DNA chains.

Early reports claiming that AraC was incorporated into DNA were received with scepticism partly because the separation conditions were not always ideal and only small amounts of the applied isotope were recovered in DNA (141, 156, 157). The AraC used was of high specific activity. Further cytidine deaminase and nucleoside phosphorylase could degrade AraC to uracil and cytosine significant amounts of which could enter RNA and DNA. The early results have been supported by improved chromatography and by the demonstration in vitro that AraCTP was incorporated into DNA by DNA polymerases (152, 158, 154).

Studies on mammalian DNA with AraCTP in the absence of dCTP support the hypothesis that AraC is incorporated into terminal position in nascent DNA (159). When L cells were treated with AraC

to kill a high proportion of the cells after centrifugation and washing the surviving cells were still capable of incorporating thymidine into DNA at normal rates (130). This was taken to mean that AraC did not terminate DNA chains. However, other workers found that most of the AraC was incorporated into alkali-resistant fractions (152, 158).

At present the hypothesis that AraC inhibits DNA synthesis and causes cell death by competitive inhibition of DNA polymerases and by incorporation onto DNA appears to be the most reasonable.

2.1.5. The effect of cytosine arabinoside on RNA and protein synthesis

Claims that AraC is incorporated into RNA are unconvincing. The RNA polymerases studied so far cannot incorporate AraCTP into RNA (127, 128). In murine leukaemic cells (151784) mastoma cells in culture and mouse fibroblast L cells, AraC inhibited DNA synthesis without inhibiting RNA or protein synthesis (129, 130). In Ehrlich ascites carcinoma cells inhibition of DNA synthesis by AraC was accompanied by a depression of glycine incorporation into histones (131).

2.1.6. Formation of 1-beta-D-Arabinofuranosylcytosine diphosphate choline

Paterson and co-workers (168) demonstrated that AraCTP and AraCDP-choline were the major metabolites in RPMI 6410 cells (a line of cultured human myeloblastoid cells). They showed that the formation of AraCDP-choline was preceded by the formation of AraCTP. The formation of AraCDP-choline is catalyzed by phosphorylcholine cytidylyltransferase.

$$\text{AraCTP} + \text{phosphorylcholine} \longrightarrow \text{AraCDP-choline} + PP_i$$

2.2. Effect of AraC on DNA Synthesis in Tetrahymena

Although AraC has been shown to be an inhibitor of DNA synthesis

the detailed mechanism of its action remains unclear. An inhibitor of DNA synthesis could interfere with the initiation of replication units, the initiation of Okazaki fragments, the elongation of nascent chains, the joining of Okazaki fragments or any of the reactions involved in the biosynthesis of DNA precursors. In this chapter studies on the effects of AraC on DNA synthesis in Tetrahymena are described.

2.2.1 Materials and Methods

2.2.1.1. Source of materials used

Cytosine-1-β-D-arabinoside and calf thymus DNA, were obtained from Sigma Chemical Company. Proteose peptone and yeast extract were obtained from Difco. (Methyl-3H)-thymidine was obtained from the Radiochemical Centre at Amersham or from ICN. (2-14C)-thymidine, (5-3H)-cytosine-β-D-arabinoside, and L-(methyl-3H)-methionine were supplied by the Radiochemical Centre. NCS tissue solubilizer was bought from Hopkin and Williams. Sarkosyl was generously donated by Industrial Chemical Division of Ciba-Geigy Plastics and Additives Company. Glass Fibre filters (GF/C, 2.5 cm) were obtained from Whatman.

2.2.1.2. Culture of Cells

Tetrahymena pyriformis, amicronucleate strain W, was grown at 28°C in a defined medium (DPY) with low concentrations of phosphate (169). Uridine was omitted from the medium. Proteose peptone and yeast extract were added to a final concentration of 0.04% and 0.004% respectively (170). The detailed composition of this medium is shown in Appendix I. The medium was used for all the experiments described in this chapter except the one where the effect of AraC on cell replication was studied. In this case the cells were grown in medium YY which contained proteose peptone (2%), yeast extract (0.1%),

glucose (0.5%) and $FeCl_3.6H_2O$ (5 microgram/ml).

2.2.1.3. Cell breakage

Cells were disrupted either by mechanical homogenisation or by sonication. For homogenisation a motor-driven Potter-Elvehjem homogeniser was used. The cells were cooled in ice and homogenisation continued until most of the cells were disrupted. The extent of disruption was checked under the microscope.

Mechanical homogenisation was not always satisfactory. It was difficult to achieve complete disruption of cells, the homogenate had a tendency to foam and it was difficult to work out a standardised procedure. Sonication was adopted as a better way of breaking cells. The cells were disrupted in an MSE sonicator at maximum amplitude and medium power. Sonication was done in three bursts each lasting five seconds with thirty-second intervals in between to allow the broken cell suspension to cool in a beaker filled with ice. With this method all the cells were broken, there was no problem with foaming and it was easy to standardise working conditions. A disadvantage of this method was that the cell mixture tended to heat rather more than with mechanical homogenisation.

2.2.1.4. Determination of radioactivity

When determining radioactivity in aqueous samples, 200-400 microlitres of the sample was placed in scintillation vials containing a toluene based scintillation cocktail. The scintillation cocktail contained in one litre, toluene (400 ml), ethoxyethanol (600 ml), PPO (4 g), and POPOP (0.2 g).

For samples from alkaline sucrose gradients 4 ml of scintillation cocktail was added to the dissolved DNA protein mixtures. The scintillation cocktail contained per litre of toluene, PPO (4 g) and

POPOP (0.2 g). Radioactivity was determined on a Packard scintillation spectrometer at the $^{3}H/^{14}C$ setting. The calculation of the respective corrected counts for ^{3}H and ^{14}C in dual-labelling experiments is shown in Appendix IV.

2.2.1.5. Effect of AraC on cell multiplication

For this experiment cells were grown in standard medium YY described in the section on materials and methods. Cells of concentration 5 x 10^{5} cells/ml were divided into three portions. The first aliquot of cells was not treated with AraC and acted as the control. The other cells were treated with AraC to give final concentrations of 0.1 mM and 1mM respectively. The cells were then incubated at 28°C in an orbital shaker set at a shaking rate of 150 oscillations per minute. Every three hours the cell density was determined using a Neubauer haemocytometer.

2.2.1.6. Effect of AraC on protein synthesis

Protein synthesis was determined by measuring the incorporation of labelled methionine into trichloroacetic acid insoluble fractions. Cells were grown overnight in DPY. The cell cultures (1 x 10^{4} cells/ml) were divided into aliquots. Cells not treated with cytosine arabinoside acted as the control. The other cells were treated with AraC to give the desired final concentrations. Labelled methionine (0.6 microcurie/ml, specific activity 12 Ci/mmol) was then added and the cells incubated at 28°C in an orbital shaker. At intervals samples (one millilitre) were removed from each group of cells and added to 5% trichloroacetic acid (one millilitre). The mixture was filtered on Whatman glass fibre discs.

After washing with trichloroacetic acid and absolute ethanol the discs were dried and placed in vials containing the toluene/PPO/POPOP

scintillation cocktail used for counting the radioactivity on glass fibre discs.

2.2.1.7. Effect of AraC on the incorporation of thymidine into DNA

Cells were grown for twenty-one hours in 100 ml of defined medium, DPY. The cells used for inoculation were taken from cultures grown in standard medium, YY with cell concentrations of about 1.5×10^5 cells/ml. (Methyl-^{3}H)-thymidine (0.1 microcurie/ml, specific activity 42 Ci/mmol) was added and the cell culture divided into four 25 ml fractions. Each fraction was treated with cytosine arabinoside to give the desired final concentration. At thirty minute intervals one millilitre samples were removed from the cell cultures and added to 5% trichloroacetic acid (1 ml). The mixtures were filtered on Whatman glass fibre filter discs. The filters were washed five times with 5% trichloroacetic acid and twice with 96% ethanol. After drying the filters were placed in vials containing the toluene/PPO/POPOP scintillation cocktail used for the determination of radioactivity on glass fibre discs.

2.2.1.8. Sedimentation of DNA intermediates on alkaline sucrose gradients

For experiments in which alkaline sucrose gradients were used cells were grown in a medium containing low concentrations of proteose peptones and yeast extract, DPY (see Appendix I). In standard experiments the cells were prelabelled overnight with (^{14}C)-thymidine (0.2 microcurie /ml, specific activity 54 Ci/mmol). At the beginning of the experiments the cells were divided into three fractions. After adding AraC to each group of cells to the required concentration the cells were incubated with the drug for thirty minutes at 28°C. After this time the cells were pulsed with (^{3}H-methyl)-thymidine (1 microcurie/ml, specific activity 42 Ci/mmol) for thirty minutes. The cells were

chilled in ice and harvested in a bench centrifuge. The cells were then washed in ice-cold buffer (NaCl, 500 mM/EDTA, 50 mM/Tris HCl pH 7.2, 50 mM). In the early experiment the cell pellet was resuspended in distilled water and applied to the top of an alkaline sucrose gradient (5 - 20%). At the bottom of each gradient was a 4 ml cushion of 40% sucrose. 400 microlitres of sarkosyl (1.25% in 1 M sodium hydroxide) was added and the gradients were left at 4°C for thirty minutes.

In the later experiments, the cells suspended in distilled water were placed in a small (5 ml) wide-mouthed conical flask. After adding 400 microlitres of sarkosyl the flask was gently swirled once or twice. The lysing cells were gently applied to the top of alkaline sucrose gradients. The gradients were left for twenty minutes at 4°C. This procedure resulted in a more uniform lysis of the cells than the method described above.

The gradients prepared by the methods described above were centrifuged in a Beckman SW 25.2 rotor at 24 000 rpm for twenty-two hours at 4°C. Fractions (2.5 ml) were collected using a modification of the unit described by Oumi and Osawa (171, 172). The construction of the unit is shown in Appendix II. Into each fraction was added 400 microlitres of a solution containing calf thymus DNA (100 micrograms /ml) and bovine serum albumin (560 microgram/ml). 5% Trichloroacetic acid (1 ml) was then added to each fraction and the mixture was left to stand overnight at 4°C. Leaving the mixture to stand overnight in the cold room improved the filtration of the acid-insoluble precipitate. The precipitates were filtered by suction on Whatman glass fibre discs. The discs were washed five times with two ml volumes of 5% trichloroacetic acid. Suction was continued until most of the water was removed but the discs were still moist.

The moist discs were placed in scintillation vials and one millilitre of NCS (diluted 1 in 3 in toluene/PPO/POPOP scintillation liquid) was added. The contents were left to stand at room temperature for about two hours. When the DNA and protein precipitate had dissolved 4 ml of scintillation cocktail was added and radioactivity counted.

Sedimentation coefficients were estimated according to the method of McEwen (173). The detailed procedure for calculating sedimentation coefficients by this method is outlined in Appendix III.

2.2.1.9. Characterization of thymidine incorporation as a measure of DNA synthesis

Cells were labelled overnight with (^{14}C)-thymidine (0.02 microcurie/ml specific activity 54 Ci/mmol). The cells were harvested by centrifugation at 5 000g and the cell pellet resuspended in distilled water in a total volume of 5 ml. The cells were then broken by sonication. DNA was separated from the other cell fractions (174). 2.5 ml of ice-cold perchloric acid (0.6 M) was added to the sonicated cells and the mixture allowed to stand for ten minutes. The mixture was then centrifuged in a bench centrifuge and the supernatant discarded. The precipitate was washed twice with 5 ml cold perchloric acid (0.2 M). After decanting the perchloric acid and allowing the tube to drain for a few minutes, 4 ml of potassium hydroxide solution (0.3 M) was added and the mixture incubated at 37^{o}C for one hour to dissolve all the solid materials. The tube was then chilled in ice for fifteen minutes. After adding 5 ml perchloric acid (0.6 M) the mixture was centrifuged and the supernatant kept. The precipitate was washed twice with 5 ml cold perchloric acid (0.2 M). The three supernatants were combined. The precipitate was made up to 0.5 ml with distilled water.

The radioactivity in the supernatant and in the pellet was determined. Because RNA is sensitive to alkaline hydrolysis and DNA

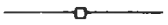
Figure 2.3

Effect of AraC on cell multiplication in Tetrahymena

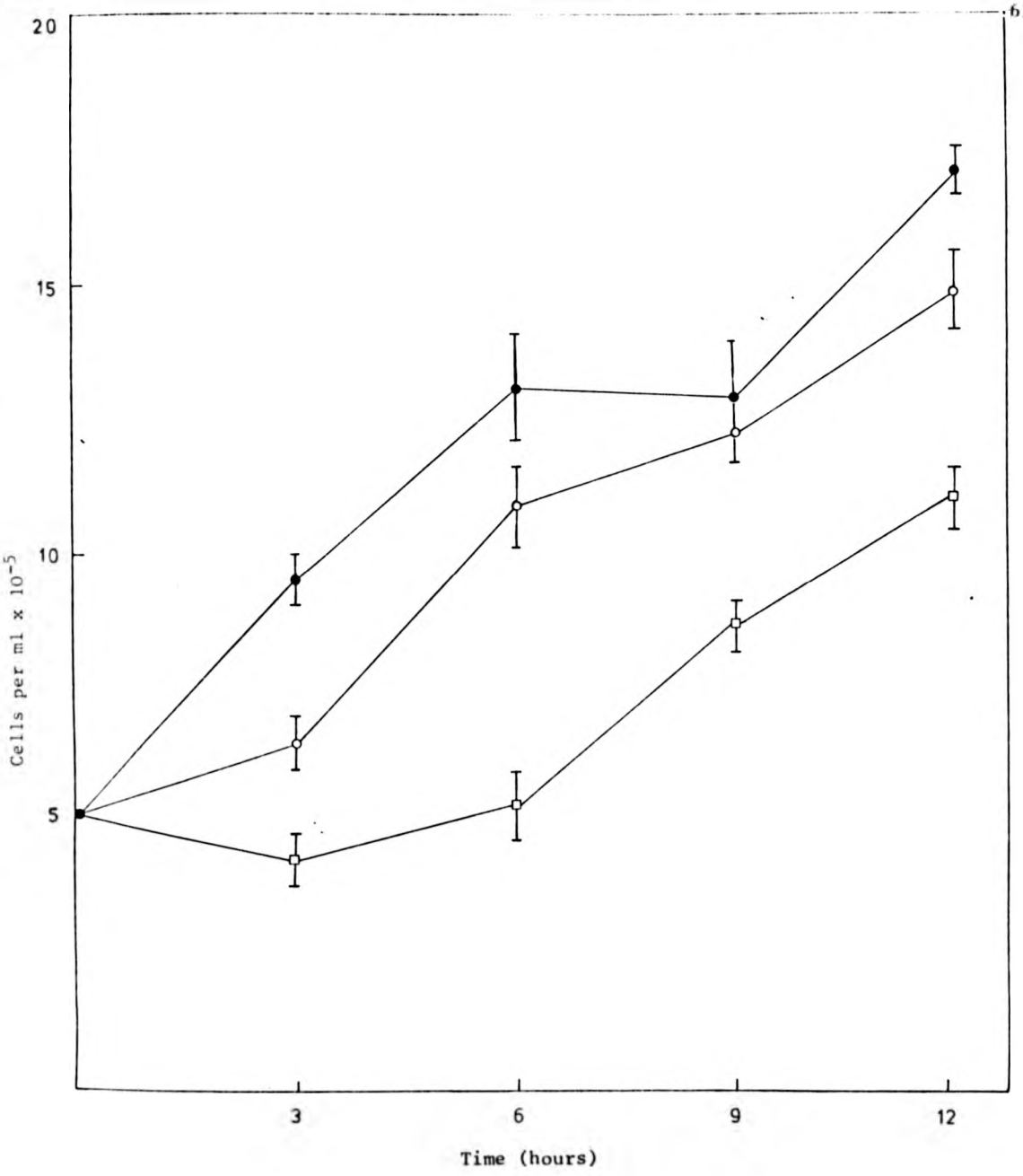
Cells were incubated with and without AraC at 28°C. At three hour intervals cells were removed and the cell density determined using a Neubauer haemocytometer.

Control

AraC (0.1 mM)



AraC (1 mM)


20
15
10
5
Cells per ml x 10^{-5}
3
6
9
12
Time (hours)

is not the radioactivity in the supernatant corresponds to RNA and that in the precipitate to DNA.

2.2.2. Results and Discussion

Unless otherwise indicated, in this and subsequent chapters all the results reported were obtained from at least two experiments each of which was done in duplicate.

2.3.1. Effect of AraC on cell multiplication

The effect of AraC on cell multiplication in Tetrahymena is shown in Figure 2.3. At concentrations below 0.1 mM, AraC did not produce noticeable inhibition of cell multiplication in Tetrahymena. At a concentration of 1 mM AraC effectively stopped cell division for about six hours (two generations). After this time the cells started to divide again at a nearly normal rate. Lower concentrations of the drug inhibited cell replication for shorter periods before division resumed at a normal rate.

For AraC to stop cell replication an inhibitory concentration of the drug must be maintained within the cell (162). The maintenance of this concentration of AraC depends on the rate at which the drug is passed through the cell membrane, the rate at which it is converted to inactive products after absorption and the rate at which it is phosphorylated.

The ability of cells to recover from the effects of even high concentrations of AraC may provide a clue to the loss of effectiveness of this drug when it is used in clinical situations. It is unlikely that recovery is associated with interference with the passage of AraC into cells. Passage of AraC and other nucleosides into cells appears to be free and unhindered and does not involve active transport (162).

The recovery could be due to an acquired resistance to the drug by cells or to the cellular concentration of the drug falling below that required to inhibit cell division. Cellular AraC could be inactivated by conversion to AraU by a cytidine deaminase (175).

2.3.2. Effect of AraC on DNA synthesis

The performance of and the interpretation of results from experiments with alkaline sucrose gradients requires some care. After lysis of cells by sarkosyl in the alkaline sucrose solution DNA is released and progressively dissociated from the other cell components. At the alkaline pH complementary strands of DNA separate. Both lysis and strand separation depend on cell type, cell concentration, time, temperature and ionic strength (176). Exposed DNA is sensitive to degradation by mechanical shearing forces. In alkaline solution DNA is also affected by visible light (177).

A completely different problem is the evaluation of sedimentation coefficients. Calculations are generally based on the method described by Studier (178). Under certain conditions drag and centrifugal forces can distort the random coil configuration of DNA. The apparent sedimentation coefficients determined will then be less than predicted by Studier's equation (179).

The effect of AraC on DNA in Tetrahymena is shown in Figures 2.4 and 2.5. When cells are pulsed with tritiated thymidine in the absence of AraC the tritium label appears in DNA of all sizes as shown in Figure 2.3. When the cells are pulsed with tritiated thymidine in the presence of AraC (0.5 mM) the label appears in DNA of high and intermediate molecular weights. In Figure 2.4 it can be seen that only a small fraction of the label is found in low molecular weight DNA.

Two apparently conflicting results have been reported of the effect of AraC on DNA synthesis. Dijkwel and Wanka (180) reported

Figure 2.4

Analysis of DNA intermediates on alkaline sucrose gradients

Cells, prelabelled with ^{14}C, were pulsed with labelled thymidine for thirty minutes in the absence of AraC. After harvesting the cells by centrifugation the cell pellet was placed onto an alkaline sucrose gradient (5 - 20%) and the cells lysed with sarkosyl. After centrifugation in an SW 25.2 rotor fractions were collected as described in the text. DNA in each fraction was precipitated with TCA and filtered onto Whatman glass fibre discs. Radioactivity was counted after dissolving the DNA precipitates in NCS. Although the experiment was done several times the results reported here are from one typical set of results. Variation in the number and size of the fractions collected made it impossible to combine results from different runs.

—●— (^{3}H)-DNA

—○— (^{14}C)-DNA

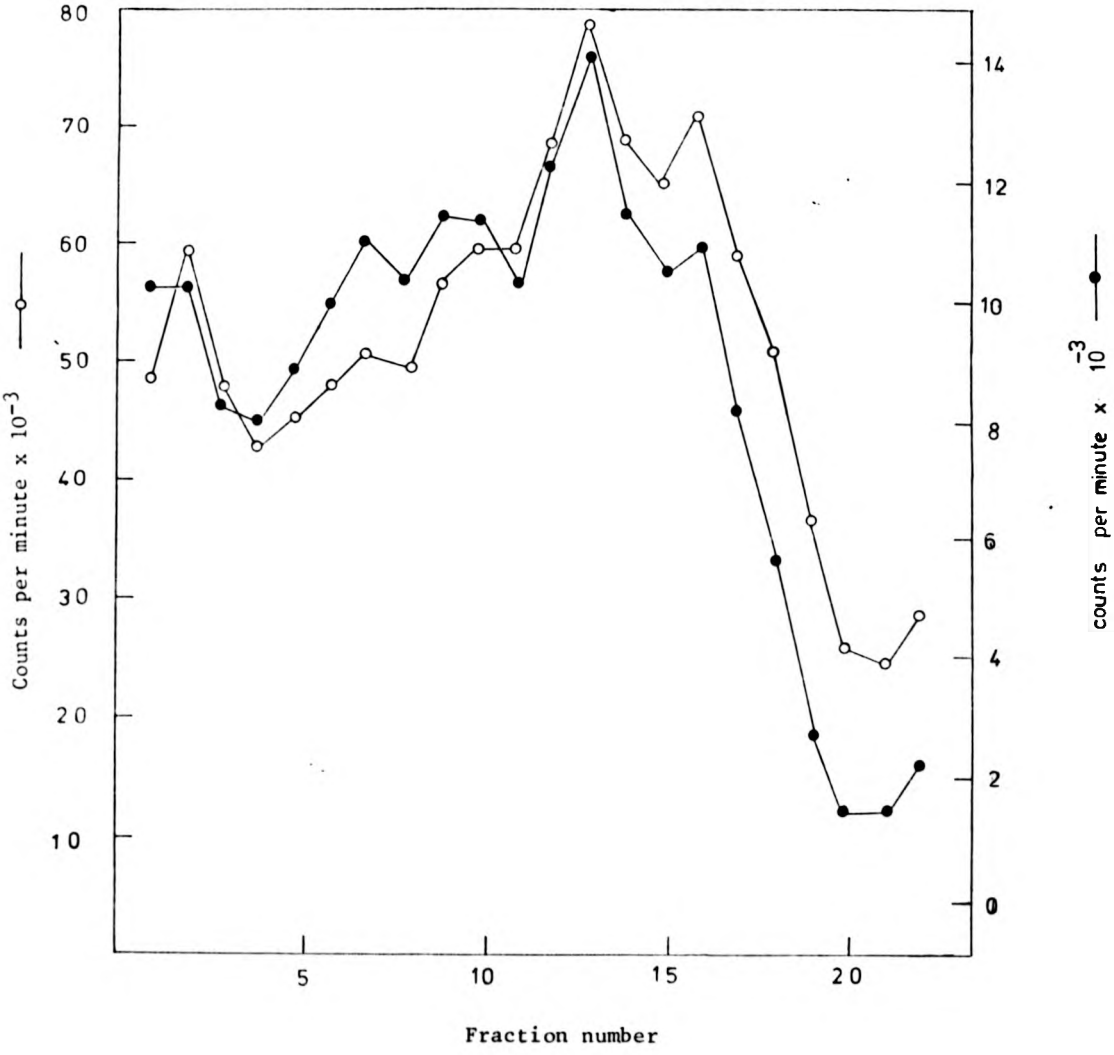

80
70
60
50
40
30
20
10
Counts per minute x 10^{-3}
14
12
10
8
6
4
2
0
counts per minute x 10^{-3}
5
10
15
20
Fraction number

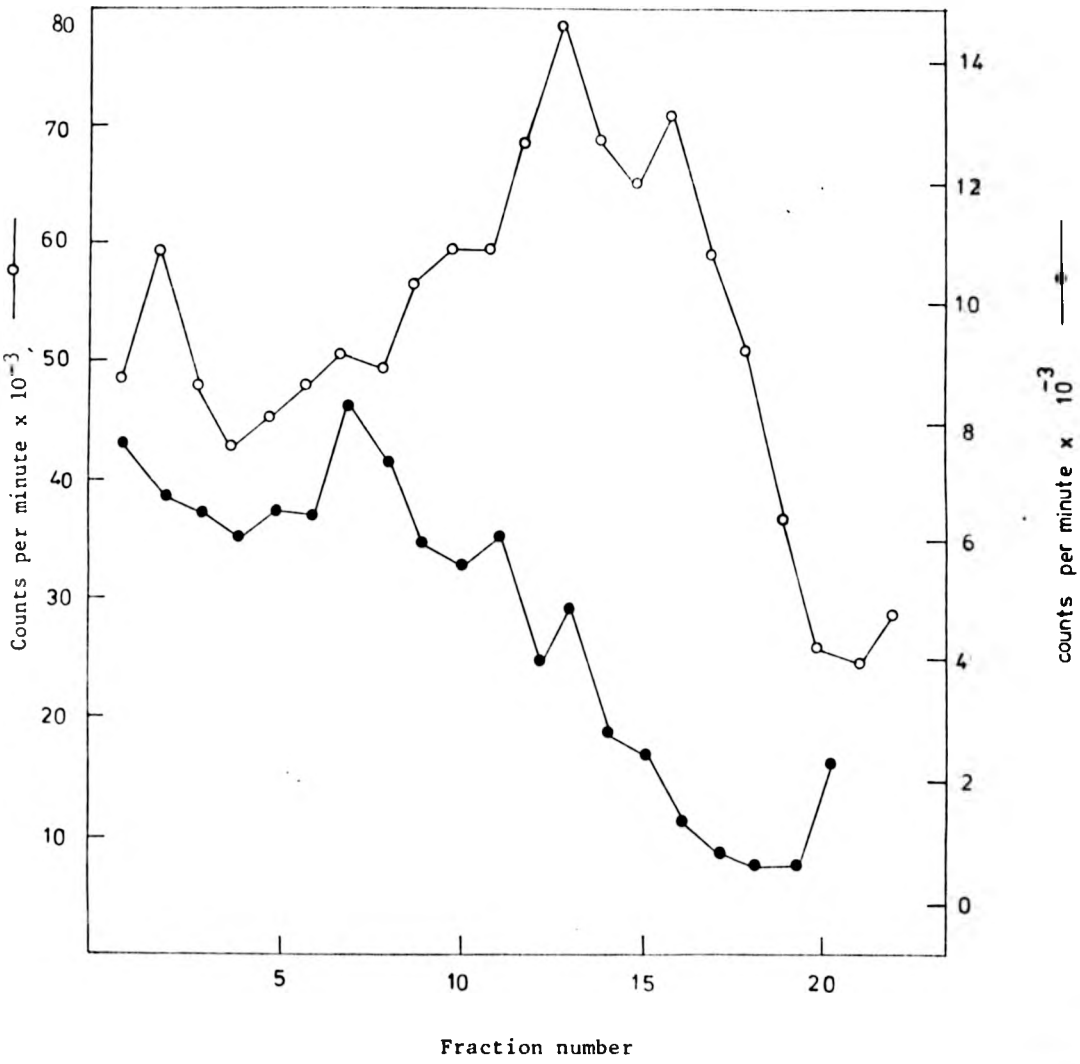
Figure 2.5

Effect of AraC on the formation of DNA intermediates

Cells prelabelled with ^{14}C were treated with AraC (1 mM) for thirty minutes. The cells were then pulsed with(^{3}H)Td for thirty minutes and then treated as described in Figure 2.4.

(^{3}H)-DNA

(^{14}C)-DNA


Counts per minute x 10⁻³
80
70
60
50
40
30
20
10
14
12
10
8
6
4
2
0
counts per minute x 10⁻³
5
10
15
20
Fraction number

that in calf liver cells AraC caused a preferential inhibition of chain polymerisation rather than termination of nascent chains. However, Fridland observed that in human lymphoblasts AraC had no effect on DNA chain elongation or the joining of molecules that had already been initiated (181). He concluded that AraC inhibited the initiation of DNA replication units.

In this work I observed the depletion of low molecular weight DNA but not of DNA of high and intermediate molecular weight. These results indicate that in Tetrahymena, AraC inhibits the initiation step of DNA synthesis. Chains that have already started to replicate do not appear to be affected by AraC.

2.3.3. Effect of AraC on the incorporation of labelled precursors into DNA and protein

Figures 2.6 and 2.7 show the effect of AraC on the incorporation of labelled thymidine and methionine into DNA and protein respectively. Low concentration of AraC did not affect the incorporation of thymidine into either DNA or protein. At higher concentration (greater than 0.25 mM) the rate of DNA synthesis was depressed. Even very high concentrations (6mg/25ml) had practically no effect on protein synthesis during the three-hour duration of the experiments. This shows that the effect of AraC is directly on the DNA replicating process rather than indirectly on protein synthesis.

2.3.4. Thymidine incorporation as a measure of DNA synthesis

From Table 2.1 it can be seen that when cells are treated with labelled thymidine about 90% of the label appears in the DNA fraction. Little of the label is found in RNA. The 10% of the labelled thymidine not recovered in DNA may be in other metabolic pools. The observed distribution of radioactivity indicates that on the whole thymidine incorporation can be taken as a reasonable measure of DNA synthesis.

Figure 2.6

Effect of AraC on the incorporation of (^{3}H)methionine into protein

Protein synthesis was measured by determining the amount of labelled methionine incorporated into acid-insoluble fractions. The incorporation of labelled methionine was measured in the presence and absence of AraC.

—o— Control

—•— AraC (1 mM)

In this graph and those that follow the experimental points plotted are means plus or minus the standard deviation which is indicated by error bars.

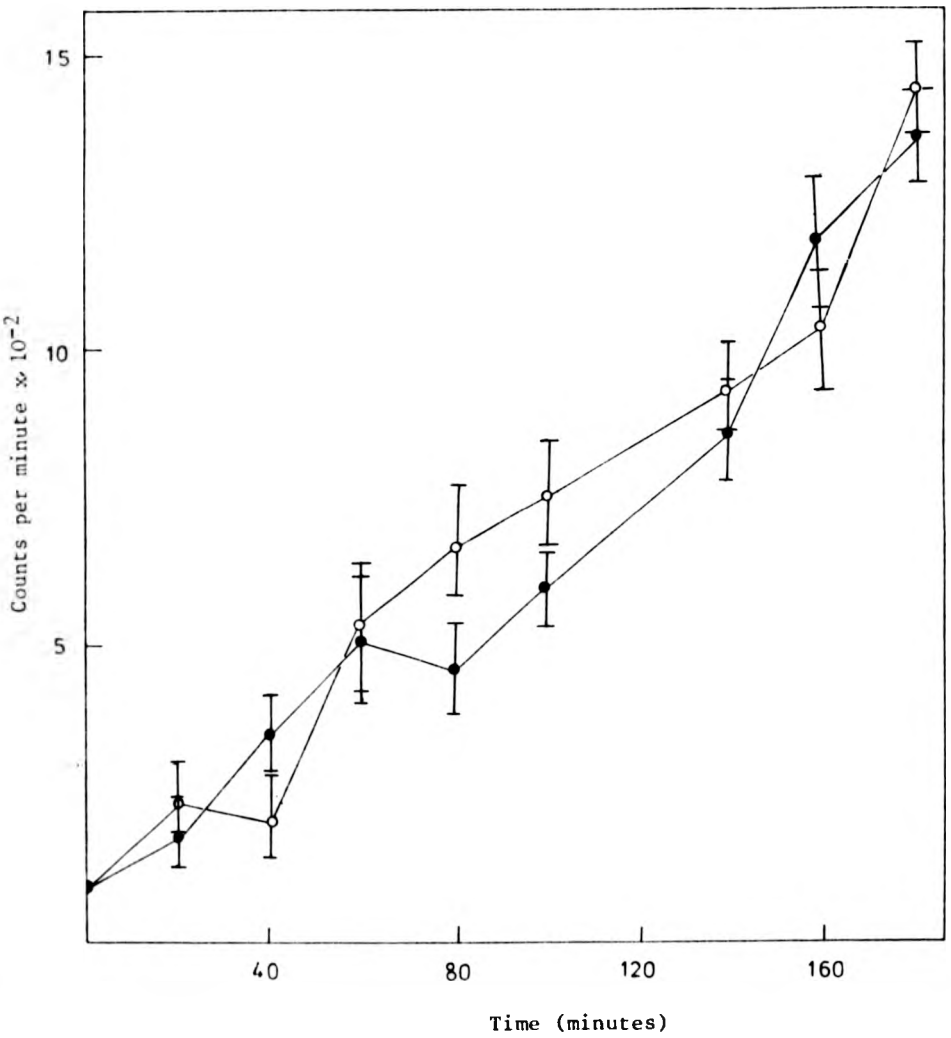
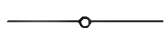

15
10
5
Counts per minute x 10⁻²
40
80
120
160
Time (minutes)

Figure 2.7

Effect of AraC on the incorporation of (methyl-^{3}H)thymidine into DNA

DNA synthesis was determined by measuring the incorporation of labelled thymidine into acid-insoluble fractions. At concentrations lower than 10^{-4}M AraC did not produce detectable inhibition of DNA synthesis as measured by thymidine incorporation.



Control

AraC (6.25×10^{-5}M)

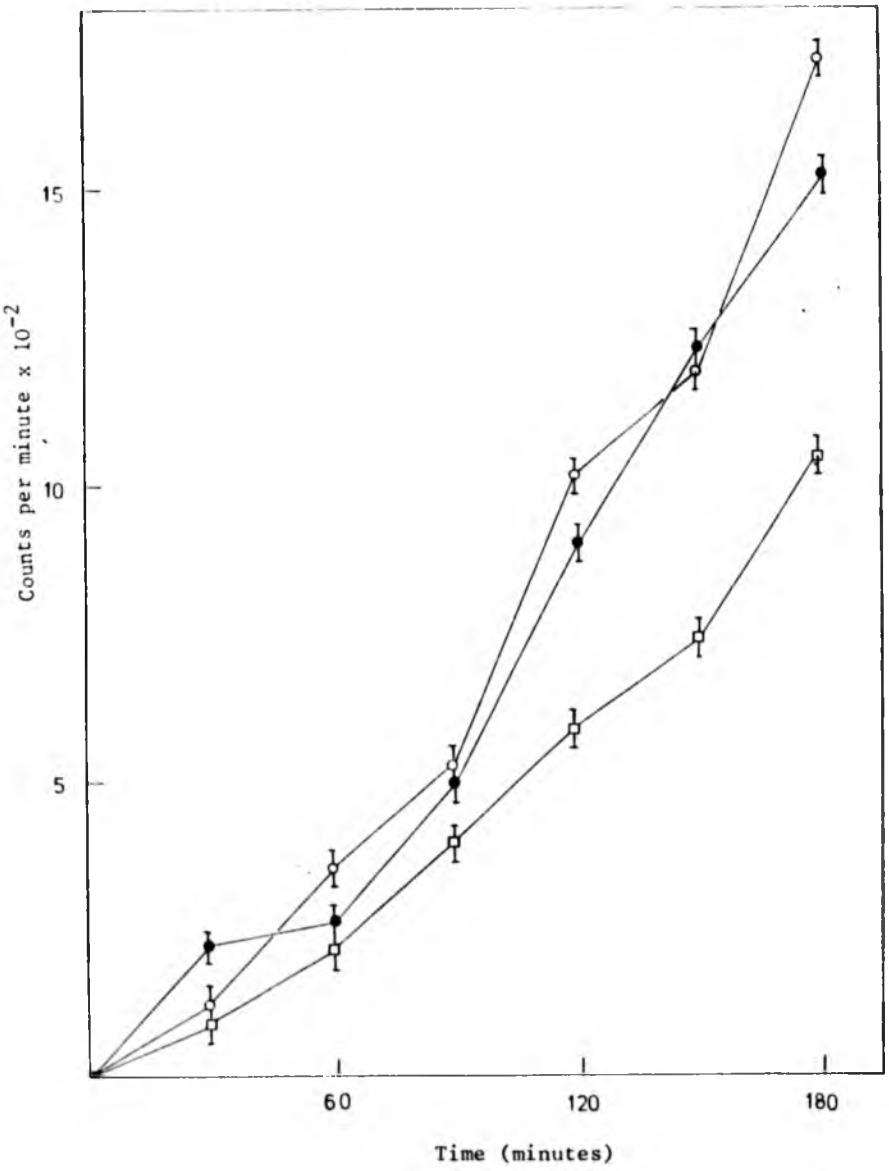
AraC (2.5×10^{-4}M)

into DNA

on of

rations

of DNA



Fraction	Percent radioactivity
Whole cells	100
DNA	89.9 ± 0.5
RNA	0.01 ± 0.002
Other (calculated)	10.1 ± 0.5

Table 2.1 Thymidine incorporation as a measure of DNA synthesis

Cells were labelled with (^{14}C)-thymidine and the DNA and RNA fractions prepared as described in the text. The radioactivity in the DNA and RNA fractions was expressed as a fraction of the total radioactivity in whole cells.

CHAPTER 3

3.1 Metabolism of Cytosine arabinoside in Tetrahymena

Although it is widely accepted that AraC stops cell division and causes cell death (162) by interfering with DNA synthesis there is disagreement about the detailed mechanism of action. There is still some confusion as to whether the primary determinant of the therapeutic efficacy of AraC is the cellular concentration of AraC itself or the phosphorylated metabolite AraCTP (175). Steuart and Burke found an inverse relationship between responsiveness to AraC and the level of cytidine deaminase in the cell-free extracts of acute leukaemia blast cells. However, other workers have found that the response to AraC was primarily correlated to the total pool of nucleotides or to cytidine kinase activities (182). Chou and co-workers found that acute myeloid leukaemic (AML) cells from patients who were sensitive to AraC treatment produced more AraCTP than cells from patients unresponsive to treatment by the drug. However, the ability to synthesize appreciable amounts of AraCTP is not sufficient to account for the therapeutic response to AraC. Samples from relapsed patients previously sensitive to AraC treatment, produced high levels of AraCTP.

In Novikoff cells it was found that the rate of transport of AraC was much higher than its phosphorylation (183). These workers concluded that phosphorylation was the limiting step in the incorporation of AraC into the nucleotide pool.

Although AraC can be converted into inactive AraU by cytidine deaminase in crude cell preparations the expression of degradative enzyme activity may not be fully expressed in intact cells. Experiments done with cell-free extracts may produce misleading results about the

metabolism of AraC in whole cells.

3.2 Materials and Methods

3.2.1. Sources of materials

(5-^{3}H)-cytosine-B-D-arabinoside was obtained from the radiochemical Centre at Amersham. Unlabelled AraC, AraCMP, AraCTP were supplied by the Sigma Chemical Company. Prefilled Econo-Columns of Agl-X8 (anion exchange agent in chloride form, 200 - 400 mesh) and AG50W-X8 (cation exchange agent in hydrogen form, 200 - 400 mesh) were purchased from BioRad. Unprocessed Dowex 50 (cation exchange resin in hydrogen form, 200 - 400 mesh) was obtained from Sigma Chemical Company. Tetrahydrouridine was obtained from Calbiochem. Proteose peptones and yeast extract were obtained from Difco.

3.2.2. Culture of cells

Tetrahymena pyriformis, amicronucleate strain W, was grown in cultures containing proteose peptones (2%), yeast extract (0.1%), glucose (0.5%), and $FeCl_3.6H_2O$ (5 microgram per ml). This medium was used for most work and is referred to in this report as the standard medium YY. For most work cells were grown in 500 ml of medium contained in 2.5 litre conical flasks.

The desired volume of cell inoc ulum was calculated from the equation

$$v = \frac{yV}{X \cdot 2^{(t/3-1)}}$$

where v is the volume of inoc ulum required, y the desired cell concentration in cells/ml, X the concentration of cells in the stock

culture, t the time in hours from the time of inoculation to the time of harvesting the cells at concentration y and V the volume of the cell culture.

The flasks were placed in an orbital shaker operating at a shaking rate of 150 oscillations per minute. The temperature was maintained at 28°C. The cells were generally harvested during the log phase when their concentration was between 2 and 4 x 10^5 cells/ml. If stationary phase cells were required, harvesting was done when the cell concentration was over 10^6 cells/ml.

To determine cell concentration the cells were first diluted with an equal volume of formalin solution (20% in 0.01 M phosphate buffer pH 7). The fixed cells were then counted in a Neubauer counting chamber.

3.2.3. Preparation of Dowex 50 resin

Dowex 50 resin, in the hydrogen form, had a yellow discolouration when supplied. The material was first washed in sodium hydroxide (1 M). After rinsing in water to remove all the sodium hydroxide the resin was washed in hydrochloric acid (1 M). After removing excess acid by rinsing in water, the resin was converted to the ammonium form by adding ammonia (1 M). Finally the resin was washed in water until the pH was between 4 and 5. The whole washing process was done batchwise. The prepared resin was stored under water at room temperature.

3.2.4. Separation of cytosine arabinoside metabolites

Cells at a concentration of about 10^6 cells per ml and grown in standard medium YY were incubated in the presence of labelled cytosine arabinoside (0.02 microcurie per ml, specific activity 24 Ci/mmol). The cells were harvested at 3000 g and then sonicated as described in Chapter 2. One volume of 5% trichloroacetic acid was added to the sonicated cells and the mixture allowed to stand for two and a half

hours. The mixture was then centrifuged in a bench centrifuge to remove acid-insoluble material. The acid-insoluble material was placed in a scintillation vial, dissolved in NCS and its radioactivity counted.

The supernatant was applied onto a pre-filled Econo-Column containing 0.7 x 4 cm of AG1-X8 anion exchange resin. The metabolites of AraC were separated by a method based on that used to separate uridine and its metabolites (140, 184). Stepwise elution of the column with water, 0.15, 0.3 and 1 M ammonium bicarbonate separated the nucleosides (AraC and AraU), AraCMP, AraCDP and AraCTP. Samples (400 microlitres) were taken from each fraction collected and placed in the toluene/ethoxyethanol/PPO/POPOP scintillation cocktail for the counting of radioactivity.

To separate AraC from AraU the fractions containing most of the radioactivity when the AG1-X8 column is eluted with water were pooled and applied onto an AG50W cation exchange column. The column was eluted with water and then ammonia (1 M). Samples (400 microlitre) were removed from each fraction collected and radioactivity counted as above.

3.2.5. Assay for cytidine deaminase activity with cytosine arabinoside as the substrate

The cytidine deaminase activity of Tetrahymena was determined using tritiated cytosine arabinoside as the substrate. At first cytidine deaminase activity was assayed following the method described by Rothman and co-workers (185). Enzyme activity was estimated by measuring the amount of labelled uracil arabinoside formed by the deamination of cytosine arabinoside.

Cells were grown in standard medium YY and harvested when the cell concentration was about 10^6 cells per ml. The cell pellet was resuspended

in buffer (Tris-HCl 0.5 M, pH 8) and sonicated. The sonicated cells were used in enzyme assays. The assay mixture contained in a total volume of 150 microlitres, 100 microlitres enzyme mixture, 30 microlitres Tris-HCl pH 8, labelled cytosine arabinoside (1.25 microcurie, specific activity 24 Ci/mmol). The mixture was incubated for twenty minutes at 28°C. The reaction was stopped by adding 50 microlitres hydrochloric acid (3M). Ten microlitres each of unlabelled marker AraC (10 mM) and AraU (41 mM) was added to the mixture. After centrifugation 150 microlitres of the supernatant was applied onto a Dowex 50 column (1 x 10 cm). In later assays the prefilled AG50W-X8 columns were used. The column was eluted with water and 2.5 ml fractions collected. When the first radioactivity had been eluted and the counts were close to background the column was eluted with an ammonia solution (1 M) and fractions collected again. 400 microlitres of each fraction was added to the toluene/ethoxyethanol/PPO/POPOP scintillation cocktail for the counting of radioactivity.

The radioactivity coming out when the column was eluted with water corresponded to uracil arabinoside which carries no charge. The radioactivity which was eluted by ammonia corresponded to cytosine arabinoside whose amino group carries a positive charge at low pH but which loses its charge in ammonia.

For routine work the method just described was too long to be practicable. A simple and quicker method was developed. Essentially the new method was batch operation which involved mixing the resin and the supernatant from the reaction mixture and allowing equilibrium to be established. Enzyme, buffer and labelled AraC were incubated at 28°C for twenty minutes as in the previous method. After stopping

the reaction with acid the reaction mixture was centrifuged and 150 microlitres of the supernatant were removed and placed into test tubes containing 1 gram of moist resin. One ml of distilled water was added and the mixture agitated in a vortex mixer. The mixture was either allowed to stand for about ten minutes or centrifuged for one minute. 400 microlitre samples were then placed into vials containing scintillation liquid with ethoxyethanol. Radioactivity was counted as above.

3.3 Results and discussion

3.3.1. Separation of cytosine arabinoside metabolites

Figures 3.1 and 3.2 show the separation of AraC and its metabolites by ion exchange chromatography. When the anion exchange column (AG1-X8) was used most of the radioactivity passed through when the column was eluted with water. This radioactivity corresponded to cytosine arabinoside and uracil arabinoside. Only a small fraction of the AraC was converted to AraCMP, AraCDP or AraCTP. Only a tiny amount of the putative active agent, AraCTP, was formed.

The fractions corresponding to AraC and AraU were pooled and the two nucleosides separated on a cation exchange column as shown in Figure 3.2. Uncharged AraU was eluted from the column with water. AraC came off when the column was eluted with 1 M ammonia.

The present results show that whereas AraC was rapidly converted to AraU in cell homogenates only a tiny fraction is phosphorylated. Although results obtained with cell homogenates may not apply to intact cells it is probable that intact Tetrahymena cells also rapidly convert AraC to AraU. Most of the AraC would then be deaminated before the accumulation of AraCTP. It is possible that the

Figure 3.1

Fractionation of AraC metabolites on AG1-X8 anion exchange resin

Cells labelled with (^{3}H)cytosine arabinoside were sonicated. After addition of TCA acid-insoluble material was removed by spinning in a bench centrifuge. The supernatant was applied onto an AG1-X8 column. After elution with water AraCMP, AraCDP and AraCTP were eluted with 0.15, 0.3 and 1 M NH_4HCO_3 at a, b, and c respectively. The peaks A, B, C, and D are for AraC/AraU, AraCMP, AraCDP and AraCTP respectively. Although the experiment was done several times the results shown here and also in Figures 3.2 and 3.3 are from one typical experiment. It was not possible to collect the same number of fractions all with equal volumes all the time. The precise position of the peaks, therefore, differed slightly in different sets of the same experiment. **Cold AraC, AraU, AraCMP, AraCDP and AraCTP were used as standards in identifying radioactivity peaks.**

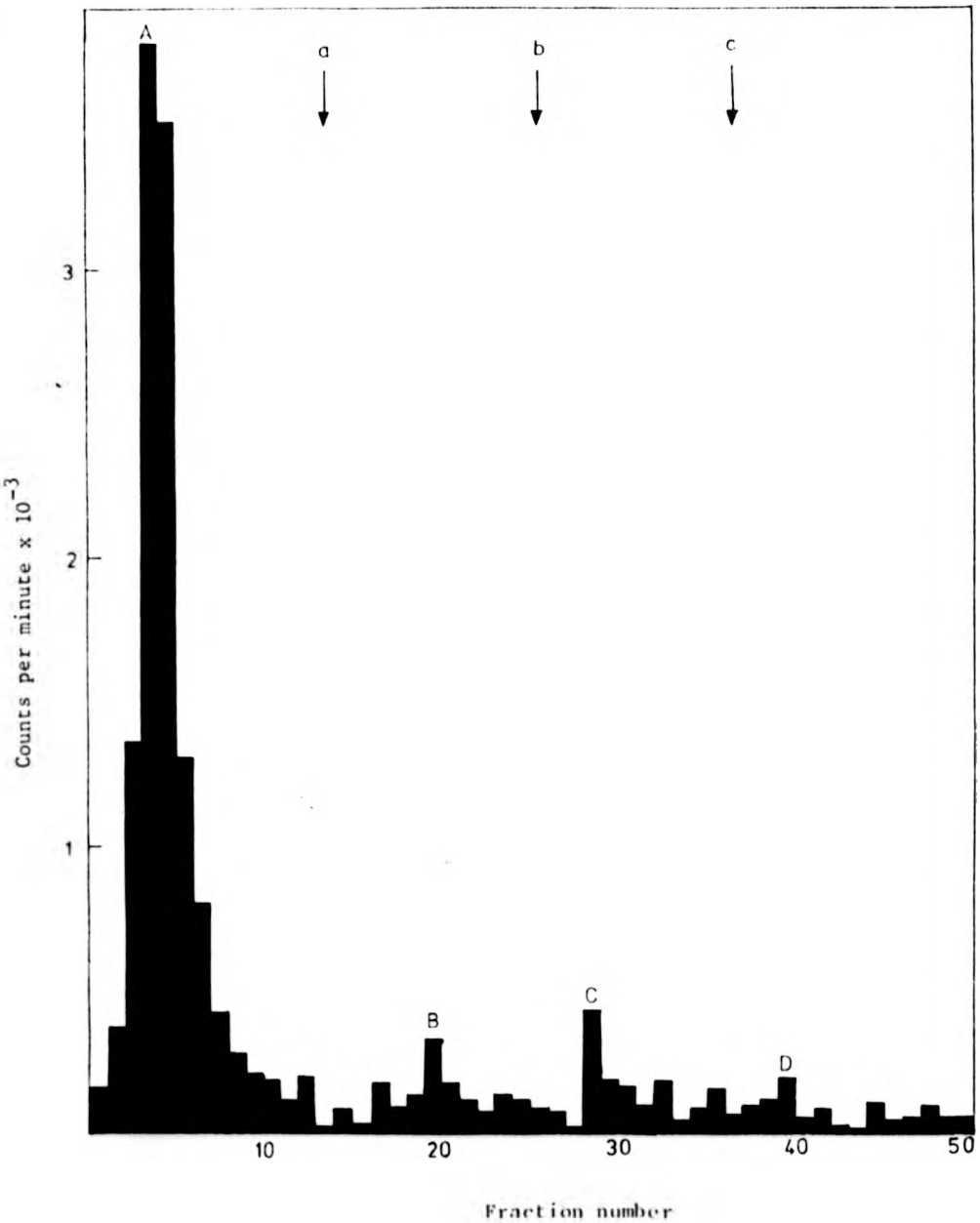
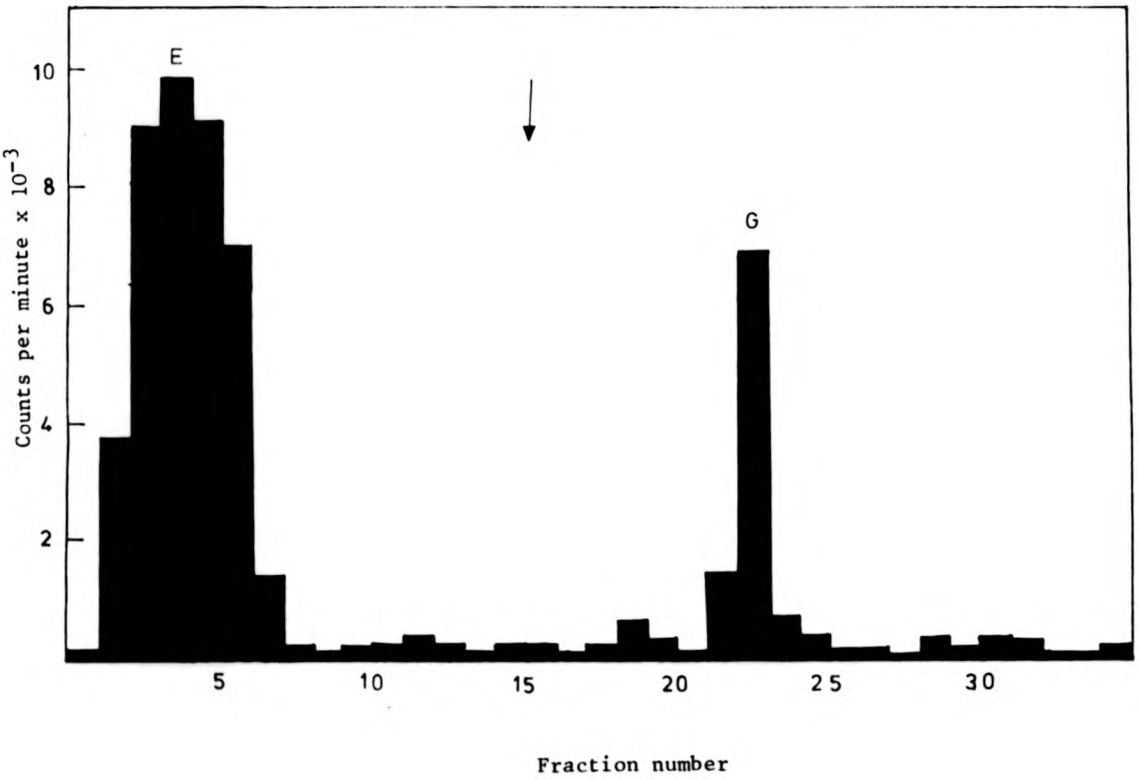

A
a
b
c
B
C
D
3
2
1
Counts per minute x 10^{-3}
10
20
30
40
50
Fraction number

Figure 3.2

Separation of AraC and AraU on AG50-X8 cation exchanger.

Fractions from peak A in Figure 3.1 were pooled and applied onto an AG50-X8 column. The column was first eluted with water and then with ammonia (1 M) at the point indicated by the arrow. Peaks E and G represent AraU and AraC respectively.


E
G
10
8
6
4
2
Counts per minute x 10^{-3}
5
10
15
20
25
30
Fraction number

amount of AraCTP formed is even less than indicated in Figure 3.1. The peak for the triphosphate could also include AraUTP which presumably would not be effective in inhibiting cell division and causing cell death.

Figure 3.10 and 3.11 show the separation of AraC metabolites from cell preparations incubated with labelled cytosine arabinoside and THU (3×10^{-6}M). It can be seen in Figure 3.10 that the presence of THU did not lead to any increase in the amount of phosphorylated AraC formed. When fractions from the nucleoside peak were analysed on the cation exchanger most of the radioactivity was found in the AraC peak (Figure 3.11). Only 2.5% of the total nucleoside radioactivity was recovered in the AraU fraction. The presence of THU markedly reduces the deamination of AraC to AraU. However, the increased cytosine arabinoside pool does not lead to a corresponding increase in the amount of phosphorylated AraC formed. It appears that phosphorylation is the critical step in the effectiveness of AraC in inhibiting DNA replication.

3.3.2. Cytidine deaminase

Figure 3.3 shows the result of a cytidine deaminase assay on an anion exchange column using cytosine arabinoside as the substrate. By eluting the column with water and ammonia clean separation of AraC and AraU was effected. Although the procedure was simple, it was time-consuming for routine use. The assay for cytidine deaminase by this method was not, therefore, used for routine purposes.

The determination of the optimum amount of resin required for the assay of cytidine deaminase by the batch process is shown in Figure 3.4. Nearly 60% of the AraC in the supernatant was bound by 0.2 g of resin. The amount of AraC bound by the resin then increases

Figure 3.3

Cytidine deaminase assay on an ion exchanger with cytosine arabinoside as the substrate

Labelled cytosine arabinoside was incubated with enzyme preparations as described in the text. The reaction was stopped by addition of acid. After centrifugation the supernatant was applied onto an AG50-X8 column which was then eluted with water and then with ammonia (1 M) at the point indicated by the arrow.

(A) Column eluted after AraC was incubated with enzyme.

(B) No enzyme control.

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n with

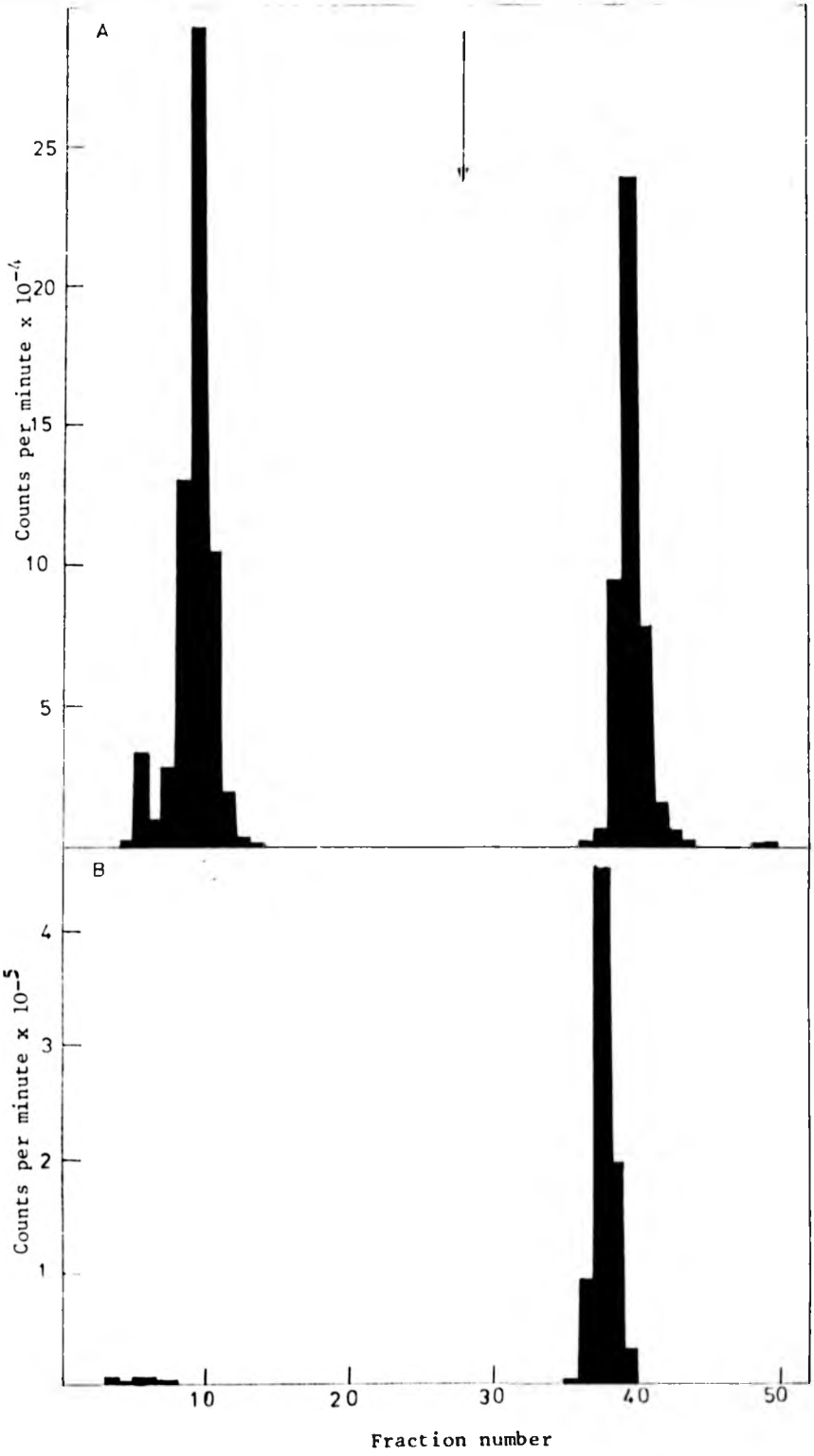
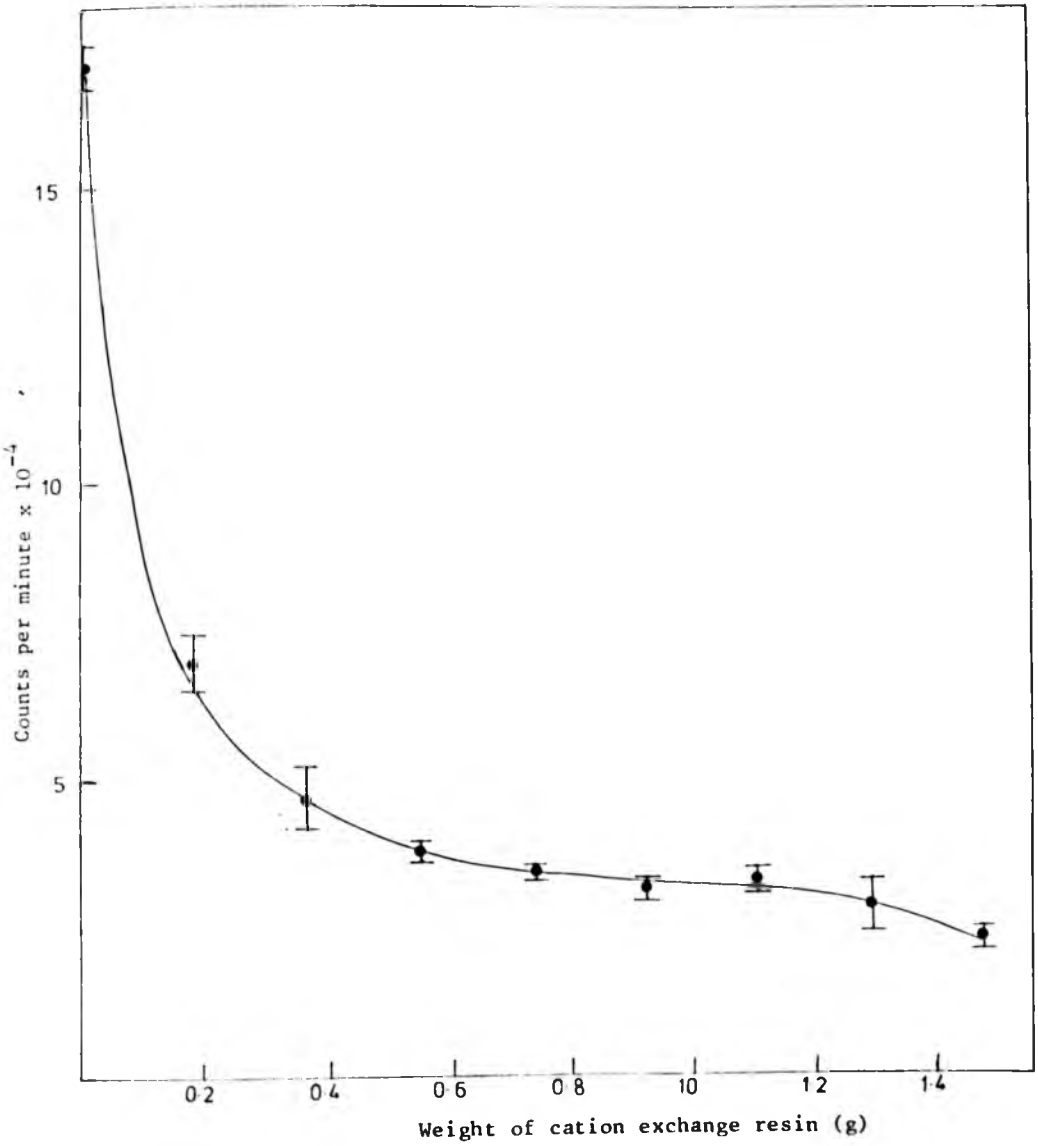


Figure 3.4

Determination of optimum amount of resin required for cytidine deaminase assay.

Labelled cytosine arabinoside was added to tubes containing increasing amounts of AG50-X8 cation exchanger and one millilitre of water. After agitation to ensure thorough mixing the tubes were centrifuged for about a minute in a bench centrifuge. The radioactivity in the supernatant was then counted and was a measure of the cytosine arabinoside that remained unbound.


15
10
5
Counts per minute x 10^{-4}
0·2
0·4
0·6
0·8
1·0
1·2
1·4
Weight of cation exchange resin (g)

slowly to about 80% for 0.8 g resin. After this an increase in the amount of resin does not result in a substantial increase in the amount of AraC bound. For routine purposes one gram of resin was used. Although it was difficult to transfer reproducibly equal amounts of resin into the assay tubes it can be seen from Figure 3.4 that a 40% variation of the amount of resin from 1 g should not result in more than 8% variation in the amount of AraC bound.

Figure 3.5 shows the time course for the cytidine deaminase reaction assayed by the new method. The reaction is nearly linear for about twenty minutes. A reaction time of twenty minutes was used for the assays described below. The effect of AraC concentration on the rate of reaction is shown in Figure 3.6.

The inhibition of Tetrahymena cytidine deaminase by tetrahydrouridine is shown in Figure 3.8. The deaminase activity is almost totally inhibited by 4 x 10^{-6}M tetrahydrouridine. This result is similar to the findings of Camiener who reported that tetrahydrouridine concentrations of 10^{-6} to 10^{-5}M inhibited 95% of the deaminase activity in preparations of mouse kidney, rhesus monkey liver and actinomycete mycelium (186). The K_i for tetrahydrouridine was determined by measuring deaminase activity at two concentrations of AraC in the presence of increasing amounts of inhibitor. The Dixon plot for these results is shown in Figure 3.9. The K_i value obtained (8 x 10^{-8}M) is considerably lower than the values reported by Camiener (10^{-4} - 10^{-5}M) but somewhat closer to the values reported by Furner and Mellett (10^{-7} - 10^{-8}M)(187).

The assay developed for the assay of cytidine deaminase was simple,

rapid and convenient for most routine purposes. Only in cases where it is necessary to recover unreacted cytosine arabinoside is the column procedure worthwhile.

Figure 3.5

Time course of the cytidine deaminase reaction with cytosine arabinoside as substrate

Labelled cytosine arabinoside was incubated with enzyme as described in the text. At five minute intervals TCA was added to some of the reaction mixtures. After removal of acid-insoluble material by centrifugation AraC was then separated from AraU on AG50-X8 resin by the batch operation described in the text.

When the supernatant from the centrifuged reaction mixture is added to the ion exchange resin most of the AraC is bound to the resin. A small fraction of the total AraC and all the AraU remains in the supernatant. In the no enzyme control all the radioactivity in the supernatant is due to AraC only. The difference between assays in which the enzyme is present and the no enzyme control is a measure of the amount of AraU formed from the deamination of AraC.

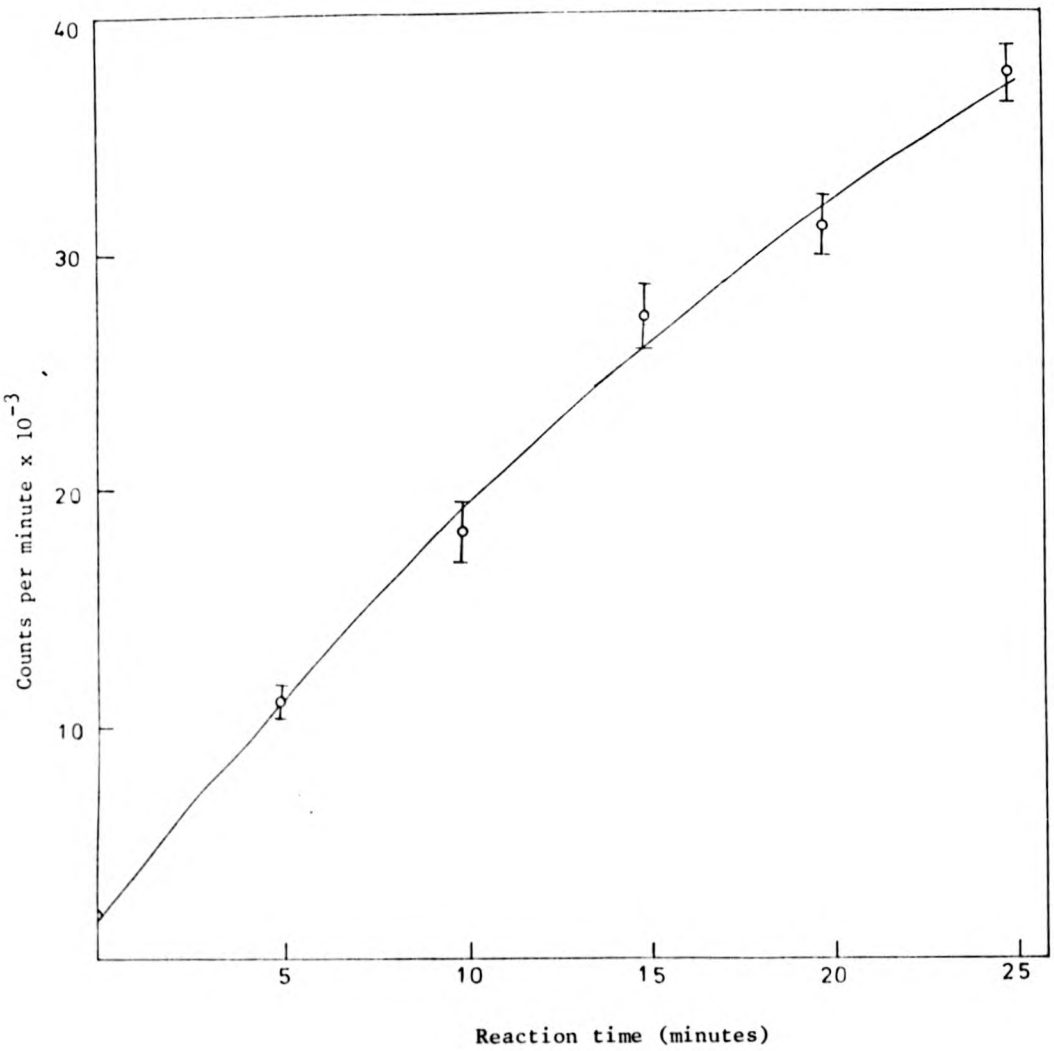

40
30
20
10
Counts per minute x 10^{-3}
5
10
15
20
25
Reaction time (minutes)

Figure 3.6

Effect of AraC concentration on the rate of cytidine deaminase reaction

Cytidine deaminase activity was determined following the batch procedure developed. Assay mixtures were incubated for twenty minutes at 28°C.

action

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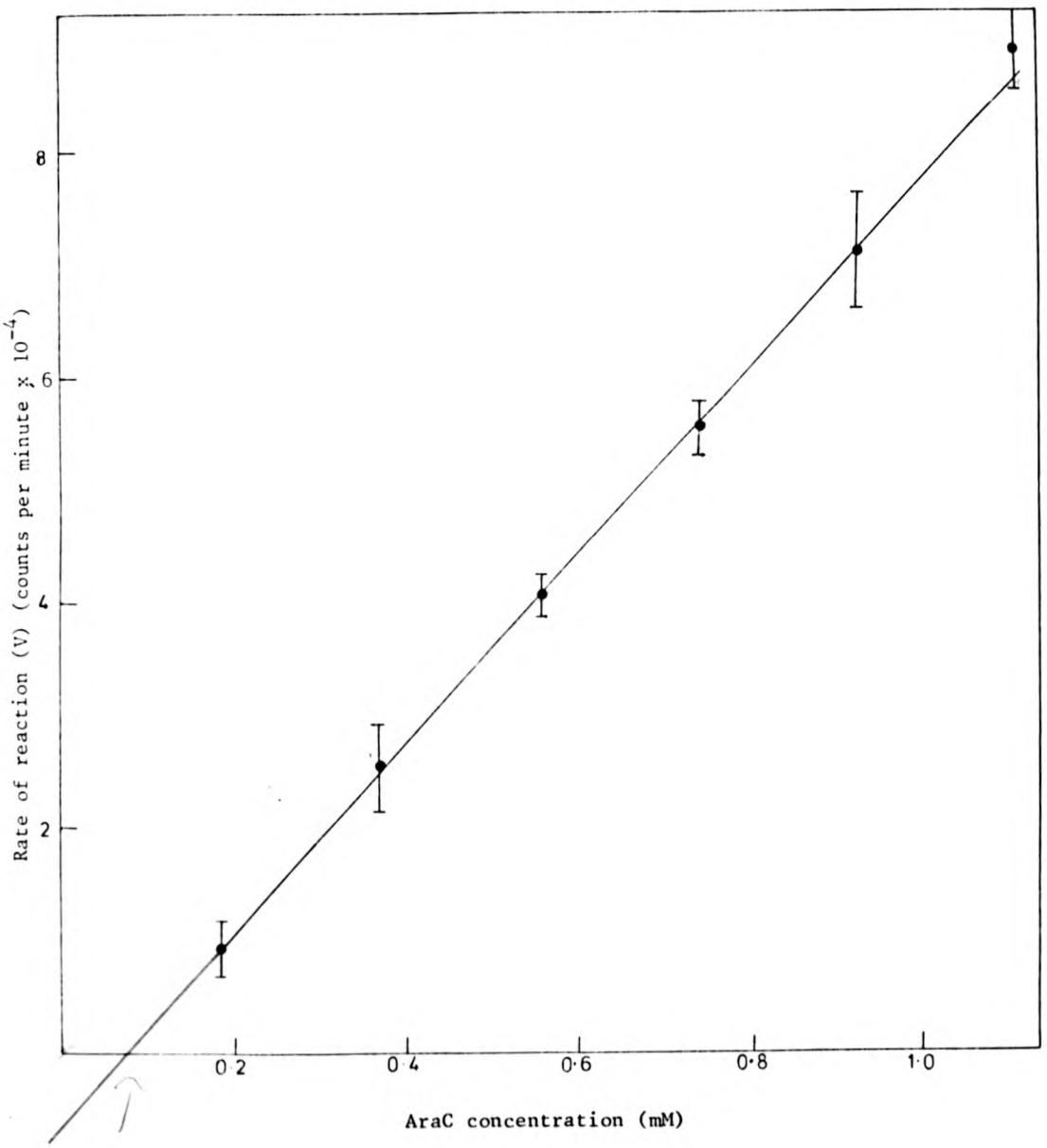


Pages 96 and 97 were removed after the examination

Figure 3.8

Effect of tetrahydrouridine on the deamination of AraC by cytidine deaminase

Labelled cytosine arabinoside was incubated with enzyme preparations in the presence of increasing amounts of tetrahydrouridine. The deamination of cytosine arabinoside was followed by measuring the amount of AraU produced.

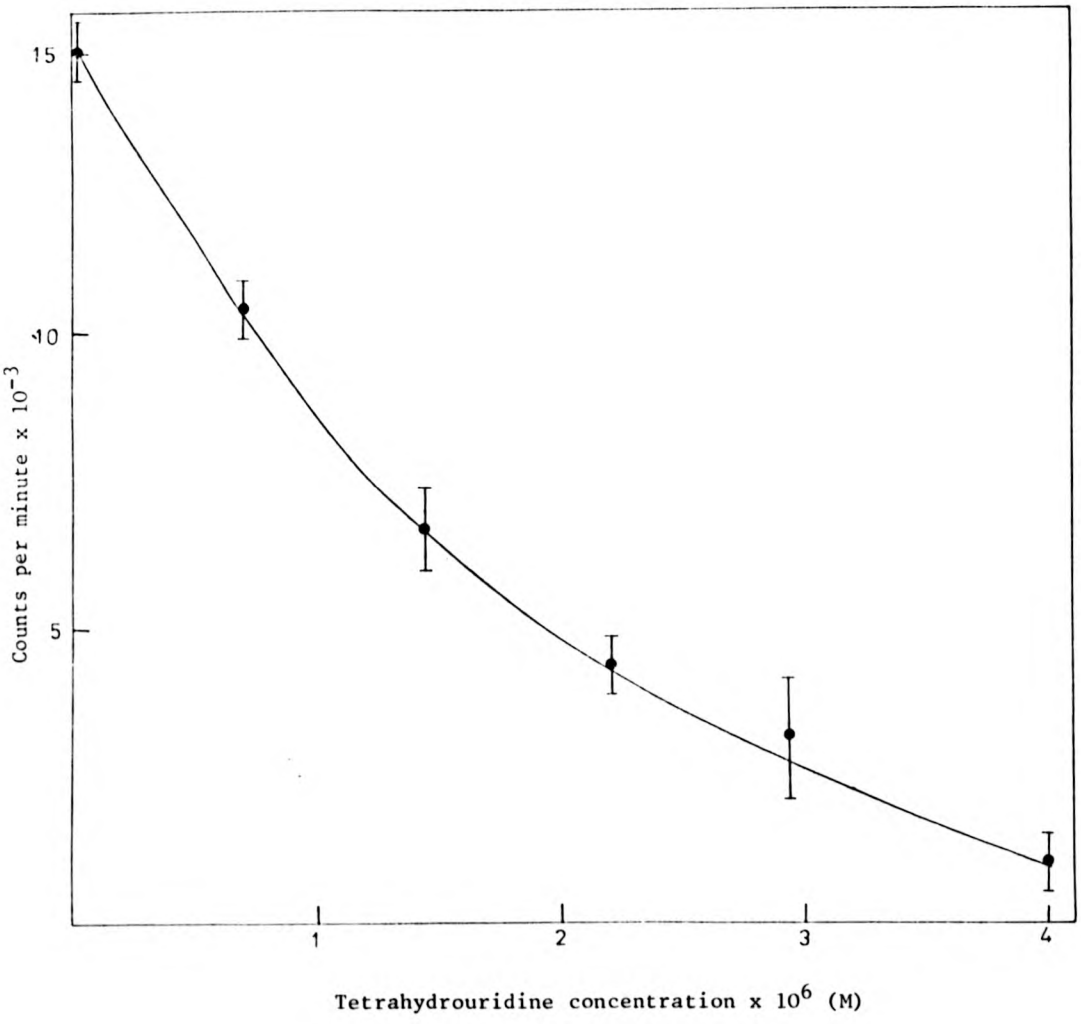

15
10
5
Counts per minute x 10^{-3}
1
2
3
4
Tetrahydrouridine concentration x 10^6 (M)

Figure 3.9

Determination of K_i for tetrahydrouridine in the deamination of AraC

The rate of deamination of AraC was measured at two concentrations of AraC (3.8 & 7·6μM)in the presence of increasing amounts of tetrahydrouridine. For the two separate sets of experiments 1/V was plotted against the tetrahydrouridine concentration. K_i was then obtained from the intersection of the two experimental lines as shown on the graph.

3.8 micromolar AraC

7.6 micromolar AraC

AraC

rations

etra-

n

shown

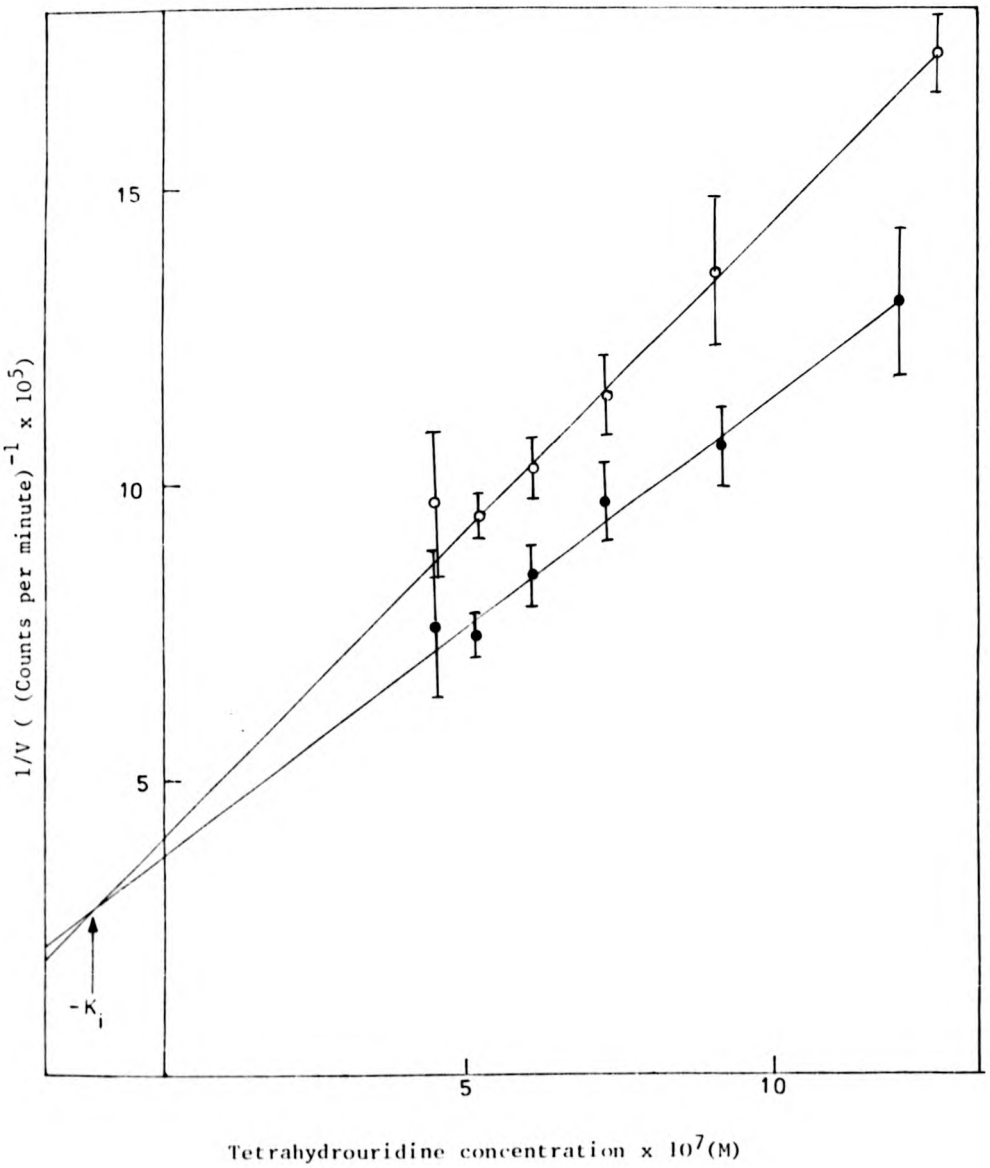


Figure 3.10

Effect of tetrahydrouridine on the metabolism of AraC in Tetrahymena

Cytosine arabinoside was incubated with enzyme preparations in the presence of tetrahydrouridine (3×10^{-6}M). After adding acid and removing acid-insoluble fractions by centrifugation the supernatant was applied onto an AG1-X8 column. The column was washed with water to elute AraC and AraU. AraCMP, AraCDP and AraCTP were then separated by eluting with 0.15, 0.3 and NH_4HCO_3 at A, B, and C respectively. The experiment was done several times but the results shown here and in Figure 3.11 are from one typical experiment.

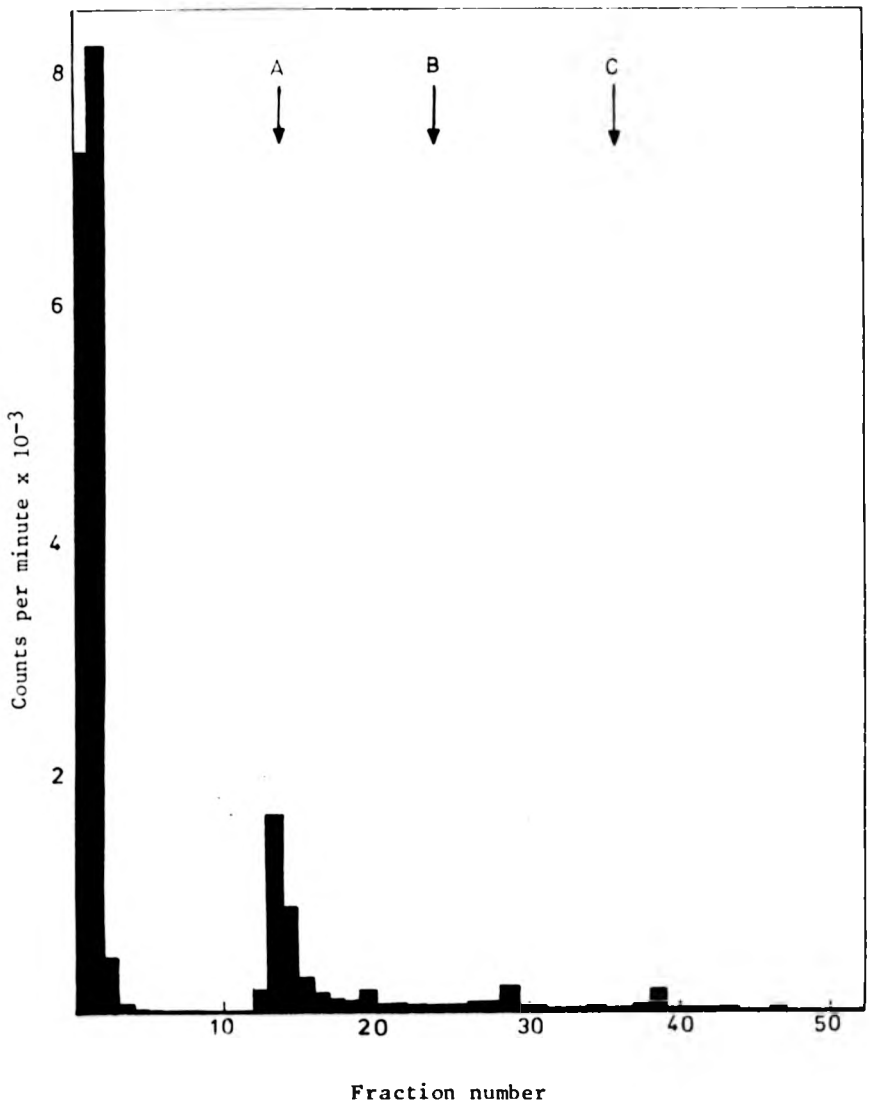
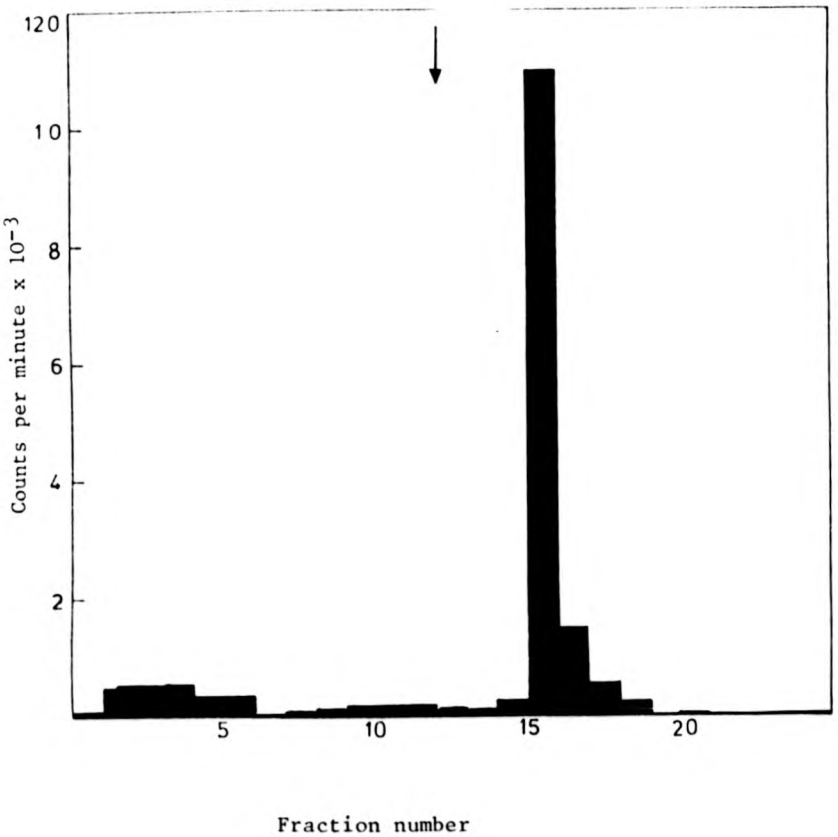

A
B
C
8
6
4
2
Counts per minute x 10^{-3}
10
20
30
40
50
Fraction number

Figure 3.11

Fractionation of the nucleoside peak from Figure 3.10 on AG50-X8 cation exchange resin

Fractions containing the nucleoside peak shown in Figure 3.10 were pooled and applied onto an AG50-X8 column. The column was washed with water to elute any AraU present and with ammonia (1 M) at the point shown by the arrow to elute cytosine arabinoside.


120
10
8
6
4
2
Counts per minute x 10^{-3}
5
10
15
20
Fraction number

CHAPTER 4

4.1 The use of synthetic template-primers

In bacteria numerous mutants defective in various aspects of DNA synthesis have been used to determine the function of DNA polymerases. Except for a few exceptions no such mutants are available for most eukaryotic cells. In these organisms less direct methods for studying the role of DNA polymerase have had to be used. One such approach has been to investigate the use of synthetic templates and primers by DNA polymerases.

All DNA polymerases described require a preformed initiation site. In addition to a requirement for 3'-hydroxyl termini the enzymes need some double-stranded structure for maximal activity. This may be provided by using degraded DNA or oligonucleotide initiated homopolymers (188). Work in many laboratories suggests that RNA fragments may be involved in the initiation of DNA synthesis (189).

In DNA polymerising systems four classes of template-primers can be considered:

(a) polyribonucleotide template-ribonucleotide primer,

(b) polyribonucleotide template-deoxyribonucleotide primer,

(c) polydeoxyribonucleotide template-deoxyribonucleotide primer, and

(d) polydeoxyribonucleotide template-ribonucleotide primer (188).

The ability of DNA polymerases to use these template-primers might provide a clue to the cellular role of these enzymes. Class (a) template-primers would be used by DNA polymerases which are able to copy an RNA strand and extend an RNA primer. Such enzymes would be reverse transcriptases. As far as I am aware no reverse transcriptases of this type have been described.

The reverse transcriptases that have been described use class (b) type template-primers. Ever since the discovery of reverse transcriptase associated with RNA tumour viruses (190, 191) and in human leukaemia cells (192) many workers have studied the ability of normal eukaryotic DNA polymerases to copy RNA templates. In addition to reverse transcriptase, DNA polymerase beta and DNA polymerase gamma can also use class (b) template-primers. Some confusion has arisen as a result of workers prematurely claiming to be able to distinguish the enzymes that can copy RNA by the use of synthetic template-primers. At present the general picture seems to be as follows. Some class (b) template-primers are used by both reverse transcriptases and normal DNA polymerases. Poly(A).poly(dT) is an effective template primer for both viral and cellular DNA polymerases (193). Because it is non-specific poly(A).poly(dT) cannot be used to distinguish between reverse transcriptase and the other polymerases.

Reverse transcriptases and other DNA polymerases can be distinguished by the use of oligo homopolymers such as poly(A)oligo-$(dT)_{12-18}$. Whereas viral polymerases prefer poly(A).oligo(dT), DNA polymerase from normal human lymphocytes showed no detectable activity (193). Although DNA polymerase beta is able to use primed poly(A) it cannot copy natural RNA or rC_ndG_5. Poly(C).oligo(dG) has been considered to be specific for viral transcriptases (194, 195, 196). However, Spadari and Weissbach have purified gamma DNA polymerase from HeLa cells which effectively uses poly(C).oligo(dG) (42). Poly(2'-O-methylcytidylate).oligodeoxyguanylate ($polyC_m$.oligo dG) is an effective template-primer for RNA tumour virus reverse transcriptases which promises to be a specific one. It has been

found that nuclear DNA polymerase gamma can copy poly(C).oligo(dG) but not poly(C_m).oligo(dG) (197).

Some care is required when using synthetic templates to characterise the different DNA polymerases. In the presence of Mn, for example, at lease one cellular DNA polymerase will use poly(A).$(dT)_{12-18}$ more effectively than poly(dA).oligo$(dT)_{12-18}$. There is no indication, however, that this is a true reverse transcriptase (194).

DNA polymerases which use class (c) template-primers extend a DNA primer and copy a DNA template. This class of template-primer includes activated DNA, denatured DNA, polyd(A-T), and oligodeoxynucleotide-initiated polydeoxynucleotides such as poly(dA).oligo(dT).

Polyd(A-T) is interesting because it can be synthesized de novo by DNA polymerase alpha (64). This synthesis is inhibited by dGTP and dCTP and is stimulated by unwinding protein. Polyd(A-T) is able to act as template-primer even though it is double-stranded at the salt concentration and temperature used. It could provide a single strand template by one or both of two mechanisms.

Polyd(A-T) can be converted into a single strand form by unwinding protein. The maximum amount of single strand template is provided when the polyd(A-T) is fully complexed to the unwinding protein. This happens when the unwinding protein/polyd(A-T) ratio is about 8 to 1 (64). An amount of unwinding protein beyond that required to convert all the template to a single-stranded form is inhibitory.

A second method by which polyd(A-T) can provide a single-stranded template is by strand slippage. The short repeating sequence allows movement of either the 3'-OH or the 5' end along the chain to expose a single-stranded template. As strand slippage cannot occur under conditions which favour breaking and reforming of hydrogen bonds it

cannot occur under conditions which favour the stability of the double helix.

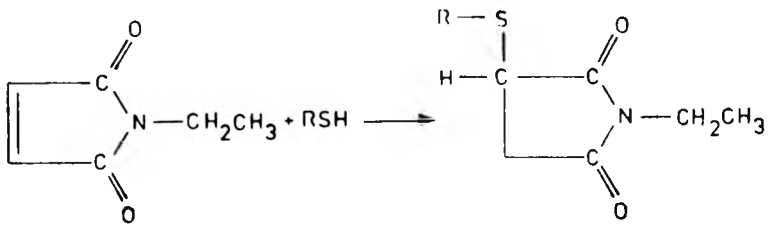
Since magnesium ions and low temperatures favour the stability of the double helix this could explain the lower magnesium optimum and the higher temperature optimum for polyd(A-T) replication as compared to that for activated DNA replication (198).

Although polyd(A-T) synthesis can occur in the absence of unwinding protein, the absence of unwinding protein results in longer lag periods before synthesis than when the protein is present. A number of polynucleotides systems can stimulate the de novo synthesis of polyd(A-T). Activated and native DNA are very effective in reducing the lag period before synthesis. The lag periods are a little longer with denatured DNA. $(dT)_{12-18}$·poly(dA) or polyribonucleotides are without effect.

In this work denatured calf thymus DNA, polyA.polydT, polyA.dT_{10}, polydA.dT_{10} and polyd(A-T) were used. These template-primers were chosen on the basis of their usefulness in assigning roles to DNA polymerase activities and cost. Denatured DNA, polydA.dT_{10}, and polyd(A-T) were used as general template-primers for DNA polymerases (52). PolyA.polydT was used as the template-primer used by DNA polymerases similar to DNA polymerase beta and gamma or to reverse transcriptases (21).

4.2. N-ethylmaleimide and DNA polymerases

A number of eukaryotic DNA polymerases are inactivated by thiol reagents (53). This has generally been interpreted to mean that sulphydryl groups are required for polymerase activity. N-ethylmaleimide (NEM) is the best known of a number of N-substituted maleimides which react with thiol groups as shown below.



Although it is probable that NEM has a high selectivity for thiol groups the formation of adducts with sulphide, sulphite and thiosulphate and the slow reaction with peptide amino end groups, the amino group of lysine and the imidazole group of histidine, limit its use as a specific reagent (199).

4.3 Materials and methods

4.3.1. Sources of materials

NEM, AraCTP, calf thymus DNA, the deoxyribonucleotides dATP, dCTP, dGTP and unlabelled TTP were obtained from Sigma Chemical Company. PolyA.polydT was bought from P. L. Biochemicals. PolydA.dT_{10}, polyd(A-T) and poly(dA-(methyl-^{3}H) dT) were supplied by Miles Laboratories. (methyl-^{3}H)TTP was supplied by the Radiochemical Centre at Amersham. Glass fibre discs (GF/C, 2.5 cm) and DEAE-cellulose (microgranular, pre-swollen DE52) were obtained from Whatman. Nonidet P40 was supplied by the British Drug Houses. PolyA.dT_{10} was obtained from Boehringer Manheim.

4.3.2. Preparation of denatured DNA template-primer

Calf thymus DNA (1.5 mg/ml in 0.02 M Tris and 0.02 M NaCl) was heated in a boiling water bath for ten minutes (200). The container was then quickly cooled in ice to obtain denatured DNA. Denaturation

of DNA was done immediately before each DNA polymerase assay.

4.3.3. Nuclease assays

Nuclease activity was determined by measuring the amount of acid-soluble products formed from poly (dA-(methyl-^{3}H)dT) (201, 202). In a total volume of 90 microlitres the assay mixture contained glycine-KOH buffer pH 8.9 (3 micromoles), dithiothreitol (0.1 micromoles), magnesium chloride (0.5 micromoles), poly (dA-(methyl-^{3}H)dT) (0.2 microcurie, specific activity 18.6 Curie/mol.). Reaction was started by adding 10 microlitres enzyme preparation and the mixtures incubated at 28°C.

At the end of the reaction 100 microlitres of a solution containing DNA (1 mg/ml) and BSA (56 mg/ml) was added. The reaction was then stopped by adding 150 microlitres of TCA (0.5 M) The mixtures were allowed to stand in an ice bath for about five minutes. Acid-insoluble material was removed by spinning in a bench centrifuge for one minute. 300 microlitres of the supernatant were then removed and placed in vials containing the toluene/ethoxyethanol/PPO/POPOP scintillation cocktail for the counting of radioactivity.

4.3.4. DNA polymerase assays

DNA polymerase activity was assessed by measuring the amount of (methyl-^{3}H)TTP incorporated into acid-insoluble material (203). In a total volume of 200 microlitres the assay mixture contained HCl (4 micromoles), Tris-HCl pH 7.6 (11 micromoles), magnesium chloride (1.8 micromoles), 2-mercaptoethanol (0.6 micromoles), EDTA (0.03 micromoles), dATP, dCTP, dGTP (24 nmoles each, (methyl-^{3}H)TTP (0.36 nmoles, specific activity 2.5 Ci/nmol), denatured calf thymus DNA (15 microgram). In some experiments manganese replaced magnesium as the metal ion and synthetic polynucleotide systems replaced DNA as the template-

primer. Reaction was started by adding 100 microlitres of enzyme preparation. The mixture was incubated at 28°C for twenty minutes.

After incubation, 50 microlitre samples were removed and spotted onto Whatman glass fibre discs which were immediately immersed in ice-cold TCA (5%). The discs were then washed five times in fresh TCA and twice in cold absolute ethanol.

Alternatively 50 or 100 microlitre samples were spotted onto glass fibre discs in a Buchner funnel. The discs were then washed with TCA and ethanol with suction.

The washed discs were dried and placed in a scintillation cocktail containing toluene, PPO/POPOP for the counting of radioactivity on glass fibre discs. The specific activity of the enzyme was calculated as nmoles (methyl-^{3}H)TTP incorporated per minute per mg protein.

When the effect of NEM and AraCTP on DNA polymerase activity was studied, quantities of these substances were added to the DNA polymerase cocktail in a total volume of 240 microlitres.

4.3.5 Assay for terminal deoxynucleotidyl transferase activity

Terminal deoxynucleotidyl transferase activity was assayed by measuring DNA polymerase activity in the presence of TTP only as the deoxynucleotide substrate (204). Otherwise the assay was done in the same way as the DNA polymerase assay.

4.3.6. Preparation of enzyme fractions

Cells at a concentration of 2 to 4 x 10^5 cells/ml were harvested by centrifugation at 5000 g for 10 minutes. The cell pellet was resuspended in 0.25 M sucrose/10 mM $MgCl_2$ to give a final volume of 5 ml. Nuclei were then prepared by a modification of the method of Mita (205). The cell suspension was treated with one volume of Nonidet P40 (1% in 0.25 M sucrose/10 mM $MgCl_2$) for one minute. In

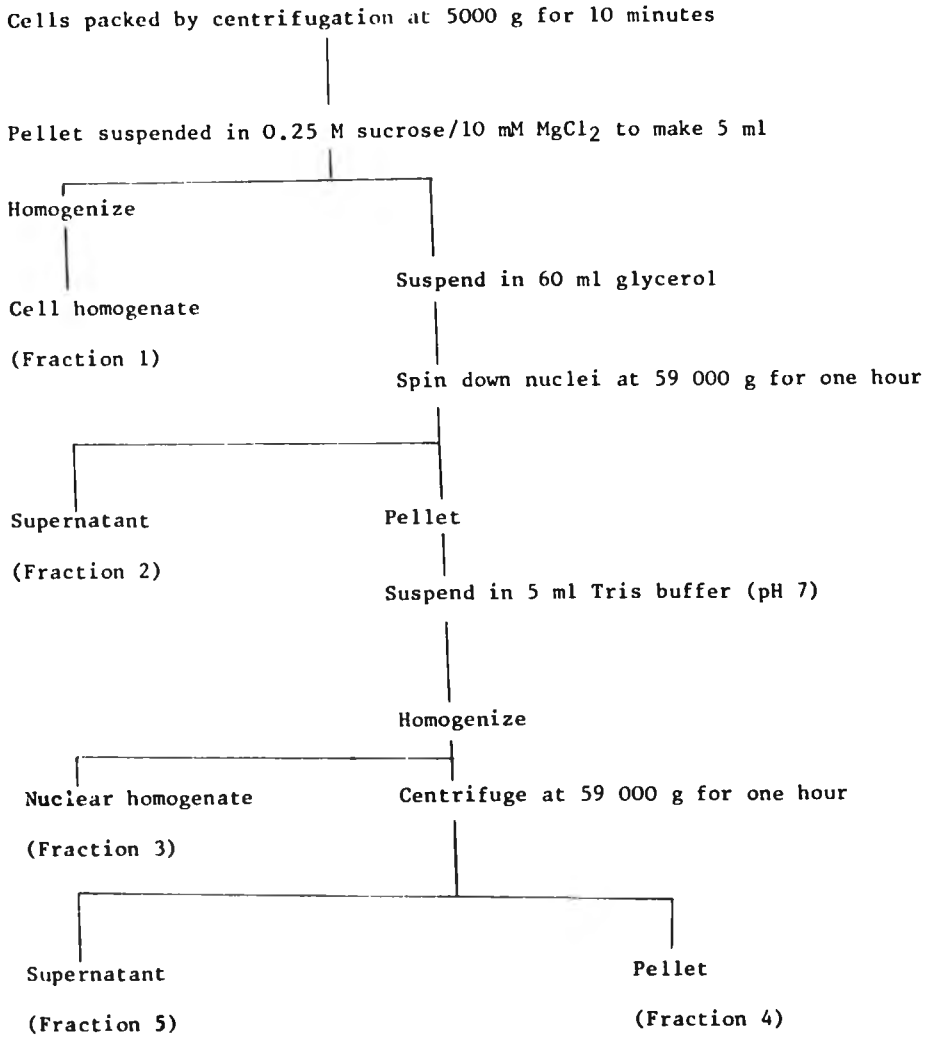


Figure 4.1 Preparation of cytoplasmic and nuclear DNA polymerase activities from *Tetrahymena pyriformis*

this time the cell membrane dissolved away but the inner nuclear membrane remained intact as seen under the microscope. The mixture was then suspended in 60 ml glycerol. Nuclei were obtained by centrifugation at 59 000 g for one hour. The supernatant (Fraction 2) contained the cytoplasmic activity. The nuclear pellet was suspended in 5 ml Tris-HCl buffer (Tris-HCl, $MgCl_2$, and KCl 10 mM each, pH 7.9) and homogenized. The homogenate was centrifuged at 59 000 g for one hour to remove unbroken nuclei. The supernatant (Fraction 5) was used as the nuclear preparation.

The preparation of enzyme fractions is shown in Figure 4.1. The DNA polymerase activity was unstable in glycerol. All experiments with Fraction 2 were done on fresh preparations. If necessary Fraction 5 was divided into several portions which were stored at $-20^{o}C$. These were then thawed and used as required. Stored in this way the preparations maintained their activity for about a week.

An attempt was made to prepare nuclei using concentrated sucrose solutions (2.4 M) instead of glycerol. Cells were harvested by centrifugation as described above. The cell pellet was suspended in 30 ml sucrose (2.4 M). The cells were then broken in a motor-driven homogenizer until all the cells were disrupted.

The homogenized cells were then centrifuged at 59 000 g for one hour. The supernatant was designated Fraction 2. The pellet was resuspended in buffer and homogenized to break up nuclei. After centrifugation for one hour at 59 000 g the supernatant was used for assays as Fraction 5.

4.3.7 Fractionation of *Tetrahymena* DNA polymerase activities by DEAE-cellulose chromatography

Log phase cells were harvested and resuspended in buffer (Tris-HCl, $MgCl_2$, and KCl, 10 mM each, pH 7.9). The cells were then homogenized

in a motor-driven homogenizer to disrupt most of the cells. The homogenate was centrifuged at 59 000 g for one hour to remove unbroken cells, nuclei and mitochondria. Homogenization broke up most of the macronuclei of the cells. The supernatant was then fractionated with ammonium sulphate. The 30 to 70% ammonium sulphate pellet was dissolved in Tris buffer and dialysed overnight against buffer A (10 mM Tris-HCl pH 7.9, 1 mM EDTA, 0.1 mM dithiothreitol, and 20% glycerol). The enzyme preparation was then fractionated on a DEAE-cellulose column (206). The dialysate was applied onto a DEAE-cellulose column (1 x 21.5 cm). The column was washed to remove unbound protein (as measured by absorbance at 280 nm). The protein bound to the column was then eluted with a gradient between 0.05 and 0.8 M KCl. Fractions (3.5 ± 0.4 ml or 65 drops) were collected on an LKB Redirac fraction collector in the "drop" mode. Protein was monitored by measuring absorbance at 280 nm in a Unicam SP 1500 spectrophotometer.

Enzyme fractions prepared by the method above were generally unstable. Adding BSA (100 microgram/fraction) improved the stability a little but not for very long. In an attempt to overcome this problem, I decided to carry out the fractionation of enzyme activity on the anion exchanger in a batch process (207). Log phase cells were sonicated in an MSE sonicator at maximum amplitude and medium power in three bursts each lasting for five seconds. Between sonication bursts the cell mixture was allowed to cool in ice for thirty seconds. The sonicated cells (4 ml, 10mg/ml protein) were mixed with DEAE-cellulose to form a thick paste. The mixture was stirred for about two minutes. The ion exchanger was separated from the liquid by centrifugation. The supernatant was treated with a further quantity

of DEAE-cellulose and the exchanger again separated from the liquid. The pellets were combined and treated with 8 ml KCl (0.3 M in buffer A). After centrifugation the pellet was treated with 8 ml of more concentrated KCl (0.8 M in buffer A). After centrifugation the pellet was discarded. The supernatants from the 0.3 M and 0.8 M salt treatment were dialysed overnight against buffer A.

DNA polymerase activity was determined by measuring the incorporation of (^{3}H)TTP into acid-insoluble material. The sensitivity of enzyme preparations to NEM and AraCTP and the use of different template-primers were studied. When studying the use of synthetic template-primers by DNA polymerase preparations 0.1 absorbance units of each synthetic template-primer were used in assays instead of denatured calf thymus DNA. The total number of absorbance units of each template-primer delivered by the supplier was dissolved in water to give a concentration of 10 units per ml. For assays ten microlitres of the resulting solution were used.

4.4 Results and Discussion

4.4.1 Preliminary studies on the DNA polymerase activity in Tetrahymena

In the early stages of the work a preliminary study on the DNA polymerase activity of Tetrahymena was made. Figure 4.2 shows the effect of pH on the DNA polymerase activity of cell homogenates. In Tris buffer the enzyme had a broad optimum with maximal activity occurring at a pH of about 7.9.

The time course of the polymerase reaction is shown in Figure 4.3. Increasing the reaction time leads to increased incorporation of labelled TTP for about half an hour. After this time there is a decrease in the amount of label incorporated. This fall in the

incorporation of TTP is probably due to nuclease activity. In order to achieve as much incorporation of label as possible it was decided to incubate the enzyme preparations with TTP for twenty five minutes.

The low incorporation of labelled TTP observed in DNA polymerase assays led to an investigation of the nuclease activity of Tetrahymena enzyme preparations. The nuclease activity of cell homogenates is shown in Figures 4.4 and 4.5. The high nuclease activity observed was certainly responsible for the low polymerase activities observed. The nuclease activity is decreased by increasing the amount of denatured calf thymus DNA added as template-primer (Figure 4.5). I was unable to find an agent which selectively inhibits nuclease activity without affecting polymerase activity. There is some evidence that the polymerase and nuclease activities are contained in the same protein. Crerar and Pearlman observed that nuclease activity accompanied polymerase activity in all the fractionation procedures they used (102).

on DNA polymerase activity

The effect of increasing the amount of denatured calf thymus DNA template-primer is shown in Figure 4.6. Although maximal activity was obtained with about 30 micrograms DNA per assay this was only slightly different from the activity obtained with half that amount of DNA per assay. For an unknown reason the amount of label incorporated decreases when more than 30 micrograms of DNA is used.

4.4.2. Terminal deoxynucleotidyl transferase

The DNA polymerase activities of Tetrahymena cell homogenates, Fraction 2, and Fraction 5 in the presence of TTP as the only deoxynucleotide substrate are shown in Table 4.1. The little activity that is observed could be due to the presence of deoxynucleotides in the enzyme preparations. In my opinion Tetrahymena does not

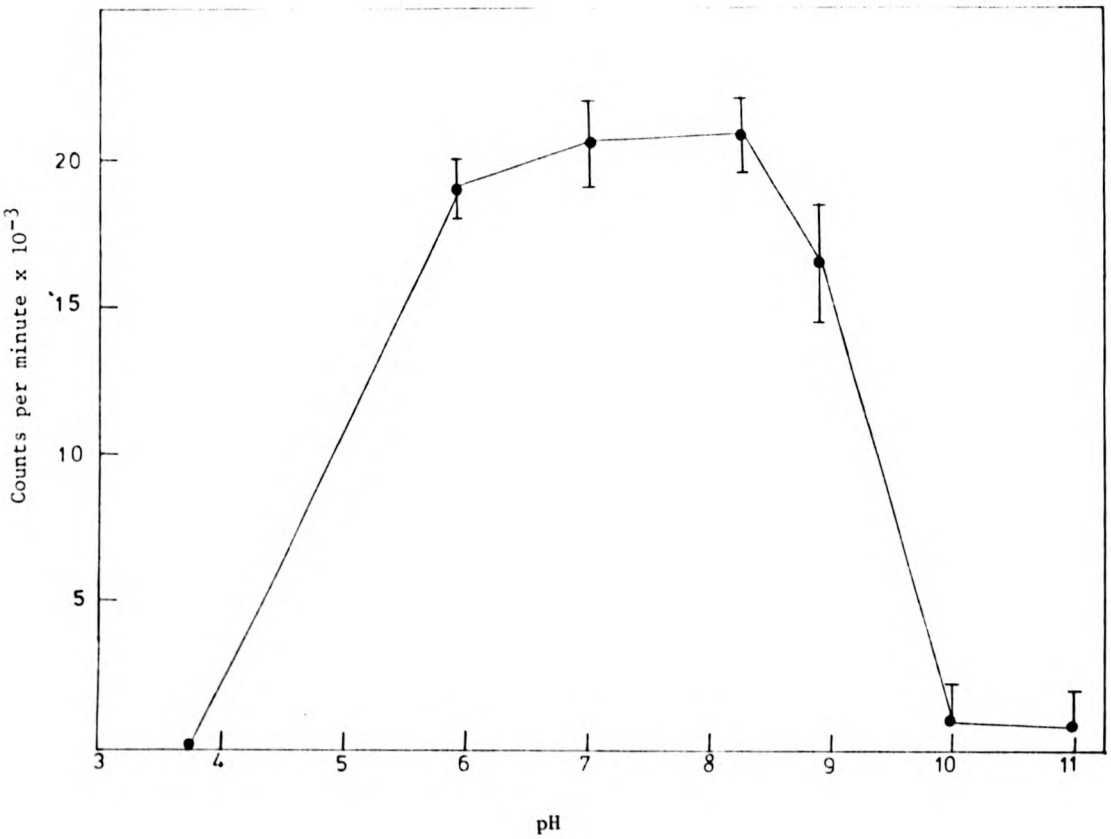

Counts per minute x 10^{-3}
20
15
10
5
3
4
5
6
7
8
9
10
11
pH

Figure 4.2

Effect of pH on the DNA polymerase activity of crude homogenates of Tetrahymena

DNA polymerase activity was determined by measuring the incorporation of labelled TTP into acid-insoluble material. The determination of enzyme activity was done as described in Section 4.3.4 except that buffers of different pH were used as required.

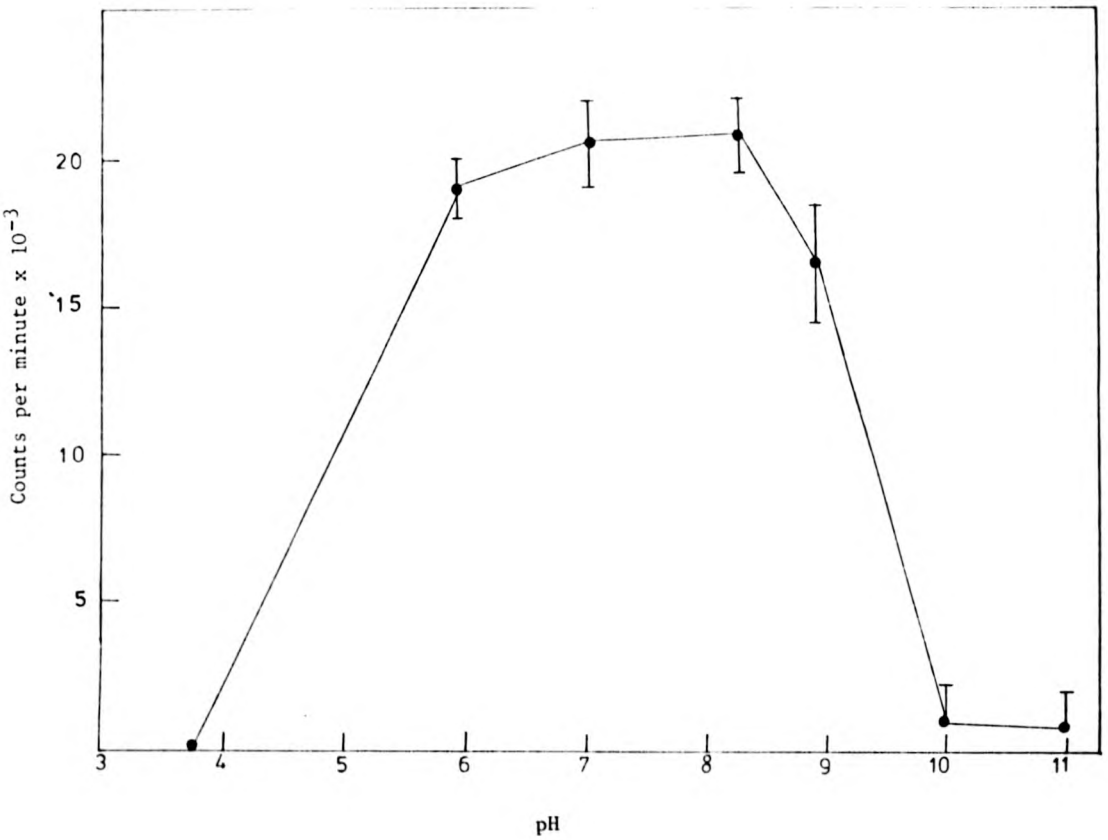

Counts per minute x 10^{-3}
20
15
10
5
3
4
5
6
7
8
9
10
11
pH

Figure 4.3

Time course of the DNA polymerase reaction

DNA polymerase activity was determined by measuring the incorporation of TTP into acid-insoluble material as described in the text. The reaction was stopped with acid at different times.

in the

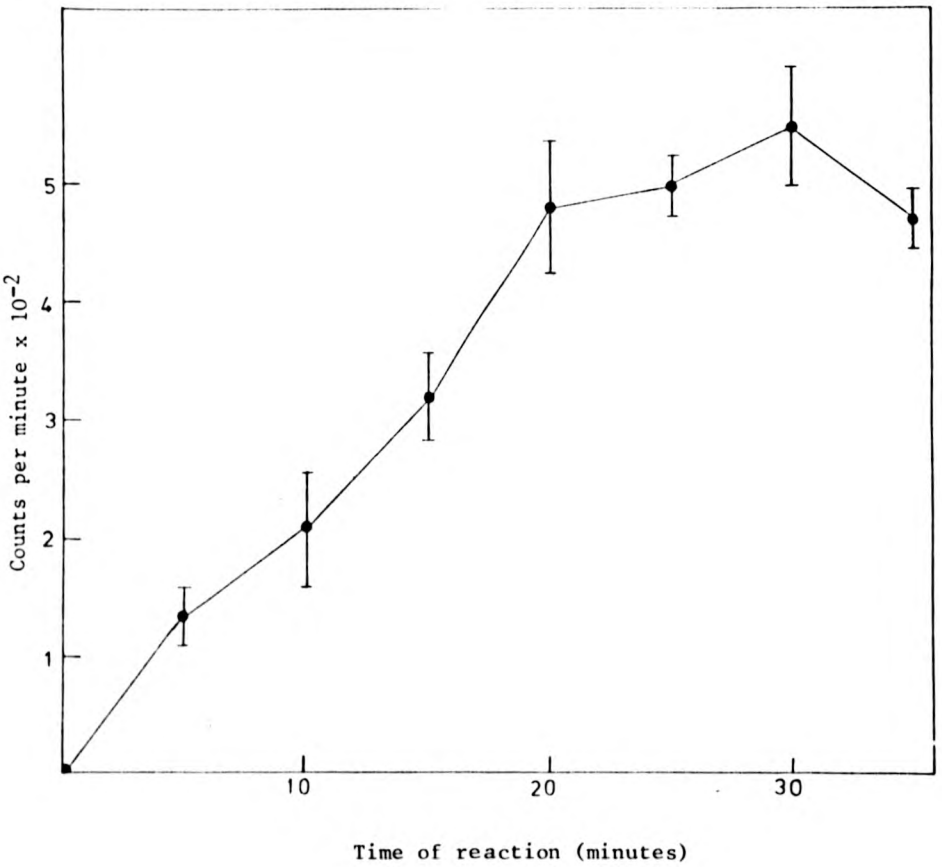

Counts per minute x 10^{-2}
1
2
3
4
5
10
20
30
Time of reaction (minutes)

Figure 4.4

Time course of the nuclease activity of Tetrahymena

Nuclease activity was measured by following the release of acid-soluble products from poly(dA-(methyl-^{3}H)dT. After incubation of labelled polyd(A-T), buffer and enzyme extract, the reaction was stopped by adding TCA. After centrifugation to remove acid-insoluble material the radioactivity in the supernatant was counted.

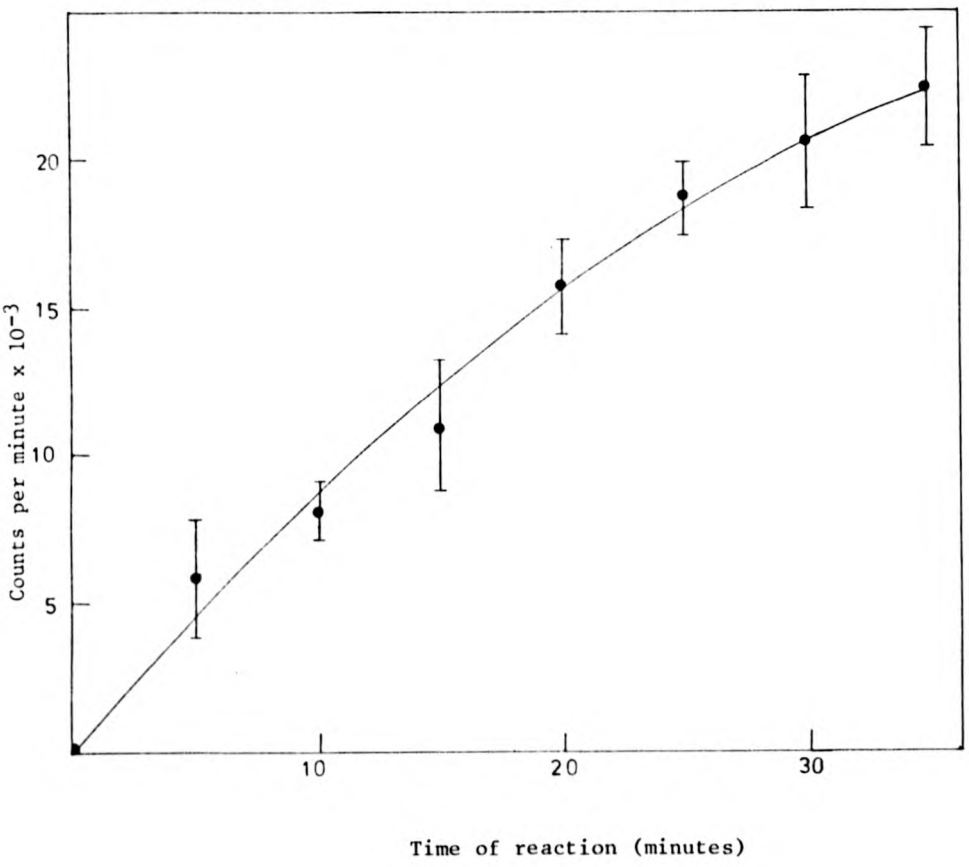
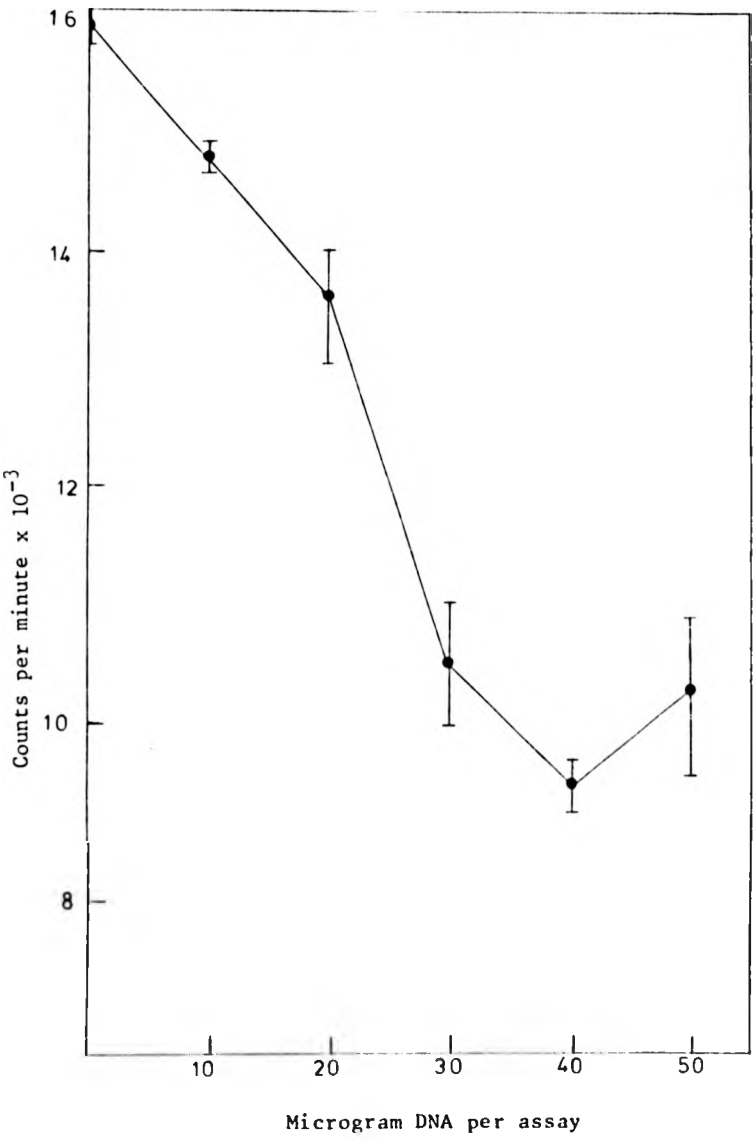

Counts per minute x 10^{-3}
20
15
10
5
10
20
30
Time of reaction (minutes)

Figure 4.5

Effect of added calf thymus DNA on the nuclease activity of Tetrahymena cell homogenates

Nuclease activity was measured by following the release of acid-soluble products from poly(dA-(methyl-^{3}H)dT) in the presence of increasing amounts of added calf thymus DNA.


16
14
12
10
8
Counts per minute x 10^{-3}
10
20
30
40
50
Microgram DNA per assay

have a terminal deoxynucleotidyl transferase activity. Fractions collected during the fractionation of the DNA polymerase activity of Tetrahymena on DEAE-cellulose columns did not show polymerase activity when TTP was the only deoxynucleotide substrate. This is probably due to the removal of any endogenous deoxynucleotides that might have contributed to the small activity observed with crude preparations.

4.4.3. Fractionation of DNA polymerase activities on DEAE-cellulose

Figure 4.7 and Table 4.2 show the fractionation of DNA polymerase activities of Tetrahymena on DEAE-cellulose columns. Two major DNA polymerase activity peaks, A and B, were observed. The activities were eluted with 0.25 and 0.6 M salt respectively. Other smaller peaks which were not consistently observed in duplicate experiments may be associated with experimental error in the fractionation and assay procedures. Because of the instability of enzyme preparations from DEAE-cellulose columns it was not possible to perform meaningful studies of the effect of using different template-primers and inhibitors.

The fractionation of DNA polymerase on DEAE-cellulose was more successful when performed as a batch process. Because the process was done in a shorter time and with fewer steps the enzyme preparations did not lose their activity during fractionation. As the final volumes occupied by enzyme samples were much smaller than in the column method there was less dilution of any activity that might be present. As can be seen from Table 4.3 a thirty-fold purification of enzyme activity was achieved. This compares favourably with the two-fold purification achieved by the initial ammonium sulphate step of the column method.

	percent activity		
	homogenate	Fraction 2	Fraction 5
Complete cocktail	100	100	100
TTP only	8.1 ± 1.3	2.7 ± 0.6	5.6 ± 1.4

Table 4.1 Terminal deoxynucleotidyl transferase activity of Fraction 2 and Fraction 5

The DNA polymerase activity of the preparations was measured with only TTP as the deoxynucleotide substrate. Polymerase activity was determined by measuring the incorporation of labelled TTP into acid-insoluble material. The activity with only TTP as the deoxynucleotide was expressed as a percentage of the DNA polymerase activity with all the four deoxynucleotides present in the assay mixture.

FRACTION	Volume	Total Protein (mg)	Total Units	Specific activity nmol/min/mg protein x 10^3
Homogenate	5	29.5	91.5	3.1 ± 0.2
59 000 g supernatant	5.6	8.1	28	3.5 ± 0.3
0-30% NH_4SO_4 pellet	1.8	2.8	4	1.7 ± 0.1
30-70% NH_4SO_4 pellet	1.8	4.2	31	7.6 ± 0.2

Table 4.2 Fractionation of Tetrahymena DNA polymerase activities

Cell homogenates were centrifuged at 59 000 g and solid ammonium sulphate added in two steps to give final concentrations of 30% and 70%. The pellets from the 30% and 70% ammonium sulphate cuts were dialysed against buffer and the DNA polymerase activity of the dialysate measured. The specific activity was expressed as nmoles TTP incorporated per minute per milligram of protein.

Figure 4.6

Effect of increasing the amount of denatured DNA template-primer on DNA polymerase activities of cell homogenates.

DNA polymerase activity was determined by determining the amount of labelled TTP incorporated into acid-insoluble material as described in Section 4.3.4 of the text. The assay mixtures contained increasing amounts of denatured calf thymus DNA template-primer.

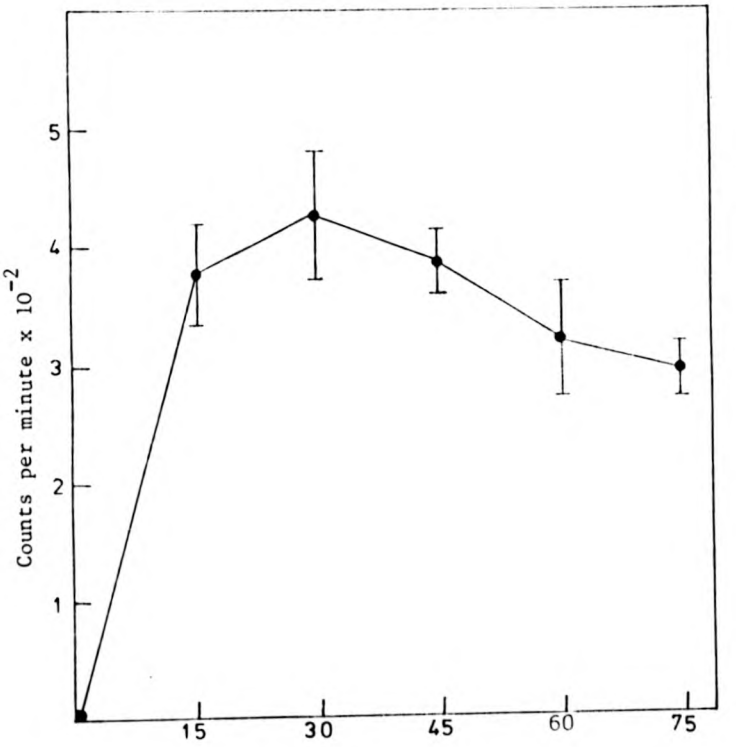

Counts per minute x 10^{-2}
5
4
3
2
1
15
30
45
60
75
Microgram DNA per assay

Figure 4.7

Fractionation of the DNA polymerase activity of Tetrahymena on DEAE-cellulose

Cell homogenates were centrifuged at 59 000g and the supernatant applied onto a DEAE-cellulose column. The column was washed with buffer and then eluted with a gradient of 0.05 to 0.8 M KCl in buffer. Fractions (3.5 ml) were collected using a fraction collector which could be set to deliver a known number of drops per fraction. The DNA polymerase of each fraction was measured as described in the text. Protein was monitored by measuring the absorbance at 280 nm.

—o— OD_{280}

—●— DNA polymerase activity

- - - - - - - - KCl gradient

na

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0 nm.

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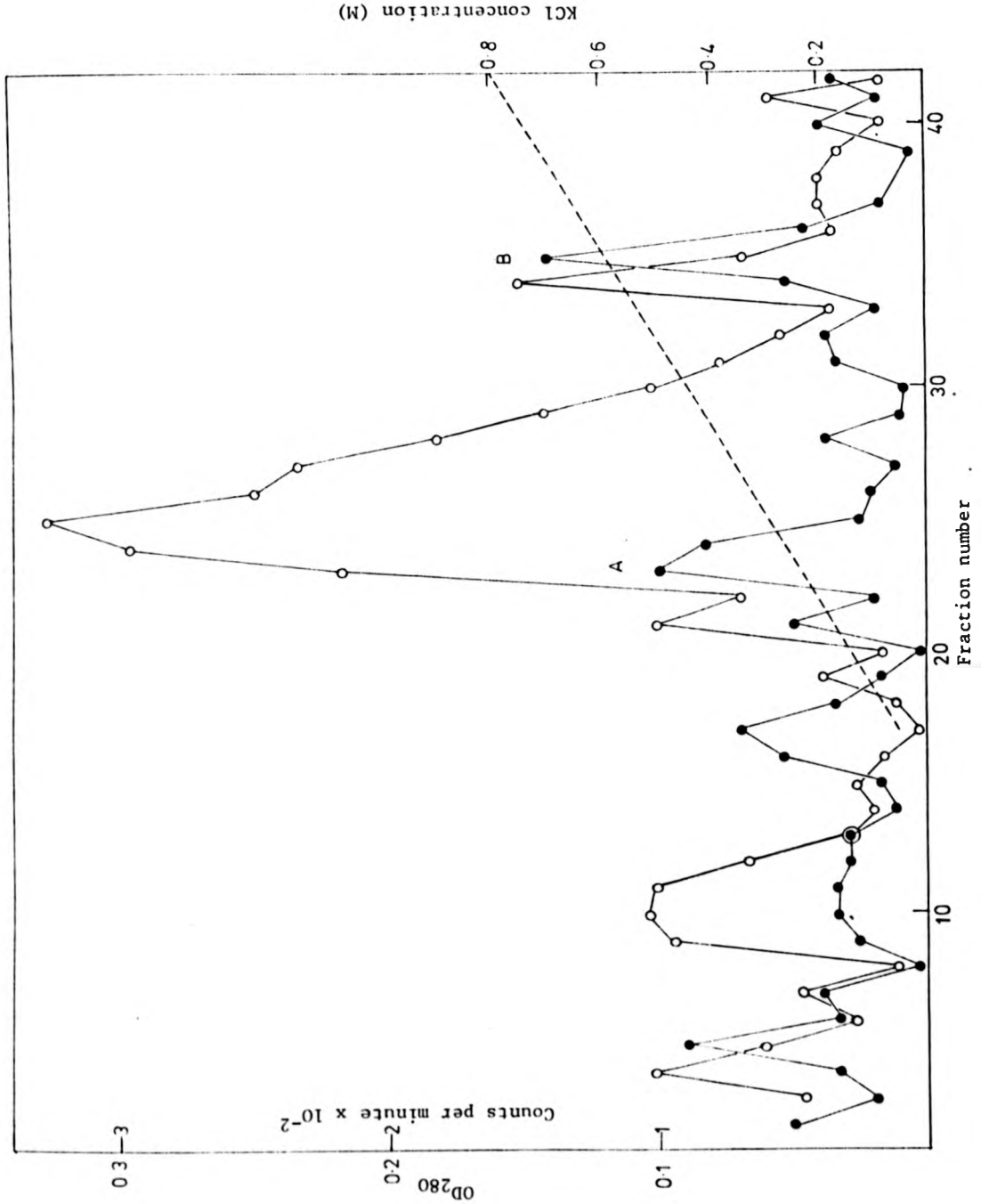


4.4.4. The use of synthetic template-primers by DNA polymerase preparations

Table 4.4 shows the use of DNA and synthetic template-primers by cell homogenates. In the presence of magnesium as the metal ion, maximal activity occurs with polyd(A-T) as the template-primer. The activity with polyd(A-T) is twice as high as that with denatured calf thymus DNA. PolyA.dT_{10} was used nearly as well as denatured DNA. It is interesting that Tetrahymena also possessed a polymerase activity which was able to copy the RNA-like template, polyA, in polyA.polydT.

In the presence of manganese as the metal ion all the template-primers were poorly used. It may be significant that with manganese as the metal ion polyA.polydT was the template-primer with which all the preparations showed maximal activity.

The use of DNA and synthetic template-primers by enzyme fractions prepared by batch DEAE-cellulose fractionation is shown in Table 4.3. The activities of the fraction that came off the exchanger in 0.3 M salt (fraction A) and 0.8 M salt (fraction B) were studied. Fraction A used denatured DNA better than all the other template-primer systems. Polyd(A-T) and polyA.dT_{10} were poorly used by this fraction. PolydA.dT_{10} was used at a rate only slightly lower than that obtained with denatured DNA. PolyA.polydT was the template-primer with which fraction B showed the highest activity. This fraction used both polyA.polydT and polydA.dT_{10} better than denatured DNA.

Table 4.5 shows the use of synthetic template-primers by fractions 2 and 5. In this experiment polyA.dT_{10} was used as the RNA-like template-primer. Both enzyme preparations used the RNA-like polyA.dT_{10} at a lower rate than denatured DNA. PolydA.dT_{10}, which gave high activities with fraction 2, was used at a lower rate than

Table 4.3

The use of DNA and synthetic template-primers by Fraction A and B

The DNA polymerase activity of fraction A and fraction B was studied with DNA and synthetic polynucleotide systems as template-primers. The specific activity was expressed as nmoles of TTP incorporated per minuted per milligram of protein. The last two rows show ratios of activities with some of the synthetic template-primers to highlight differences between fraction A and B. The activity of the two fractions in the presence of 80 mM AraCTP is also shown.

	Specific activity: nmol/min/mg protein x 10^5	
	Fraction A	Fraction B
DNA	147.0 ± 0.2	131.0 ± 1
polyd(A-T)	23.9 ± 0.4	35.2 ± 0.5
polyA.polydT	39.1 ± 0.3	195.0 ± 2
polydA.dT_{10}	126.0 ± 2	163.0 ± 3
AraCTP	108.0 ± 2	149.0 ± 3
Ratio $\frac{\text{PolyA.polydT}}{\text{Polyd(A-T)}}$	1.6	5.5
Ratio $\frac{\text{PolyA.polydT}}{\text{PolydA.}dT_{10}}$	0.3	1.2

Table 4.4

The use of denatured DNA and synthetic template-primers by crude enzyme preparations of Tetrahymena

The DNA polymerase activities of cell homogenates with denatured calf thymus DNA and synthetic polynucleotide systems as template-primers was studied. The polymerase activity with each template-primer was measured with magnesium or manganese as the divalent cation.

Template-primer	Metal ion	Specific activity nmoles/min/mg protein x 10^4
denatured DNA	Mg	6.2 ± 0.2
denatured DNA	Mn	1.1 ± 0.2
polyA.polydT	Mg	3.2 ± 0.3
polyA.polydT	Mn	1.9 ± 0.4
polydA.dT_{10}	Mg	5.1 ± 0.3
polydA.dT_{10}	Mn	1.1 ± 0.2
polyd(A-T)	Mg	12.6 ± 0.3
polyd(A-T)	Mn	1.5 ± 0.2

Table 4.5

The use of DNA and synthetic template-primers by Fractions 2 and 5

The DNA polymerase activity of fraction 2 and fraction 5 was studied with DNA and synthetic polynucleotide systems as template-primers. The specific activity was expressed as nmoles of TTP incorporated per minute per milligram of protein. The last two rows show ratios of activities with some of the synthetic template-primers to highlight differences between fractions 2 and 5.

	Specific activity nmol/min/mg protein x 10^3	
	Fraction 2	Fraction 5
DNA	14.3 ± 0.3	284.0 ± 2
polyd(A-T)	21.0 ± 0.2	237.0 ± 1
polyA.dT_{10}	12.6 ± 0.3	273.0 ± 3
polydA.dT_{10}	22.6 ± 0.1	201.0 ± 2
Ratio $\frac{\text{PolyA.dT}_{10}}{\text{Polyd(A-T)}}$	0.6	1.2
Ratio $\frac{\text{PolyA.dT}_{10}}{\text{PolydA.dT}_{10}}$	0.5	1.3

denatured DNA by fraction 5. The lack of a clear trend in these results probably indicates that the two enzyme activities, sensitive and insensitive to NEM, are not clearly resolved in fractions 2 and 5. Taking this into account it seems that most of the activity in the nuclear preparation, fraction 5, is the same as activity B. The enzyme activity in the cytoplasmic preparation, fraction 2, behaves like activity A.

4.4.5. The effect of AraCTP and NEM on DNA polymerase activities of preparations from Tetrahymena

The effect of NEM on the DNA polymerase activities of fraction 2 and fraction 5 is shown in Figure 4.10. In the presence of 1.5 mM NEM, the activity of fraction 2 is inhibited by 70% whereas that of fraction 5 is inhibited by only 30%. The differences in sensitivity to NEM probably reflect differences in the number of sensitive sulphydryl groups in the two enzymes.

The activity of fraction 2 is reduced by about 90% in the presence of 80 mM AraCTP as shown in Figure 4.8. By contrast, this concentration of AraCTP increased the activity of fraction 5 by about 50% (Figure 4.9). The increased activity of fraction 5 may indicate that this enzyme is able to use AraCTP in replicating DNA. However, this conclusion needs further examination in view of the observation by Yoshida's group that AraCTP did not support DNA synthesis by substituting for dCTP (166).

Figure 4.8

Effect of AraCTP on the DNA polymerase activity of fraction 2

The DNA polymerase activity of fraction 2 was determined in the presence of increasing concentrations of AraCTP. The activity of preparations in the presence of AraCTP was expressed as a percentage of the activity with no inhibitor. The activity with no AraCTP added was taken to be 100%

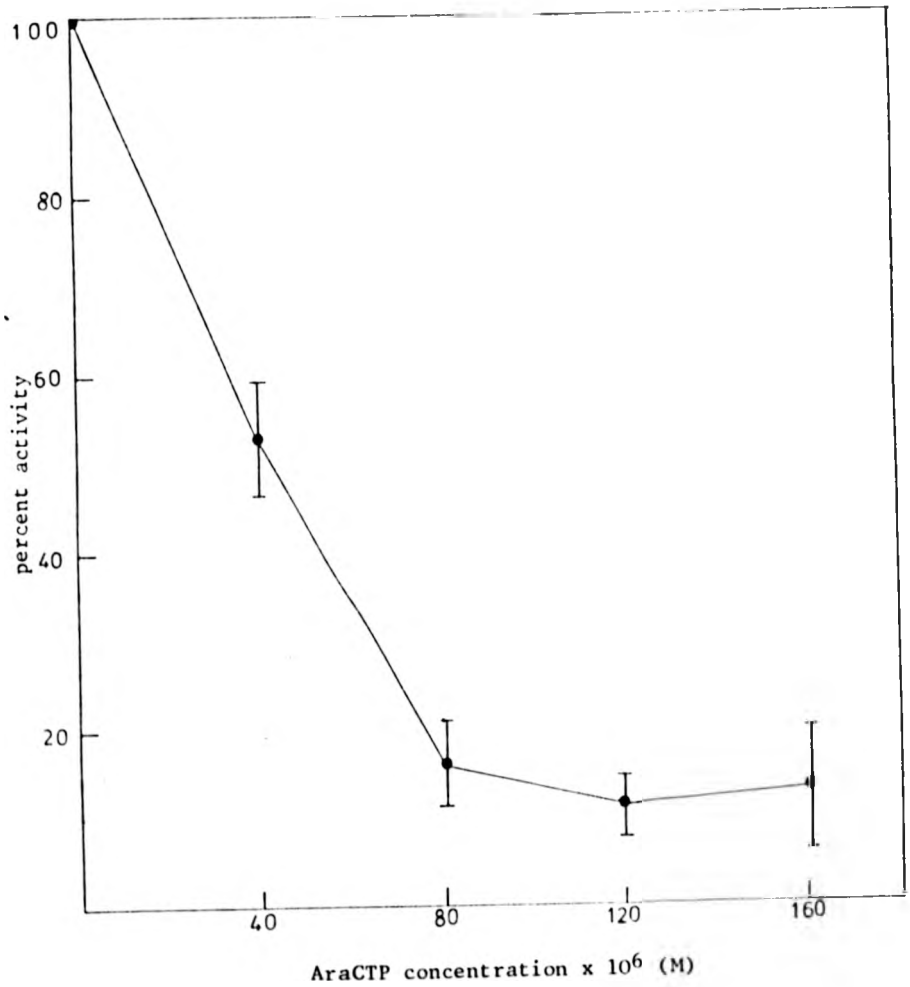

100
80
60
40
20
percent activity
40
80
120
160
AraCTP concentration x 10⁶ (M)

Figure 4.9

Effect of AraCTP on the DNA polymerase activity of fraction 5

The DNA polymerase activity of fraction 5 was measured in the presence of AraCTP and expressed as a percentage of the activity without AraCTP added. The graph shows the percentage increase in activity over that obtained without AraCTP.

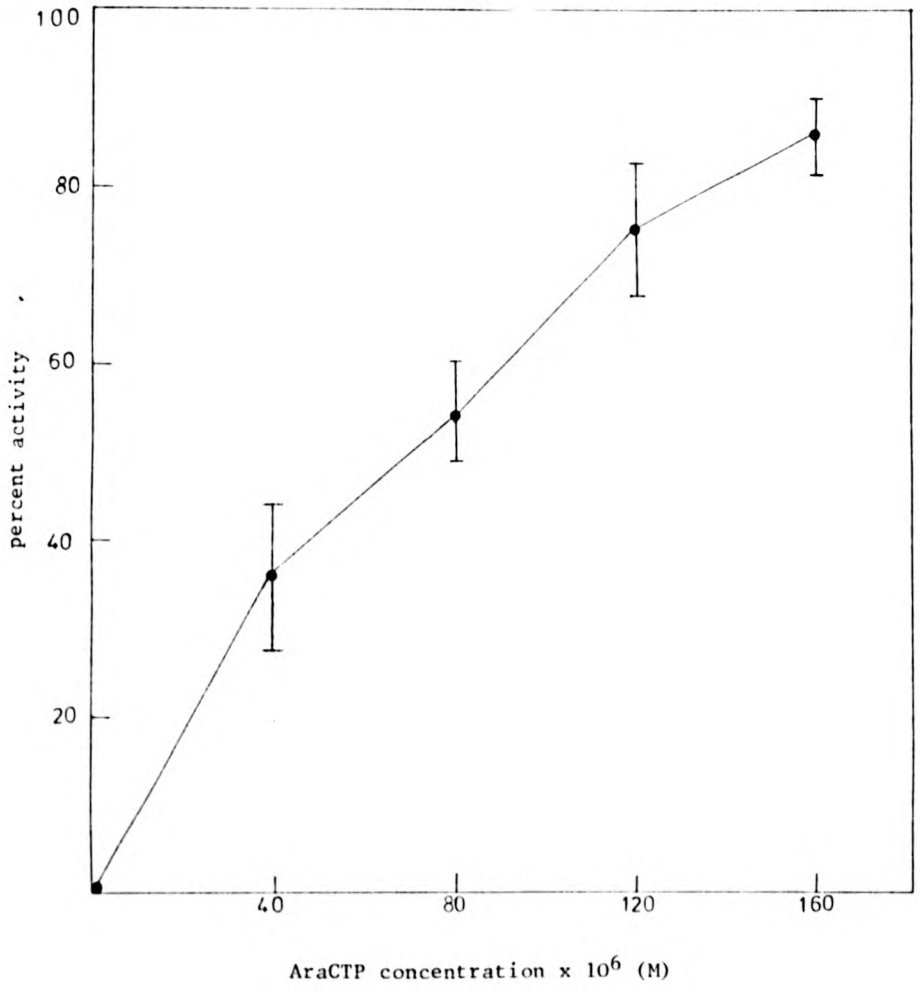

100
80
60
40
20
percent activity
40
80
120
160
AraCTP concentration x 10^6 (M)

Figure 4.10

Effect of NEM on the DNA polymerase activities of fractions 2 and 5

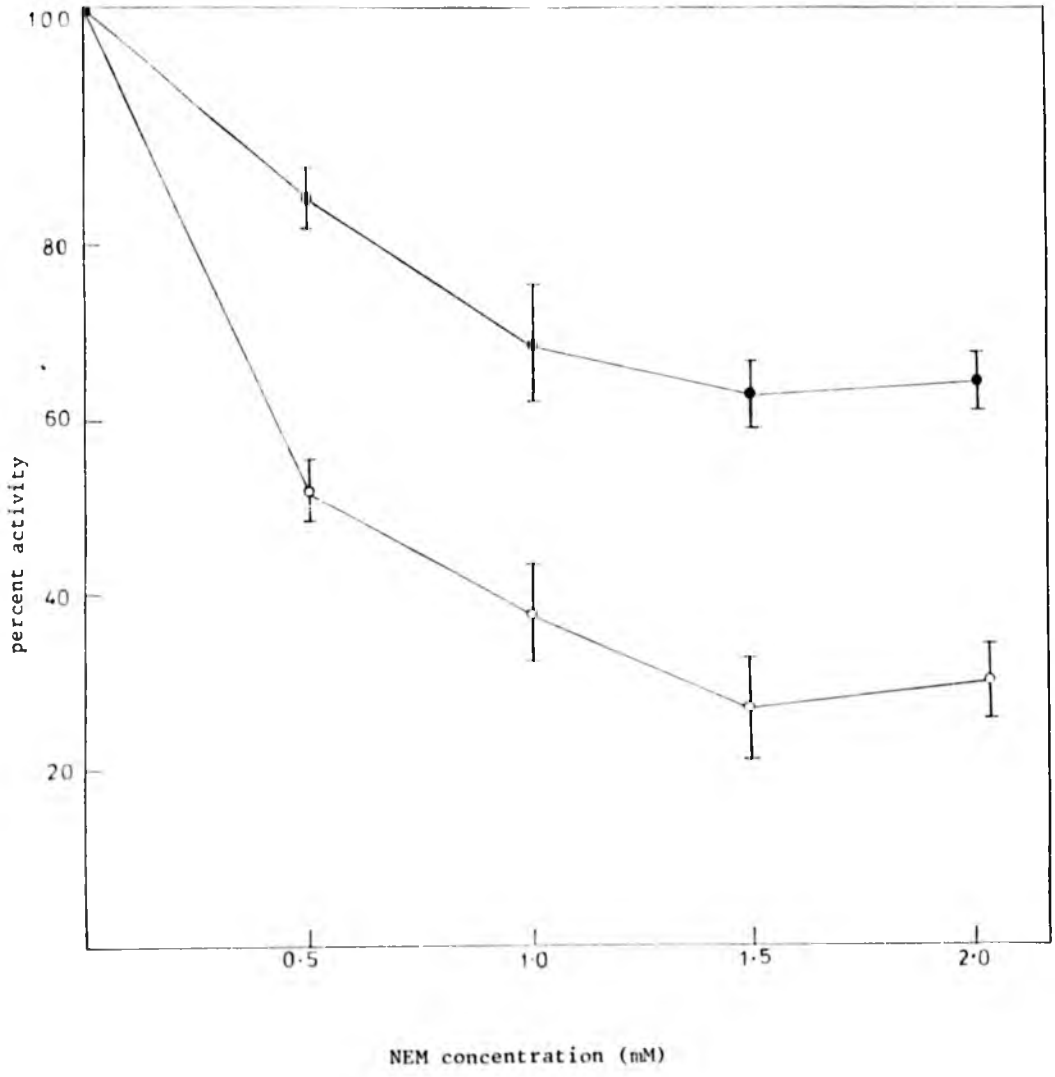
The DNA polymerase activities of fractions 2 and 5 were determined in the presence of NEM. The activity with NEM was expressed as a percentage of that without the drug in the assay mixture.

—●— Fraction 5

—○— Fraction 2

and 5

pressed



CONCLUSION

Work on DNA replication in eukaryotes has been strongly influenced and sometimes dominated by ideas from studies on bacterial and viral systems. The concept of replicons, well characterised in the lower systems, is only poorly understood in eukaryotes. This has led to some confusion in the literature especially with respect to the problem of the initiation of DNA synthesis. It is not always clear whether what is referred to is the beginning of the replication of whole chromosomes, the initiation of individual replication units or the initiation of Okazaki fragments. Although it is usually assumed that the above are three distinct aspects in the initiation of DNA replication, in practice it has not been easy to observe the processes separately. This limits the usefulness of distinguishing these facets of the initiation process.

In this work I observed that in the presence of AraC, (methyl-^{3}H)thymidine was incorporated into DNA of high molecular weight but that the initiation of new small molecular weight DNA was inhibited. When lysed cells that had been incubated with labelled thymidine were applied onto alkaline sucrose gradients and centrifuged, the peak of maximum radioactivity was recovered from fractions corresponding to a sedimentation coefficient of 20S. In the presence of AraC (0.5 mM) this peak shifted to the higher sedimentation coefficient of 30S. This result is similar to that reported by Fridland (181) who observed that in human lymphoblasts, in the presence of AraC peaks of radioactivity were associated with progressively higher S values. Fridland concluded that AraC inhibited the formation of new replicons without affecting elongation or other steps in the replication of DNA.

By contrast Dijkwel and Wanka (180), working with calf liver cells, found that in the presence of AraC short chains of DNA accumulated. It may be significant that the AraC concentrations used by Fridland (3×10^{-8} M) were much lower than those used by Dijkwel and Wanka (10^{-4} M) and those used in the work described in this thesis (5×10^{-4} M). However, this variation could also be a reflection of differences between the species from which the experimental cells were obtained. The results reported in this thesis indicate that in Tetrahymena, AraC at the concentrations used inhibited the early stages of DNA synthesis. If AraC acted on DNA synthesis by reducing the rate of chain elongation, the sedimentation profiles of pulse-labelled DNA from cells treated with AraC would have shifted to lower S values than those observed with untreated cells. It is not possible from the results obtained, to state whether the initiation stage affected is that of whole chains, replication units or Okazaki-type fragments. As protein synthesis was found to be insensitive to AraC it can be concluded that AraC does not act by inhibiting the production of the enzymes required for the initiation of DNA replication.

To test the conclusion arrived at here it would be necessary to perform pulse-chase experiments in which cells would be pulsed with bromodeoxyuridine and labelled thymidine followed with a chase of unlabelled thymidine as described by Gautschi and co-workers (209). The density of DNA formed in the presence of AraC would then be analysed by equilibrium centrifugation on caesium chloride gradients. A reduction in the density of DNA in the presence of inhibitor would indicate that chain elongation was inhibited whereas no change would indicate that elongation was not affected (181).

It does not seem that there will be an early answer to the

problem of the detailed mechanism of how AraC causes cell death. Although there is general agreement that AraCTP is the active agent in causing cell death there is disagreement as to whether it is incorporated into DNA. Reports claiming the incorporation of AraCTP are not always convincing. In many cases only a tiny fraction of the total radioactivity in AraCTP is observed to enter into DNA.

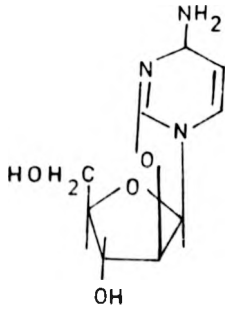
The effect of AraC in inhibiting DNA synthesis and causing cell death would be expected to depend, at least in part, on the cellular concentration of AraC itself. The rate of transport of AraC and other nucleosides into cells is higher than the rate of phosphorylation (183). In this work I have described how AraC is rapidly deaminated to AraU in Tetrahymena. In the presence of low concentrations of tetrahydrouridine the deamination of AraC by Tetrahymena cytidine deaminase is totally inhibited. Although the levels of AraC remain high in the presence of tetrahydrouridine no increase in the levels of the putative active agent, AraCTP, was observed. The levels of AraCMP and AraCDP also remained low in the presence of tetrahydrouridine. It appears, therefore, that phosphorylation is the limiting step in the incorporation of AraC into the nucleotide pool. The results described in this thesis indicate that the low incorporation of AraC into the nucleotide pool does not result from slow transport into cells but rather from a low rate of phosphorylation. The slow phosphorylation of AraC could be due to feedback inhibition of the cytidine kinase by intracellular dCTP (183).

During the course of this study it was realised that Tetrahymena cells were much less sensitive to the effects of AraC than mammalian cells. For example, whereas 0.018 mM AraC produced 20% inhibition of DNA synthesis as measured by thymidine incorporation in human

leukaemic leukocytes (210), about 100 times this amount of AraC is required to produce detectable inhibition of thymidine incorporation Tetrahymena cells. It is not clear why Tetrahymena should have this high threshold AraC concentration before DNA synthesis and cell replication are affected.

Further work in this area should include more detailed study of the phosphorylation of AraC in target cells and the examination of the possibility that deamination can occur after the formation of AraCTP or other phosphorylated products of AraC. Since AraC is rapidly inactivated, in clinical use continuous infusion or multiple repeated doses of the drug are required to produce toxicity. It would be useful to synthesize and investigate the use of AraC derivatives which retain the cell-killing properties of AraC but whose amino groups are modified to make them poor substrates for cytidine deaminase. Such compounds would be expected to retain the chemotherapeutic properties of AraC and to remain in cells longer than the parent compound. For example, cyclocytidine has been used to circumvent the need for continuous infusions of AraC. This drug, another cytidine derivative, is slowly hydrolysed in cells and thus acts as a steady source of AraC (211). However, this drug has undesirable effects in addition to those observed with AraC.

cyclocytidine



The enzymology of DNA replication in eukaryotes is still in its early stages. Because of the lack of suitable mutants it has been necessary to rely on the use of synthetic template-primers and analyses of DNA polymerase activities during the cell cycle in

assigning biological roles to the various DNA polymerase activities discovered. It is generally assumed that the DNA polymerases whose activities rise during the S phase of the cell cycle are the ones involved in DNA replication. Those DNA polymerases whose activities remain constant throughout the cell cycle are presumed to perform special functions like DNA repair.

One must be careful in interpreting results obtained when using purified or partially purified enzyme preparations. It is always possible that units which are stable in vivo disintegrate during fractionation procedures to yield separate proteins or protein fragments which retain some enzyme activity. Study of the properties of these disordered proteins would lead to erroneous conclusions about the behaviour of the enzymes in intact cells. Procedures like salt treatment may disrupt weakly associated proteins. Disruption of cells could also lead to the attack of DNA polymerizing enzymes by proteolytic enzymes. As DNA polymerases tend to lose activity when protein concentration is low (206) I added BSA to dilute fractions to improve stability of the enzymes. Glycerol, dithiothreitol and mercaptoethanol were also added to provide further stabilisation.

In this work I attempted to study the biological function of Tetrahymena DNA polymerases by investigating the use of some synthetic template-primers by enzyme preparations from this organism. Four template-primer systems, denatured calf thymus DNA, polyd(A-T), polyA.polydT or polyA.dT_{10} and polydA.dT_{10} were used. The four enzyme fractions, the nuclear preparation (fraction 5), the cytoplasmic fraction (fraction 2), the activity eluted from DEAE-cellulose by 0.3 M salt (fraction A) and the activity eluted from DEAE-cellulose by 0.8 M salt (fraction B) could use all the four template-primer

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Property	Fraction 2 (A)	Fraction 5 (B)
Sensitivity to AraCTP	Inhibited	Stimulated
Sensitivity to NEM	Sensitive	Resistant
Preferred synthetic template-primer	polydA.dT_{10}	polyA.polydT or polyA.dT_{10}
Cellular location	Cytoplasm and nucleus (2 only)	Nucleus (5 only)
Salt concentration for elution from DEAE-cellulose	0.3 M (A only)	0.8 M (B only)

A comparison of the DNA polymerase activities *Tetrahymena* described in this thesis

systems above in incorporating TTP into acid-insoluble material. Fraction 2 and fraction A showed high activities with the DNA-like template-primers polydA.dT_{10} and polyd(A-T). Fraction A showed maximal activity with denatured calf thymus DNA whereas fraction 2 had the highest activity with polydA.dT_{10}. In contrast fraction 5 and fraction B were able to use polyA.dT_{10} and polyA.polydT respectively better than polydA.dT_{10}. This means that these enzyme preparations were able to copy the RNA-like template polyA in polyA.polydT of polyA.dT_{10}. This finding is in disagreement with that of Crerar and Pearlman (102) who found that activity with polydA.dT_{10} but not with polyA.dT_{10} in the presence of magnesium as the metal ion. Working with crude homogenates from Tetrahymena, these workers found only a small activity with poly.dT_{10} with manganese as the divalent cation.

Fraction 2 and fraction A were found to be sensitive to AraCTP whereas fraction 5 and fraction B showed increased activity in the presence of this drug. Differential sensitivity to AraCTP has also been observed between DNA polymerase alpha and DNA polymerase beta of the higher eukaryotes. DNA polymerase is found to be less sensitive than DNA polymerase alpha (166). On the basis of the differences reported alone, it would be difficult to assign specific functions to the enzyme activities that were studied. It would be useful to study the ability of these enzyme preparations to extend RNA primers in copying DNA-like templates as enzymes that can extend RNA primers would probably be DNA replication rather than repair enzymes.

APPENDIX I

Preparation of defined medium DYY

The following stock solutions were made:

Stock 1

D-L alanine	1500 mg	6g
L-arginine HCl	1500 mg	6g
L-asparagine H2O	1000 mg	4g
L-glutamic acid	2000 mg	8g
L-glutamine	500 mg	2g
Glycine	2000 mg	8g
L-histidine HCl,H2O	1000 mg	4g
L-isoleucine	1000 mg	4g
L-leucine	1000 mg	4g
L-lysine HCl	1000 mg	4g
D-L methionine	1500 mg	6g
D-L phenylalanine	1500 mg	6g
L-proline	1000 mg	4g
D-L serine	1500 mg	6g
D-L threonine	2000 mg	8g
L-tryptophan	750 mg	3g
D-L valine	1000 mg	4g
Total	250 ml water	1000 ml water

Stock 2

Guanosine	500 mg

Total: 250 ml water, add KOH to dissolve

Stock 3

Nicotinic acid	45.0 mg
d-pantothenate Ca	37.5 mg
Thiamine HCl	25.0 mg
Riboflavine 5'P Na	22.5 mg
pyridoxamine 2 HCl	2.5 mg
D-L thioctic acid	0.5 mg
Folinic acid, Calcium salt	0.5 mg
pyridoxal HCl	2.5 mg
Biotin	0.05 mg
Total	250 ml

Thioctic acid should be dissolved first.

Stock 4

10280 mg K_2HPO_4 Total 250 ml

Stock 5

"Salts". Add citric acid first and ensure that it is dissolved before adding the other salts.

A:	citric acid H_2O	15000 mg
B:	$CaCl_2 2H_2O$	331 mg (anhydrous 250 mg)
	$MgSO_4\ 7H_2O$	12500 mg
Total 250 ml		

Stock 6

$Fe(NH_4)_2\ (SO_4)_2 6H_2O$	284 mg
$ZnSO_4.7H_2O$	90 mg
$MnSO_4.4H_2O$	32 mg
$CuSO_4.5H_2O$	6 mg
$Co(NO_3)_2.6H_2O$	10 mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	2 mg

Total: 100 ml adjust to pH 2.00 with conc. HCl.

All stock solutions are stored in the freezer.

For one litre of medium the following amounts from stock solutions were used:

(1) 50 ml amino acids

(2) 25 ml guanosine

(3) 5 ml vitamins

(4) 10 ml phosphate

(5) 10 ml of both A and B

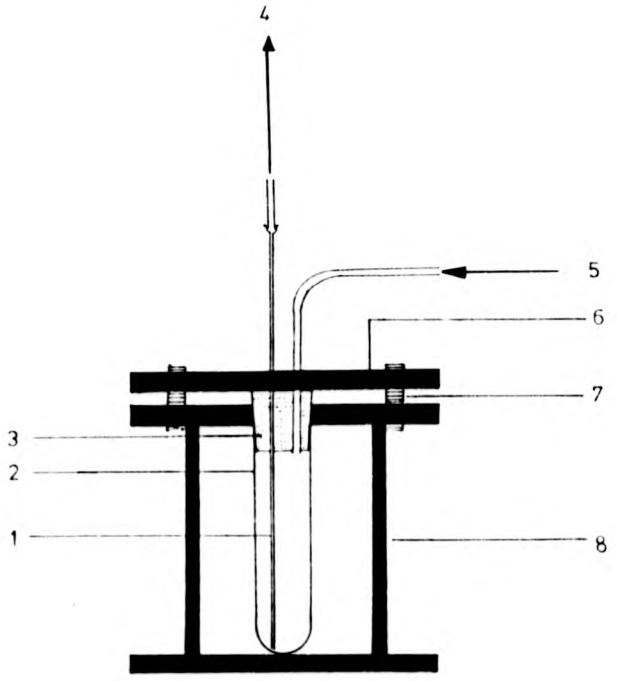
(6) 5 ml trace elements

The quantities were made up to one litre with water and the pH was adjusted to 7.1 with 20% KOH. Glucose was added to a final concentration of 1 g/100 ml of medium. The medium was then autoclaved for ten minutes at a pressure of 15 lb/sq. in.

APPENDIX II

Apparatus for collecting fractions from density gradients

1. Long injection needle
2. Centrifuge tube
3. Rubber stopper
4. Outlet to receiving tube
5. Inlet from air supply
6. Perspex plate to keep rubber stopper in place
7. Rubber band
8. Perspex stand


4
5
6
7
3
2
1
8

APPENDIX III

Calculation of sedimentation coefficients

The sedimentation coefficient, s, is defined as the sedimentation velocity in unit field strength, that is

$$s = \frac{1}{\omega^2 r} \cdot \frac{dr}{dt} \qquad (1)$$

where r is the distance of the molecule from the axis or rotation at time t and ω is the angular velocity in radians/second. Sedimentation coefficients determined in density gradient media are converted to standard coefficients which would be obtained for a given molecule in water at 20°C ($s_{20,w}$). The $s_{20,w}$ of a particle sedimenting at a temperature T, through a medium m of density $\rho_{T,m}$ and viscosity $\eta_{T,m}$ is given by

$$s_{20,w} = \frac{1}{\omega^2 r} \cdot \frac{dr}{dt} \cdot \frac{\eta_{T,m}(\rho_p - \rho_{20,w})}{\eta_{20,w}(\rho_p - \rho_{T,m})} \qquad (2)$$

where $\rho_{20,w}$ and $\eta_{20,w}$ are the density and viscosity of water at 20°C and ρ_p is the density of the macromolecule in that medium.

Integration of (2) yields

$$s_{20,w}\int \omega^2 dt = \int \frac{\eta_{T,m}(\rho_p - \rho_{20,w})}{\eta_{20,w}(\rho_p - \rho_{T,m})} \qquad (3)$$

Assuming that acceleration and deceleration times are negligible the time integral on the left becomes $S_{20,w}\omega^2 t$ where t is the total time of the sedimentation run. If the gradient is linear the radius may be expressed in terms of the sucrose concentration (Z)

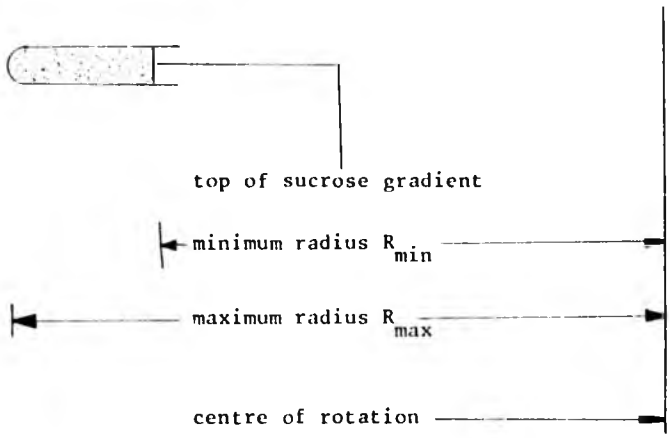
$$\frac{dZ}{dr} = \frac{Z - Z_o}{T} \qquad (4)$$

where Z_o is the extrapolated concentration at the centre of rotation. The right side of (3) can be expressed solely in terms of concentration and may be evaluated if the temperature, particle density and Z_o are known. The values of this integral for particle densities of 1.1 to 1.9 g/ml, temperatures of 0°, 5°, and 20°C and for Z_o values from 5 to -100 have been computed by McEwen.

If during centrifugation a particle travels from Z_1 at t_1 to Z_2 at t_2 then equation (3) can be written as

$$s_{20,w}\omega^2(t_2 - t_1) = I_{(Z_2)} \quad I_{(Z_1)} \qquad (5)$$

$I_{(Z_1)}$ and $I_{(Z_2)}$ are obtained from McEwen's tables from a knowledge of the particle density, temperature and Z. Z_o is evaluated from the dimensions of the rotor used.



A typical calculation for the SW25.2 rotor is shown below.

(A) First the distance of each fraction from the centre of rotation was evaluated. If 25 fractions are collected and each occupies

$$\frac{R_{max} - R_{min}}{25} = \frac{7.53}{25} = 0.30 \text{ cm} \qquad (6)$$

r_x for each fraction can now be obtained.

(B) Z_o is now evaluated using

$$Z_o = \frac{Z_1 r_x - Z_x r_1}{r_x - r_1} \qquad (7)$$

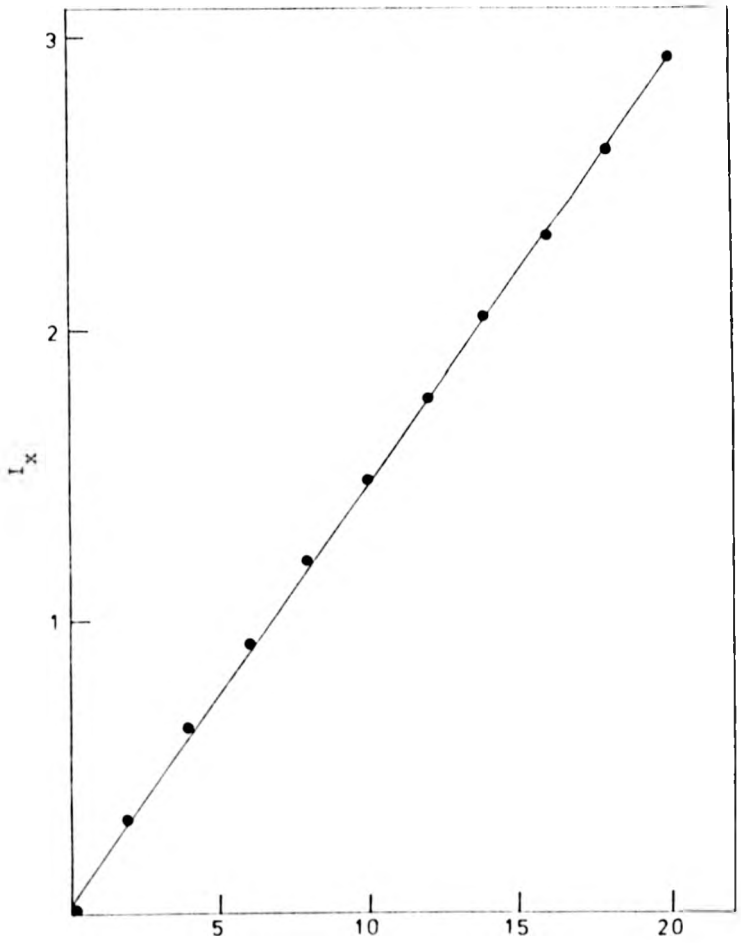
Z_1 is taken to be 5% and r_1 8 cm. Evaluation Z_o for any Z, R pair yields 11.67.

(c) Knowing Z_o $I_{(x)}$ values are now looked up from McEwen's tables. A particle density of 1.4 is assumed. For ease of use a plot of Z_x against $I_{(x)}$ is made.

(D) $I_{(x)} - I_{5\%}$ is calculated for each fraction.

Finally sedimentation coefficients are calculated from

$$s_{20,w} = \frac{I_{(x)} - I_{5\%}}{\omega^2 t}$$


3
2
1
I_x
5
10
15
20
Sucrose concentration (%)

Fraction	r_x(cm)	Z_x	Z_o	I_x	$I_x - I_{4\%}$	S(x 10^{-13})
1	8	5	-11.67	-	-	-
2	8.3	5.625	-11.67	0.88	0.2349	4.7
3	8.6	6.25	-11.67	0.98	0.3349	6.7
4	8.9	6.875	-11.67	1.06	0.4149	8.3
5	9.2	7.5	-11.67	1.16	0.5149	10.3
6	9.5	8.125	-11.67	1.22	0.5749	11.5
7	9.8	8.75	-11.67	1.32	0.6749	13.5
8	10.1	9.375	-11.67	1.42	0.7749	15.5
9	10.4	10.0	-11.67	1.5	0.8549	17.1
10	10.7	10.625	-11.67	1.58	0.9349	18.7
11	11.0	11.25	-11.67	1.66	1.0149	20.3
12	11.3	11.875	-11.67	1.75	1.1049	22.1
13	11.6	12.5	-11.67	1.83	1.1849	23.7
14	11.9	13.125	-11.67	1.91	1.2649	25.3
15	12.2	13.75	-11.67	2.0	1.3549	27.1
16	12.5	14.375	-11.67	2.1	1.4549	29.1
17	12.8	15.0	-11.67	2.18	1.5349	30.7
18	13.1	15.625	-11.67	2.26	1.6149	32.3
19	13.4	16.25	-11.67	2.34	1.6949	33.9
20	13.7	16.875	-11.67	2.45	1.8049	36.1
21	14.0	17.5	-11.67	2.53	1.8849	37.7
22	14.3	18.125	-11.67	2.62	1.9749	39.5
23	14.6	18.75	-11.67	2.70	2.0549	41.1
24	14.9	19.375	-11.67	2.82	2.1749	43.5
25	15.1	20.0	-11.69	2.93	2.2849	45.7

APPENDIX IV

Calculation of counts for each radionuclide in double-isotope experiments.

When measuring radioactivity in a sample containing two radionuclides each of the radionuclides contributes to the scintillations and produces pulses. Two counting channels are required to separate the pulses from each radionuclide, although each can produce counts in both channels. If the counting efficiencies of each radionuclide in each channel are known the activity of each of the two radionuclides can be calculated (208).

The contribution of each isotope to the total counts observed was evaluated from the simultaneous equations given below.

C	^{14}C counts in sample
H	^{3}H counts in sample
c_1	^{14}C efficiency in red channel
c_2	^{14}C efficiency in green channel
h_1	^{3}H efficiency in red channel
h_2	^{3}H efficiency in green channel
N_1	total counts observed in red channel
N_2	total counts observed in green channel

$$N_1 = C(c_1) + H(h_1) \qquad (1)$$

$$N_2 = C(c_2) + H(h_2) \qquad (2)$$

Solving for C and H we have

$$C = \frac{N_1 - N_2(h_1/h_2)}{c_1 - c_2(h_1/h_2)} \qquad (3)$$

$$H = \frac{N_2 - N_1(c_1/c_2)}{h_2 - h_1(c_2/c_1)} \qquad (4)$$

when all the ^{3}H counts are excluded from the ^{14}C channel the equations become

$$N_1 = C(c_1) \tag{5}$$

$$N_2 = C(c_2) + H(h_2) \tag{6}$$

Solving these we have

$$C = N_1/C_1 \tag{7}$$

$$H = \frac{N_2 - C(c_2)}{h_2} \tag{8}$$

The efficiencies C_1, C_2, h_1, and h_2 were determined by measuring the radioactivity of ^{14}C and ^{3}H standards in both the red and the green channel.

REFERENCES

1. Wintersberger, E. (1978) Rev. Physiol. Biochem. Pharmacol. 84, 93 - 142.

2. Huberman, J. A. and Riggs, A. D. (1968) J. Mol. Biol. 32, 327 - 341.

3. Weintraub, P., Palter, K., van Lente, F. (1975) Cell, 6, 85 - 110.

4. Shaw, B. R., Herman, T., Kovacic, R. T., Beaudreau, G. S., van Holde, K. E. (1976) Proc. Natl Acad. Sci. U.S. 73, 505 - 509.

5. Kornberg, R. O. (1977) Ann. Rev. Biochem. 46, 931 - 954.

6. Bradbury, (1976) Trends in Biochemical Sciences 1, 7 - 9.

7. Kornberg, A. DNA Synthesis, W. H. Freeman, N.Y. 1974.

8. Ioannou, P. (1973) Nature New Biol. 244, 257 - 280.

9. Okazaki, R., Okazaki, T., Iakabe, K., Sugimoto, K. (1968) Proc. Natl Acad. Sci. U.S. 59, 598 - 605.

10. Hewish, S. P. (1976) Nucl. Acid Res. 3, 69 - 79.

11. Geider, K., Kornberg, A. (1974) J. Biol. Chem. 249, 3999 - 4005.

12. Brynolf, K., Eliasson, R., Reichard, P. (1978) Cell 13, 573 - 580.

13. Wickner, W., Brutlag, R., Sheckman, R., Kornberg, A. (1972) Proc. Natl. Acad. Sci. U.S., 965 - 969.

14. Wells, R. D., Flügel, R. M., Larson, J. E., Schendel, P. F., Sweet, R. W. (1972) Biochemistry, 11, 621 - 629.

15. Slater, J. P., Tamir, I., Loeb, L. A., Mildvan, A. S. (1972) J. Biol. Chem. 247, 6784 - 6794.

16. Hand, R. (1979) Cell, 15, 317 - 425.

17. Kornberg, T., Kornberg, A., (1971) The Enzymes 10, 119 - 144.

18. Slater, J. P., Mildvan, A. S., and Loeb, L. A. (1971) Biochem. Biophys. Res. Comm. 44, 37 - 43.

19. Coleman, J. E. (1967) J. Biol. Chem., 242, 5212 - 5219.

20. Bollum, F. J., (1975) Prog. Nucl. Acid Res. Mol. Biol. 15, 109 - 144.

21. Weissbach, A. (1975) Cell 5, 108 - 109.

22. Smith, R. G., Gallo, R. C. (1972) Proc. Natl Acad. Sci. U.S., 2879 - 2884.

23. Hunton, G. R., Kalf, G. T. and Morris, H. P. (1973) Cancer Res. 33, 987 - 992.

24. Wu, A. M. and Gallo, R. C. (1975) Critical Rev. Biochem. 3, 289 - 347.

25. Stenstrom, M. L., Edelstein, M. and Grisham, J. W. (1974) Exptl Cell Res. 89, 439 - 442.

26. Tompson, F. M., Tischler, A. N., Adams, J. and Calvin, M. (1974) Proc. Natl Acad. Sci. U.S. 71, 107 - 109.

27. Loeb, L. A., Agarwal, S. S., Dube, D. K., Gopinathan, K. P., Travaglini, E. C., Seal, G., Sirover, M. A. (1977) Pharmac. Ther. A. 2, 171 - 193.

28. Modak, M. J. (1976) Biochemistry 15, 3620 - 3626.

29. Salvo, R.A., Serio, G. F., Evans, J. E. and Kimball, A. P. (1976) Biochemistry. 15, 493 - 497

30. Bardos, T. J. (1974) Topics in Current Chemistry, 52, 63 - 98.

31. Srivastava, B. I.S . (1973) Biochim. Biophys. Acta 335, 77 - 84.

32. Abrell, J. W., Smith, R. G., Robert, M. S. and Gallo, R. C. (1972) Science 177, 1111 - 1114.

33. Neidle, S., Achari, A., Taylor, G. L., Berman, H. M., Carrot, H. L., Glusker, J. B. and Stallings, W. C. (1977) Nature, 269, 304 - 307.

34. Lerman, L. S. (1964) J. Cell Comp. Phy. 64, Suppl. 1, 1 - 13.

35. Reich, E., Franklin, R. M., Shatkin, A. J. and Tatum, E. L. (1961) Science, 134, 556 - 557.

36. Goldberg, I. H., Rabinovitz, M., Reich, E., Proc. Natl Acad. Sci. U.S. 48, 1238 - 1245.

37. Nass, M. M. K., (1970) Proc. Natl Acad. Sci. U.S., 67, 1926 - 1933.

38. Meyer, R. R. and Simpson, M. V. (1969) Biochem. Biophys. Res. Comm. 34, 238 - 244.

39. Sirover, M. A., and Loeb, L. A. (1976) Biochem. Biophys. Res. Comm. 70, 812 - 817.

40. Linford, J. H., Froese, A., Israels, L. G. (1963) Nature 197, 1068 - 1070.

41. Sedwick, W. D., Wang, T. S-F., Korn, D. (1972) J. Biol. Chem. 247, 5026 - 5033.

42. Spadari, S. and Weissbach, A. (1974) J. Biol. Chem. 249, 5809 - 5815.

43. Byrnes, J. J., Downey, K. M., Black, V. L., So., A. G. (1976) Biochemistry 15, 2817 - 2825.

44. Hecht, N. B. (1975) Biochim. Biophys. Acta, 383, 388 - 398

45. Baril, E.F.(1975) Cancer Res. 35, 1 - 5.

46. Di Cioceio, R.A. and Srivastava, B.I.S. (1976) Cancer Res. 36, 1664 - 1668.

47. Loeb, A. A. (1974) The Enzymes 10, 173 - 209.

48. Bollum, F. J. (1975) Prog. Nucl. Acid Res. Mol. Biol. 15, 109 - 144.

49. Weissbach, A. (1975) Cell 5, 101 - 108.

50. Allaudeen, H. S. (1978) Pharmac. Ther. A2, 447 - 476.

51. Weissbach, A. (1977) Ann. Rev. Biochem. 46, 25 - 47.

52. Sarngadharan, M. G., Robert-Guroff, M., Gallo, R. C. (1978) Biochim. Biophys. Acta, 516, 419 - 487.

53. Holmes, A. M. and Johnston, I. R. (1975) Febs Letters 60, 233 - 243.

54. Wintersberger, E. (1977) TIBS 2, 58 - 61.

55. Weissbach, A., Baltimore, D., Bollum, F., Gallo, R., Korn, D. (1975) Science 190, 401 - 402.

56. Hecht, N. B. (1975) Biochim. Biophys. Acta 414, 59 - 70.

57. Bollum, F. J. (1960) J. Biol. Chem. 235, 2399 - 2403.

58. Weissbach, A., Schlabach, A., Fridlender, J. and Bolden, A. (1971) Nature New Biol., 231, 167 - 170.

59. Holmes, A. M., Hesslewood, I. P. and Johnston, I. R. (1974) Eu. J. Biochem. 43, 487 - 499.

60. Wang, T. S-F., Sedwick, W. D. and Korn, D. (1974) J. Biol. Chem. 249, 841 - 850.

61. Brun, G., Rougeon, F., Lauber, M., Chappe ville, F. (1974) Eu. J. Biochem. 41, 241 - 251.

62. Craig, R. K. and Keir, H. M. (1975) Biochem. J. 145, 225 - 232.

63. Chang, L. M. S., Brown, M., Bollum, F. J. (1973) J. Mol. Biol. 74, 1 - 8.

64. Henner, D. and Furth, J. J. (1977) J. Biol. Chem. 252, 1932 - 1937.

65. Momparler, R. L., Rossi, M. and Labitan, A. (1973) J. Biol. Chem. 248, 285 - 293.

66. Spadari, S., Weissbach, A. (1975) Proc. Natl Acad. Sci. U.S. 72, 503 - 507.

67. Foster, D. N. and Gurney, J. Jr. (1976) J. Biol. Chem. 251, 7893 - 7898.

68. Lynch, W. E., Surrey, S. and Lieberman, I. (1975) J. Biol. Chem. 250, 8179 - 8183.

69. Smith, R. G., and Gallo, R. C. (1972) Proc. Natl Acad. Sci. U.S. 69, 2879 - 2884.

70. Chang, L. M. S. (1973) J. Biol. Chem. 248, 2789 - 2795.

71. Wang, T. S-F., Sedwick, W. D. and Korn, S. (1975) J. Biol. Chem. 250, 7040 - 7044.

72. Hecht, N. B. (1973) Biochim. Biophys. Acta 312, 471 - 483.

73. Schlabach, A., Fridlender, B., Bolden, A. and Weissbach, A. (1971) Biochem. Biophys. Res. Comm. 44, 878 - 885.

74. Chang, L. M. S. (1973) J. Biol. Chem. 248, 6983 - 6992.

75. Mosbaugh, D. W., Stalker, D. M., Probst, G. S., Meyer, R. R. (1977) Biochemistry 16, 1512 - 1517.

76. Fridlender, B., Fry, M., Bolden, A. and Weissbach, A. (1972) Proc. Natl Acad. Sci. U.S. 71, 57 - 62.

77. Bolden, A., Fry, M., Muller, R., Citarella, R. and Weissbach, A. (1972) Arch. Biophys. Biochem. 153, 26 - 33.

78. Lewis, B. J., Abrell, J. W., Smith, R. G. and Gallo, R.C . (1974) Biochim. Biophys. Acta 349, 148 - 160.

79. Spadari, S., Muller, R Weissbach, A. (1974) J. Biol. Chem. 249, 2991 - 2992.

80. Matsukage, A., Bohn, E. W. and Wilson, S. H. (1974) Proc. Natl Acad. Sci. U.S. 71, 578 - 582.

81. Knopf, K. W., Yamado, M., Weissbach, A. (1976) Biochemistry 15, 4540 - 4548.

82. Meyer, R. R. and Simpson, M. V. (1970) J. Biol. Chem. 245, 3426- 3435.

83. Bolden, A., Noy, G. P. and Weissbach, A. (1977) J. Biol. Chem. 252, 3351 - 3356.

84. Radsak, K., Seidel, G. (1976) Biochem. Biophys. Res. Comm. 71. 1259 - 1268.

85. Coleman, M. S., Hutton, J. J., De Simoni, P., Bollum, F. J. (1974) Proc. Natl Acad. Sci. U.S. 71, 4404 - 4408.

86. Chang, L. M. S. (1971) Biochem. Biophys. Res. Comm. 44, 124 - 131.

87. Marcus, S. L., Smith, S. W., Jarowski, C. I. and Modak, M. J. (1976) Biochem. Biophys. Res. Comm. 70, 37 - 44.

88. Coleman, M. S. (1977) Arch Biochem. Biophys. 182, 525 - 532.

89. Eichler, D. C., Fisher, P. A., Korn, D. (1977) J. Biol. Chem. 252, 4011 - 4014.

90. Katouzian, M. and David, J. C. (1978) Biochem. Biophys. Res. Comm. 82, 1168 - 1175.

91. Söderhall, S. (1975) Eu. J. Biochem. 51, 129 - 136.

92. Söderhall, S. and Lindahl, T. (1973) J. Biol. Chem. 248, 672 - 675.

93. Herrick, G. and Alberts, B. (1976) J. Biol. Chem. 251, 2124 - 2132.

94. Herrick, G., Delius, H., Alberts, B. (1976) J. Biol. Chem. 251, 2142 - 2146.

95. Jeggo, P. A., Unrau, P., Banks, G. R., Holliday, R. (1973) Nature New Biol. 242, 24 - 16.

96. Banks, G. R., Holloman, W. K., Kairis, M. V. and Yarranton, G. T. Spanos,A (1976) Eur. J. Biochem. 62, 131 - 142.

97. McLennan, A. G., and Keir, H. M. (1975) Nucl. Acids Res. 2, 223 - 237.

98. McLennan, A. G., and Keir, H. M. (1975) Biochim. Biophys. Acta 407, 253 - 262.

99. Ross, C. A. and Harris, W. J. (1976) Biochem. Soc. Trans. 4, 806 - 807.

100. Wintersberger,U . (1974) Eur. J. Biochem. 50, 197 - 202.

101. Chang, L. M. S. (1977) J. Biol. Chem. 252, 1872 - 1880.

102. Crerar, M. and Pearlman, R. E. (1974) J. Biol. Chem. 249, 3123 - 3133.

103. Loomis, L. W., Rossomando, E. F. and Chang, L. M. S. (1976) Biochim. Biophys. Acta 425, 469 - 477.

104. Schönherr, O. Th., and Wanka, F. (1971) Biochim. Biophys. Acta 232, 83 - 93.

105. Holley, R. W. (1975) Nature, 258, 487 - 490.

106. Shields, R. (1977) Nature, 267, 704 - 707.

107. Jazwinski, S. M., Edelman, G. M. (1976) Proc. Natl Acad. Sci. U.S. 73, 3933 - 3936.

108. Ford, C. C. (1975) Proc. Natl Acad. Sci. U.S. 72, 2437 - 2441.

109. Krokan, H., Wist, E., Prydz, H. (1977) Biochem. Biophys. Res. Comm. 75, 414 - 419.

110. Holz, G. G. (1964) Biochemistry and Physiology of Protozoa 3, 199 - 242.

111. Mitchison, J. M. (1971) The Biology of the Cell Cycle, Cambridge University Press.

112. McLennan, A. G., Keir, H. M. (1977) Biochem. Soc. Symp. 42, 55 - 73.

113. Cohen, S. S. (1966) Prog. Nucleic Acid Res. Mol. Biol. 5, 1 - 88.

114. Saran, A., Pullman, B., Perahia, D. (1974) Biochim. Biophys. Acta 349, 189 - 203.

115. Bergmann, W., Burke, D. (1955) J. Org. Chem. 20, 1501 - 1507.

116. Bergmann, W., Teeney, R. (1951) J. Org. Chem. 16, 981 - 987.

117. Evans, S., Musser, E. A., Mengel, G. D., Forsblad, K. R., Hunter, J. H. (1961) Proc. Soc. Exp. Biol. Med. 106, 350- 353.

118. Underwood, G. E. (1962) Proc. Soc. Exp. Biol. Med. 111, 660 - 664.

119. Plunkett, W., Lapi, L., Ortiz, P., Cohen, S. S. (1974) Proc. Natl Acad. Sci. U.S. 71, 73 - 77.

120. Chu, M. Y., Fischer, G. A. (1962) Biochem. Pharmacol. 11, 423 - 430.

121. Evans, J. S., Mengel, G. D. (1964) Biochem. Pharmacol. 13, 989 - 994.

122. Livingston, R. B., Carter, S. K. (1968) Cancer Chemotherapy Reports 1, 179 - 205.

123. Talley, R., Vaitkevicius, V. (1963) Blood 21, 352 - 362.

124. Hanze, A. R. (1960) J. Amer. Chem. Soc. 89, 6720 - 6725.

125. Pizer, L. I., Cohen, S. S. (1960) J. Biol. Chem. 235, 2387 - 2392.

126. Lark, C. L., Lark, K. G. (1964) J. Mol. Biol. 10, 120 - 136.

127. Cardeilhac, P. T., Cohen, S. S. (1964) Cancer Res. 24, 1595 - 1603.

128. Furth, J. J., Cohen, S. S. (1968) Cancer Res. 28, 2061 - 2067.

129. Camiener, G. W., Smith, C. G. (1965) Biochem. Pharmacol. 14, 1405 - 1416.

130. Doering, A., Keller, J., Cohen, S. S. (1960) Cancer Res. 26, 2444 - 2450.

131. Chae, C. B., Williams, A., Krasny, H., Irvin, J. L., Piantadosi, C. (1970) Cancer Res. 30, 2652 - 2660.

132. Kim, J. H., Eidinoff, M. L. (1965) Cancer Res. 25, 698 - 702.

133. Wheeler, G. P., Browdon, B. J., Adamson, D. J., Vail, M. H. (1972) Cancer Res. 32, 2661 - 2669.

134. Karon, M., Chirakawa, G. (1970) J. Natl Cancer Inst. 45, 961 - 967.

135. Karon, M., Benedict, W., Rucker, N. (1972) Cancer Res. 32, 2612 - 2615.

136. Benedict, W., Karon, M. (1971) Science 171, 680 - 681.

137. Chaube, S., Kreis, W., Uchida, K., Murphy, M. L. (1968) Biochem. Pharmacol. 17, 1213 - 1216.

138. Kouri, R. E., Kurtz, S. A., Price, P. J., Benedict, W. F. (1975) Cancer Res. 35, 2413 - 2419.

139. Kessel, D., Shurin, S. B. (1968) Biochim. Biophys. Acta 163, 179 - 187.

140. Chou, T. C., Hutchinson, D. J., Schmid, F. A., Phillips, F. S. (1975) Cancer Res. 35, 225 - 236.

141. Chu, M. Y., Fischer, G. A. (1965) Biochemical Pharmacol. 14, 333 - 341.

142. Dechamps, M., De Saint-Vincent, B. R., Eurard, C., Sassi, M., Buttin, G. (1974) Exptl Cell Res. 86, 269 - 279.

143. Scherecker, A. W. (1970) Cancer Res. 30, 632 - 641.

144. Meuth, M., Green, H. (1974) Cell 3, 367 - 374.

145. Momparler, R. L., Chu, M. Y., Fisher, G. A. (1968) Biochim. Biophys. Acta 161, 481 - 493.

146. Ho, D.H.W., Frei, E. (1971) Clin. Pharmacol. Ther. 12, 944 - 954.

147. Meyers, R., Malathi, V. G., Cox, R. P., Silber, R. (1973) J. Biol. Chem. 248, 5909 - 5913.

148. Stewart, C. D., Burke, P. J. (1971) Nature New Biol. 233, 109 - 110.

149. Camiener, G. W. (1968) Biochem. Pharmacol. 17, 1981 - 1991.

150. Ho, D.H.W. (1972) Biochem. Pharmacol. 21, 2936 - 2940.

151. Moore, E. C., Cohen, S.S. (1967) J. Biol. Chem. 242, 2116 - 2118.

152. Graham, F. L., Whitmore, G. F. (1970) Cancer Res. 30, 2636 - 2644.

153. Kimball, A. P., Wilson, M. J. (1968) Proc. Soc. Exp. Biol. Med. 429 - 432.

154. Momparler, R. L. (1969) Biochem. Biophys. Res. Comm. 34, 465 - 471.

155. Furlong, N. B., Gresham, C. (1971) Nature New Biol. 233, 212 - 213.

156. Silagi, S. (1965) Cancer Res. 25, 1446 - 1453.

157. Drewinks, B., Ho, D. H. W., Barranco, S. C. (1972) Cancer Res. 32, 2737 - 2742.

158. Manteuil, S., Kopecka, H., Caraux, T., Prunell, A., Girard, M. (1974) J. Mol. Biol. 90, 751 - 756.

136. Benedict, W., Karon, M. (1971) Science 171, 680 - 681.

137. Chaube, S., Kreis, W., Uchida, K., Murphy, M. L. (1968) Biochem. Pharmacol. 17, 1213 - 1216.

138. Kouri, R. E., Kurtz, S. A., Price, P. J., Benedict, W. F. (1975) Cancer Res. 35, 2413 - 2419.

139. Kessel, D., Shurin, S. B. (1968) Biochim. Biophys. Acta 163, 179 - 187.

140. Chou, T. C., Hutchinson, D. J., Schmid, F. A., Phillips, F. S. (1975) Cancer Res. 35, 225 - 236.

141. Chu, M. Y., Fischer, G. A. (1965) Biochemical Pharmacol. 14, 333 - 341.

142. Dechamps, M., De Saint-Vincent, B. R., Eurard, C., Sassi, M., Buttin, G. (1974) Exptl Cell Res. 86, 269 - 279.

143. Scherecker, A. W. (1970) Cancer Res. 30, 632 - 641.

144. Meuth, M., Green, H. (1974) Cell 3, 367 - 374.

145. Momparler, R. L., Chu, M. Y., Fisher, G. A. (1968) Biochim. Biophys. Acta 161, 481 - 493.

146. Ho, D.H.W., Frei, E. (1971) Clin. Pharmacol. Ther. 12, 944 - 954.

147. Meyers, R., Malathi, V. G., Cox, R. P., Silber, R. (1973) J. Biol. Chem. 248, 5909 - 5913.

148. Stewart, C. D., Burke, P. J. (1971) Nature New Biol. 233, 109 - 110.

149. Camiener, G. W. (1968) Biochem. Pharmacol. 17, 1981 - 1991.

150. Ho, D.H.W. (1972) Biochem. Pharmacol. 21, 2936 - 2940.

151. Moore, E. C., Cohen, S.S. (1967) J. Biol. Chem. 242, 2116 - 2118.

152. Graham, F. L., Whitmore, G. F. (1970) Cancer Res. 30, 2636 - 2644.

153. Kimball, A. P., Wilson, M. J. (1968) Proc. Soc. Exp. Biol. Med. 429 - 432.

154. Momparler, R. L. (1969) Biochem. Biophys. Res. Comm. 34, 465 - 471.

155. Furlong, N. B., Gresham, C. (1971) Nature New Biol. 233, 212 - 213.

156. Silagi, S. (1965) Cancer Res. 25, 1446 - 1453.

157. Drewinks, B., Ho, D. H. W., Barranco, S. C. (1972) Cancer Res. 32, 2737 - 2742.

158. Manteuil, S., Kopecka, H., Caraux, T., Prunell, A., Girard, M. (1974) J. Mol. Biol. 90, 751 - 756.

159. Atkinson, M. R., Deutscher, M.P.Kornberg, A., Russell, A.F., Moffat, J. G. (1969) Biochemistry 8, 4897 - 4904.

160. Nichols, W. W., Heneer, W. K., (1964) Hereditas 52, 402 - 410.

161. Cass, C. E., Paterson, A. R. P.,(1972) J. Biol. Chem. 247, 3314 - 3320.

162. Cohen, S. S. (1976) Medical Biology 54, 299 - 326.

163. Kozai, Y., Sugino, Y. (1976) Cancer Res. 31, 1376 - 1382.

164. Rossi, M., Momparler, R. L., Nucci, R., Scarano, E. (1970) Biochemistry 9, 2539 - 2543.

165. De Saint-Vincent, B. R., Buttin, G. (1973) Eu. J. Biochem. 37, 481 - 488.

166. Yoshida, S., Yamada, M., Masaki, S. (1977) Biochim. Biophys. Acta 477, 144 - 150.

167. Schrecker, A. W., Smith, R. G., Gallo, R. G. (1974) Cancer Res. 34, 286 - 292.

168. Lauzon, G. T., Paran, J. H., Paterson, A. R. P. (1978) Cancer Res. 38, 1723 - 1729.

169. Rasmussen, L., Modeweg-Hansen, L. (1973) J. Cell Sci. 12, 275 - 286.

170. Westergaard, O., Marcker, K. A., Leer, J. Chr. (1978) Eur. J. Biochem. 86, 255 - 260.

171. Oumi, T., Osawa, S. (1966) Analyt. Biochem. 15, 539 - 541.

172. Campbell, P. N., Sargent, J. R. (1967) Techniques in Protein Biosynthesis 1, 308 - 309.

173. McEwen, C. R. (1967) Anal. Biochem. 20, 114 - 149.

174. Keleti, C. and Lederer, W. H. (1974) Handbook of micromethods for the Biological Sciences, Van Nostrand, N.Y.

175. Chou, T-C., Arlin, Z., Clarkson, B. D., Philips, F. S. (1977) Cancer Res. 37, 3561 - 3570.

176. Rydberg, B. (1975) Radiation Res. 61, 274 - 287.

177. Elkind, M. (1971) Biophysical Journal 11, 502 - 520.

178. Studier, F. W. (1965) J. Mol. Biol. 11, 373 - 390.

179. Zimm, B. H., Schumaker, V. N., Zimm, C. B., (1976) Biophys. Chem. 5, 265 - 270.

180. Dijkel, P. A., Wanka, 1. (1978) Biochim. Biophys. Acta 520, 461 - 471.

181. Fridland, A. (1977) Biochem. Biophys. Res. Comm. 74, 72 - 78.

182. Kessel, D., Hall, T. C., Wodinsky, I. (1967) Science 156, 1240 - 1241.

183. Plagemann, G. W., Marz, R., Wohlheter, R. M., (1978) Cancer, 38, 978 - 989.

184. Lindsay, R-M., Wong, M. Y., Romine, C. J., Hill, J. B. (1968) Anal. Biochem. 24, 506 - 514.

185. Rothman, I. K., Malathi, V. G., Silber, R., (1978) Methods in Enzymology 51, 408 - 412.

186. Camiener, G. W. (1967) Biochem. Pharmacol. 16, 1691 - 1702.

187. Furner, R. L., Mellett, L. B. (1975) Cancer Chemotherapy Part 1, 59, 717 - 720.

188. Chang, L. M. S., and Bollum, F. J. (1972) Biochem. Biophys. Res. Comm. 46, 1354 - 1360.

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

190. Temin, H. M. and Mizutani, S. (1970) Nature 226, 1211 - 1213.

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

192. Gallo, R. C., Yang, S. S., Ting, R. C. (1970) Nature 228, 927 - 929.

193. Robert, M. S., Smith, R. G., Gallo, R. C., Sarin, P. S., Abrell, J. W., (1972) Science 176, 798 - 800.

194. Fridlender, B., Fry, M., Bolden, A., Weissbach, A. (1972) Proc. Natl Acad. Sci. U.S. 69, 452 - 455.

195. Weissbach, A., Hong, S-C. L., Aucker, J., Muller, R. (1973) J. Biol. Chem. 248, 6270 - 6277.

196. Flugel, R. M., Wells, R. D. (1972) Virol. 48, 394 - 401.

197. Gerard, G. F. (1975) Biochem. Biophys. Res. Comm. 63, 706 - 711.

198. Henner, D., Furth, J. J. (1975) Proc. Natl Acad. Sci. U.S. 72, 3944 - 3946.

199. Webb, J. L. (1966) Enzyme and Metabolic Inhibitors 3, 337 - 364.

200. Aposhian, H. V., Kornberg, A. (1962) J. Biol. Chem. 237, 519 - 525.

201. Lambert, M. W., Studzinski, G. P. (1978) Methods in Cell Biology 19, 43 - 49.

202. Klenow, H., Overgaard-Hansen, K., Patkar, S.A.(1971) Eur. J. Biochem. 22, 371 - 381.

203. Chang, L. M. S., Bollum, F. J. (1971) J. Biol. Chem. 246, 909 - 916.

204. Yoneda, M., Bollum, F. J. (1965) J. Biol. Chem. 240, 3385 - 3391.

205. Mita, T., Shiomi, H., Iwai, K. (1966) Exptl Cell Res. 43, 696 - 698.

206. Dube, D. K., Travaglini, E. C., Loeb, L. A. (1978) Methods in Cell Biology 19, 27 - 42.

207. Stanworth, D. R. (1960) Nature 188, 156 - 157.

208. Klein, P. D., Eisler, W. J. (1966) Anal. Chem. 38, 1453 - 1457.

209. Gautschi, J. R., Kern, R. M., Painter, R. B. (1973) J. Mol. Biol. 80, 393 - 403.

210. Inagaki, A., Nakamura, T., Wakisaka, G. (1969) Cancer Res. 29, 2169 - 2176.

211. Bender, R. A., Zwelling, L. A., Doroshow, J. H., Locker, G. Y., Hande, K. R., Murinson, D. S., Cohen, M., Myers, C. E., Chabper, B. A., (1978) Drugs 16, 46 - 87.