GENETIC AND BIOCHEMICAL STUDIES OF DNA REPLICATION IN *ESCHERICHIA COLI*

by

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SUMMARY

This thesis describes an attempt to identify the DNA polymerase responsible for replicative DNA synthesis in *E. coli* by isolating mutants which exhibit temperature-sensitive DNA synthesis and mutator activity as a result of a single mutation, and testing such mutants for a defect in one of the known DNA polymerases.

A procedure for the isolation of mutator mutants of *E. coli* and the properties of a number of mutator mutants obtained are described. Four mutator mutants which were temperature-sensitive for growth were isolated by selection for the mutator or temperature-sensitive characteristic; however none of these mutants proved to be of the type sought.

A number of temperature-sensitive *dna* mutants previously isolated by other workers were screened for mutator activity. All three *dnaE* mutants tested were found to exhibit mutator activity and the mutator activity was shown to result from the *dna* mutation in the case of at least one mutant, *dna*-511E. This result shows that the product of the *dnaE* gene is involved in base selection during replicative DNA synthesis.

Attempts to purify DNA polymerases II and III from crude extracts of *pol*$^-$ *E. coli* cells prepared by treatment with lysozyme followed by cycles of freezing and thawing proved unsuccessful. The properties of the DNA synthesis observed in the crude extract were studied and this system was used for preliminary tests for

a defective DNA polymerase in dnaE mutants.

DNA synthesis in crude extracts was found to be completely dependent on ATP. No synthesis was detected in freshly prepared lysates of recB$^-$ cells indicating a requirement for the ATP-dependent exonuclease coded for by the recB gene. However DNA synthesis was observed in lysates of recB$^-$ cells after storage at 4° and in freshly prepared lysates on addition of suitable primer DNA. It was concluded that the DNA synthesis observed in this system does not take place at the replicating fork which pre-existed *in vivo* but at artificial primer sites, and that the recB nuclease is required for the conversion of endogenous DNA to a suitable primer in freshly prepared lysates.

DNA synthetic activity was assayed at 30° and 40° in extracts of a pol$^-$ derivative of the group E mutant, dna-486E, and a dna$^+$ control. Synthesis appeared to be partially defective at both 30° and 40° in dna-486E. This result is consistent with the presence of a defective DNA polymerase in dna-486E. (Recently the dnaE gene has been shown to code for DNA polymerase III.)

Since it is possible that components of the replicating complex other than the polymerase are required for synthesis in the crude system, pol$^-$ derivatives of a group B mutant, dna-70B, and a group G mutant, dna-308G, were also tested for temperature-sensitive DNA synthesis in this system. The dnaB gene product does not appear to be required for synthesis in the crude system. The results of tests with dna-308G were inconclusive.

TABLE OF CONTENTS

INTRODUCTION		Page
A.	*In vivo* Replication of DNA in *E. coli*	1
	i) The replicating fork	2
	ii) Chain elongation	2
	iii) Unwinding	5
B.	*In vitro* Synthesis of DNA	6
	i) DNA synthesis in toluene-treated cells	8
	ii) DNA synthesis in crude lysates	9
	iii) DNA polymerase II and DNA polymerase III	11
C.	Genetic Approach to the Mechanism of DNA Replication	13
	i) Conditional Lethal mutants	13
	ii) Mutator mutants	13
	iii) Genetic analysis of phage T4	15
	iv) Genetic analysis of *B. subtilis*	17
	v) Genetic analysis of *E. coli*	17
MATERIALS AND METHODS		
A.	Strains	21
B.	Media	21
C.	Chemicals and Enzymes	22
D.	Other Materials	22
E.	General Methods	23
F.	Growth of Galactose Epimerase$^-$ Strains	24
G.	Preparation of P_1v Transducing Lysate	24
H.	P_1 Transduction	25

		Page
I.	Isolation of Thymine Requiring Mutants	25
J.	Mutagenesis	25
K.	Selection of Mutator Mutants	26
L.	Tests for Mutator Activity	26
M.	Determination of Mutation Frequencies	26
N.	Indoleglycerol Accumulation in *trpA*$^+$ Revertants	27
O.	Isolation of Temperature-sensitive Mutants after ^{3}H-thymine Suicide	27
P.	Identification of Temperature-sensitive *dna* Mutants by Double Labelling	28
Q.	Preparation of Extracts for Assay of DNA Polymerase Activity	28
R.	Treatment of Cells with Toluene	29
S.	Assay of DNA Polymerase Activity	29
T.	Preparation of Primer DNA	29
RESULTS		
	Introduction	31
Part I	Isolation and Characterisation of Mutator Mutants	33
A.	Isolation of Mutator Mutants	34
	i) Construction of the parent strain	34
	ii) Isolation of mutants	36
B.	Properties of the Mutator Mutants Isolated	36
	i) Mutation frequency	36
	ii) Mutator specificity	36
C.	Isolation of Mutator Mutants with an Increased Frequency of Induction of Frame-shift Mutations	38

Page

Part II Search for Mutants with a Defect in the DNA Polymerase Responsible for DNA Replication 40

A. Isolation of Temperature-sensitive Mutator Mutants 40

i) Isolation of mutator mutants at low temperature 40

ii) Isolation of temperature-sensitive mutants using ^{3}H-thymine suicide enrichment 41

B. Properties of the Temperature-sensitive Mutator Mutants Isolated 43

C. Mutator Activity of Temperature-sensitive *E. coli dna* Mutants 43

i) Screening of *dna* mutants for mutator activity 44

ii) Mutation frequency of *dnaE* mutants 45

iii) Reversion of temperature-sensitive and mutator characteristics 45

Part III DNA Synthesis in Extracts of *pol*$^{-}$ *E. coli* cells 47

A. Preparation of Crude Extract 47

i) Lysis of cells 47

ii) DNA synthetic activity in lysates 48

B. Properties of the DNA Synthetic Activity in the Supernatant 49

i) Effect of pHMB and primer DNA 50

ii) Effect of ATP and KCl 51

C. Partial Purification of the DNA Synthetic Activity from the Supernatant 51

i) DEAE chromatography 52

Page

ii) Properties of the DNA synthetic activity in the eluate 52

Part IV The Role of ATP in DNA Synthesis in Crude Extracts 54

A. The recB21 pol-4* Double Mutant 55

B. DNA Synthesis in the Supernatant Fraction of recB21 pol-4* and recB+ pol-4* Cells 55

C. DNA Synthesis in recB21 pol-4* Supernatants in the Presence of Added Primer DNA 56

Part V DNA Synthesis in Extracts of Temperature-sensitive Mutants Defective in DNA Synthesis 59

A. DNA synthesis in the Supernatant Fraction of dna-486E 60

B. DNA Synthesis in the Supernatant Fraction of dna-70B 61

C. DNA Synthesis in the Supernatant Fraction of dna-308G 61

DISCUSSION

A. Identification of DNA Polymerase Mutants by their Mutator Activity 63

B. DNA Synthesis in vitro 66

C. DNA Synthesis in Lysates of dna Mutants 68

REFERENCES 70

APPENDIX 77

INTRODUCTION

This introduction summarises the present knowledge of DNA replication. *In vivo* DNA replication, *in vitro* DNA synthesis and the genetics of DNA replication are reviewed.

A. *In vivo* Replication of DNA in *E. Coli*

DNA consists of an antiparallel double helix of two complementary polynucleotide chains, related to one another by specific base-pairing (Watson and Crick, 1953). DNA duplication is semi-conservative; the two parental polynucleotide chains separate and each acts as a template on which a new complementary strand is synthesised (Meselson and Stahl, 1958; Cairns, 1962; Baldwin and Shooter, 1963).

In *E. coli* replication is initiated at a fixed point and proceeds in both directions (Masters and Broda, 1971). The replicating chromosome has been visualised by autoradiography and found to be a closed circular structure with two forks (Cairns, 1963a), presumably the loci at which replication is taking place. The rate of DNA synthesis at a single growth point is constant throughout the life cycle (Cairns, 1963b; Bonhoeffer and Gierer, 1963; Clark and Maaloe, 1967; Helmstetter, 1967). The max-

imum rate of synthesis has been estimated to correspond to addition of approximately 1.5×10^3 bases per sec. to each growing chain, (Cairns, 1963b; Cooper and Helmstetter, 1968; Werner, 1968). The rate of cellular DNA synthesis is controlled by the rate of initiation of rounds of replication (Helmstetter, et al., 1968).

i) The replicating fork

Cytological studies have shown one point of contact between the nuclear body and the bacterial membrane (Ryter and Jacob, 1966; Ryter, 1968). Replicating forks have been isolated attached to fast-sedimenting structures (Smith and Hanawalt, 1967; Fuchs and Hanawalt, 1970), however no membranous material such as lipid was found in these structures (Fuchs and Hanawalt, 1970) and attachment of the replicating fork to the membrane is not established.

Two processes occur at the replication fork, separation of the two strands of the parental double helix and synthesis of new complementary strands. It is possible that unwinding and synthesis are concerted processes or that they occur by independent mechanisms, in which case unwinding need not necessarily be driven by a process occuring at the replicating fork.

ii) Chain Elongation

DNA phosphorus derives from the phosphorus attached to the 5'-position of the nucleotide precursor (Price et. al., 1967). The deoxynucleotide-5'-monophosphates are not the direct precursors of DNA (see Bonhoeffer and Messer, 1969). All in vitro systems studied to date require deoxynucleotide-5'-triphosphate precursors; however there is some evidence that in vivo the

precursors are not drawn directly from the triphosphate pool and may be deoxynucleotide-5'-diphosphate derivatives (Werner, 1971b).

The DNA polymerases isolated from mammalian cells, bacteria and phage-infected bacteria are capable of synthesising DNA in the 5'-to-3' direction only, by the addition of deoxynucleotide precursors to the 3'-hydroxyl end of the growing chain (Richardson, 1969). Since two strands of opposite polarity are synthesised concurrently as the replicating fork proceeds along the chromosome, a mechanism is required for synthesis of chains in the 3'-to-5' direction using 5'-triphosphates. This could be achieved by the addition of 5'-triphosphates to an activated 5'-end (fig. 1A) (Mitra and Kornberg, 1966). However, no such reaction has been found to occur in vitro (Richardson, 1969). Alternatively all synthesis could occur with 5'-to-3' polarity, the 5'-OH terminus of the growing chain being extended by joining to it polynucleotide fragments synthesised "backwards" from the fork. Models involving discontinuous synthesis of this type have been proposed (fig. 1B & 1C) (Mitra and Kornberg, 1966; Gilbert and Dressler, 1968; Okazaki et. al., 1968). Such models require de novo initiation of DNA synthesis which is difficult to reconcile with the fact that all known polymerases require a 3'-hydroxyl primer for initiation (Richardson, 1969; Kornberg, 1969). An alternative model (fig. 1D) overcomes this problem. Synthesis is initiated on the 3'-OH terminus of the chain with 5'-to-3' polarity and after a short distance the polymerase switches to the complementary parental strand as template and synthesises backwards to the free 5'-end. Nicking by an

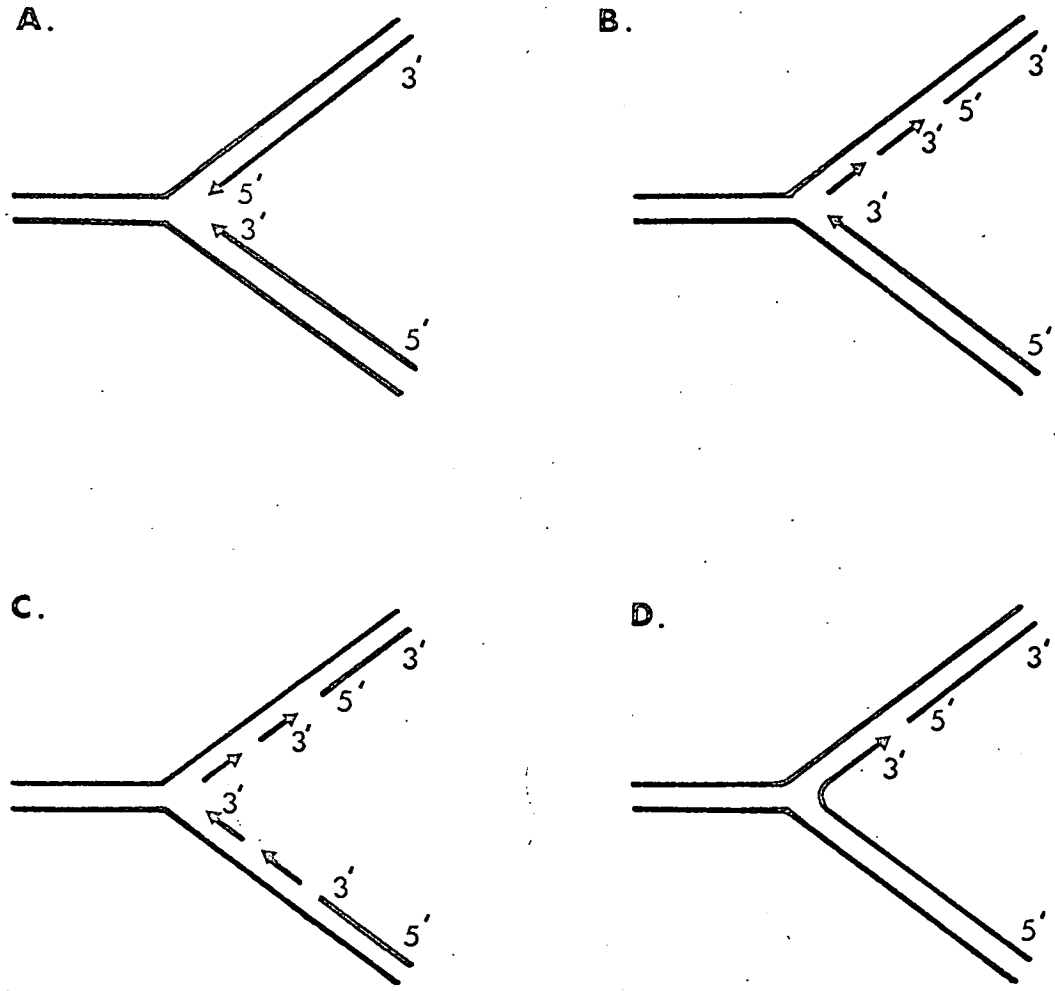

Figure 1. Models of DNA chain elongation

A. One strand is synthesised in the 5'-to-3' direction, the other in the 3'-to-5' direction.

B. Both strands are synthesised in the 5'-to-3' direction. The chain with 3'-to-5' polarity is synthesised discontinuously.

C. Both strands are synthesised discontinuously in the 5'-to-3' direction.

D. Synthesis is initiated on the strand with 5'-to-3' polarity and switched periodically to the other parental strand as template. The apex of the loop is nicked and synthesis reinitiated.

The direction of synthesis is indicated by arrows.

endonuclease at the fork makes available a new 3'-OH priming and for further extension. The 5'-OH end of the growing chain is extended by joining to it the small fragments produced (Guild, 1968; Kornberg, 1969; Richardson, 1969).

Models involving discontinuous synthesis predict the existence of short polynucleotide fragments at the growing fork. Single stranded fragments of low molecular weight (Okazaki fragments) have been recovered after extraction (in alkali or at neutral pH) of DNA synthesised during short times of pulse-labelling. After longer periods of labelling progressively more of the labelled DNA is found to be of high molecular weight. (Okazaki et. al., 1968; Oishi, 1968). These models also predict the existence of an enzyme capable of joining single strand breaks in DNA. Such an enzyme, polynucleotide ligase, has been found in E. coli (Gellert, 1967; Olivera and Lehman, 1967) and Okazaki fragments have been found to accumulate in the absence of functional polynucleotide ligase (Pauling and Hamm, 1969).

Okazaki fragments have not been shown to be essential intermediates in DNA replication and much of the available data is conflicting. The results of Okazaki et. al., (1968) and Pauling and Hamm (1969) suggest that discontinuous synthesis occurs on both strands; however Okazaki fragments from B. subtilis, which replicates its DNA in one direction, have been shown to hybridise with only one of the parental strands (Kainuma and Okazaki, 1970), and Iyer and Lark (1970) have shown that more than 50% of the labelled DNA recovered from E. coli after short pulses is in high molecular weight pieces of variable size, consistent with discontinuous synthesis on one strand only. Much of the available data

on Okazaki fragments has been obtained using pulses of ^{3}H-thymidine but the majority of DNA pulse labelled with thymidine does not originate from the growing point (Fuchs and Hanawalt, 1970; Werner, 1971a). DNA labelled with ^{3}H-thymine is preferentially associated with the replicating complex (Fuchs and Hanawalt, 1970) and most of this label appears first in large pieces of DNA (Werner, 1971a). Werner suggests that Okazaki fragments are the result of accumulation of single strand interruptions in DNA and that thymidine is preferentially used in the repair synthesis accompanying the joining of such gaps. Whether Okazaki fragments are artefacts or not, there is evidence for asymmetry at the replicating fork. Inman and Schnos (1971) have examined replicating lambda DNA molecules by electron microscopy and observed that one of the two daughter molecules at the replicating fork was frequently joined to the parental double helix by a segment of single-stranded DNA.

iii) Unwinding

The requirement of purified DNA polymerase for single-stranded template suggests that the two strands of the parental double helix may be separated in the region ahead of the DNA synthesising complex. Since double-stranded DNA is overwhelmingly more stable than single-stranded DNA under physiological conditions *in vitro*, (Marmur and Doty, 1962) a mechanism for stabilising the separated strands would seem necessary. A protein which binds co-operatively to single-stranded DNA and catalyses the denaturation and renaturation of DNA under physiological conditions *in vitro*, has been isolated from *E. coli* cells infected with T4 (Alberts *et. al.*, 1968; Alberts and Frey, 1970; Alberts, 1970). This protein is

the product of the phage gene 32 and is known to be required for DNA replication and genetic recombination in T4 (Epstein et. al., 1963; Tomizawa et. al., 1966). Gene 32 protein has a structural role in DNA replication since approximately 10000 molecules are produced per infected cell yet the amount of protein produced directly limits the number of progeny phage produced. (Snustad, 1968). Sufficient gene 32 protein is produced to provide up to 170 molecules per growing fork and a complex of 32-protein with DNA contains about one molecule of protein for every ten single-stranded DNA nucleotides. This would allow the stabilisation of approximately 1700 nucleotides at each replicating fork (Alberts and Frey, 1970).

In vitro experiments have shown that T4 DNA polymerase activity is stimulated by gene 32-protein (Alberts, 1970).

B. In vitro synthesis of DNA

In order to study the biochemistry of chromosome replication an in vitro system with the basic features of in vivo DNA replication is required. The criteria for such a system are 1) semi-conservative synthesis of DNA at a rate equivalent to replicative synthesis in vivo 2) normal biological activity of the DNA synthesised 3) synthesis at the replicating fork which pre-existed in vivo 4) expected phenotypic behaviour of mutants in which DNA synthesis is defective.

Over 90% of the DNA synthesis observed in crude extracts of E. coli is carried out by Kornberg polymerase (DNA polymerase I). This enzyme has been purified and studied extensively (Kornberg, 1969). Although polymerase I-dependent DNA synthesis satisfies

some of the above criteria, it is unlikely that this enzyme is responsible for replicative DNA synthesis *in vivo* since mutants of *E. coli* have been isolated which have little or no detectable polymerase I activity *in vitro* but nonetheless grow normally (de Lucia and Cairns, 1969). The mutation in the strain of de Lucia and Cairns is a recessive amber mutation designated *pol AI* (Gross and Gross, 1969) and the *Pol A* locus has been shown to be the structural gene for polymerase I (Kelley and Whitfield, 1971). It is probable that polymerase I is a repair enzyme since *pol*$^-$ mutants exhibit a decreased ability to repair single strand gaps in DNA (for review see Gross, 1971) and purified polymerase I has many properties consistent with an involvment in repair processes (see e.g. Kornberg, 1969; Kelly et al., 1969). Though the evidence available does not constitute conclusive proof that DNA polymerase I is not involved in DNA replication *in vivo*, the existence of *pol*$^-$ mutants with no detectable polymerase I activity *in vitro* has facilitated the study of other *in vitro* DNA synthesising systems.

In vitro DNA synthesis which is independent of polymerase I has been detected in toluene-treated cells and in crude lysate systems. An enzyme, DNA polymerase II, which catalyses the polymerisation of deoxynucleotide-5'-triphosphates has been isolated from *pol*$^-$ *E. coli*. The DNA synthesis observed requires all four deoxynucleotide-5'-triphosphates and magnesium and the product is DN'ase sensitive. The synthesis differs from polymerase I-dependent synthesis in that it is insensitive to antiserum to polymerase I and inhibited by sulphydryl-blocking agents (e.g. N-ethyl maleimide and p-chloromercuribenzoate) which do not affect

polymerase I. The same amount of activity can be detected in *pol*$^+$ and *pol*$^-$ cells, showing that the synthesis is not due to an altered DNA polymerase I, resulting from the *pol A1* mutation.

i) DNA Synthesis in toluene-treated cells

Moses and Richardson (1970a) have studied DNA synthesis in *E. coli* cells made permeable to deoxynucleotide triphosphates by treatment with toluene. The synthesis observed does not require exogenous DNA and is stimulated ten to twenty fold by ATP. In the presence of ATP synthesis proceeds for approximately one hour at a linear rate comparable to the rate of synthesis *in vivo*. There is good evidence that the synthesis observed in toluene-treated *E. coli* cells corresponds to *in vivo* replicative synthesis. DNA synthesis is semi-conservative and is thermosensitive in two genetically distinct classes of *E. coli* mutants in which *in vivo* DNA synthesis is blocked at high temperature (Moses and Richardson, 1970a; Kohiyama and Kolber, 1970; Mordoh *et. al.*, 1970). Furthermore in toluene-treated cells of *B. subtilis* it has been shown that the DNA synthesised has full biological activity when assayed by transformation and that the synthesis occurs at the fork which existed prior to toluenisation (Matsuchita *et. al.*, 1971).

Two interesting features of the "replicative" DNA synthesis observed in toluene-treated cells are the marked stimulation by ATP and the dependence on K^+ ions observed by Mordoh *et. al.*, (1970). It is unlikely that the ATP is required for the protection of the precursors against degradation since polymerase I dependent synthesis under the same conditions (see below) is not ATP dependent. Nor is ATP required for limited degradation of the template DNA by the ATP-dependent exonuclease coded for by the *rec B* gene

(Barbour and Clark, 1970; Oishi, 1969) since synthesis was shown to be normal in *rec B*$^-$ cells (Moses and Richardson, 1970a).

A second type of DNA synthesis was observed in toluene-treated *pol*$^+$ cells after activation of endogenous endonuclease or addition of exogenous pancreatic DN'ase. This synthesis is dependent on polymerase I since it is inhibited by antiserum to polymerase I and absent from *pol*$^-$ cells. DNA polymerase I dependent synthesis is not semi-conservative and is probably the result of repair activity at sites created by the DN'ase (Moses and Richardson, 1970a).

ii) DNA synthesis in Crude Lysates

DNA synthesis has been observed by several groups of workers in membrane fractions prepared after gentle lysis of spheroplasts (Smith *et. al.*, 1970; Knippers and Stratling, 1970; Okazaki *et. al.*, 1970). Synthesis is independent of added primer, semi-conservative and stimulated up to three fold by ATP. The DNA synthetic activity observed is short lived; the rate of synthesis levels off after one to two minutes at temperatures between 20° and 37°. The rate of chain elongation during the first thirty seconds was calculated to be 1.5×10^3 nucleotides / second, which is comparable to the rate of synthesis *in vivo* (Smith *et. al.*, 1970). The initial rate of synthesis observed by Knippers and Stratling was of the same order of magnitude.

It is unlikely that synthesis stops due to inactivation at the assay temperature or the depletion of precursors, since pre-incubation in the absence of precursors has no effect on subsequent synthesis and addition of more deoxynucleotides during the assay does not increase the amount of DNA synthesised (Smith *et.*

al., 1970); also purified polymerase I added to the preparation shows linear synthesis for thirty minutes (Knippers and Stratling, 1970). Synthesis is not affected by shearing with a vortex mixer, showing that the integrity of the bacterial chromosome is not required.

The membrane association of the DNA synthesising activity has been investigated by Stratling and Knippers (1971). These workers have shown that the fast-sedimenting complex with which the DNA synthetic activity is associated is comprised of cell wall fragments, membrane lipids, approximately 20% of the cellular protein and most of the bacterial chromosome. The DNA synthesising activity can be released from this complex by ultrasonication which releases the DNA but not the membrane lipid and protein from the complex. The detergent Triton X-100 (p-isoethylpolyoxyethylenephenol polymer) solubilised the DNA synthesising activity whilst leaving the DNA associated with the membrane. These results are interpreted to mean that the DNA synthesising activity is associated with the bacterial chromosome rather than the membrane and that the chromosome is attached to the membrane by structures other than the DNA synthesising complex. The study of in vitro DNA synthesis in lysates of phage-infected cells has provided evidence that the synthesis observed corresponds to in vivo replication. Knippers and Stratling (1970) found that the fraction of synthesis which was phage specific in extracts of ϕX 174-infected cells was the same in vitro as in vivo, and that the hybrid and heavy ϕX 174-specific DNA synthesised, using dBUTP to replace TTP, was biologically active in the spheroplast assay. Okazaki et. al., (1970) examined the DNA synthesis

in membrane fractions made at various times after infection of pol⁻ cells with T4. The DNA synthesising activity in the preparation changed in parallel with DNA synthesis in vivo. T4-specific DNA synthesising activity was not found when the cells were infected with T4 amber mutants unable to synthesis DNA in vivo. Much of the DNA synthesised was of low molecular weight and some single stranded (Knippers and Stratling, 1970; Okazaki et. al., 1970) and the ØX 174 DNA synthesised in vitro was linear, whilst the unreplicated infecting DNA retained its closed circular structure (Knippers and Stratling, 1970). These facts suggest a defect in joining in vitro. Addition of ligase during the reaction had no effect.

DNA synthesising activity has also been detected in the supernatant fraction of cells lysed by ultrasonication or in a pressure cell (Loeb et. al., 1971; Kornberg and Gefter, 1970). Both methods of lysis caused extensive shearing of DNA and the presence of the DNA synthetic activity in the supernatant presumably results from its association with DNA as proposed by Stratling and Knippers (1971). The DNA synthesis observed was completely dependent on added double stranded primer DNA, and stimulated up to three fold by ATP. Synthesis ceased after approximately one minute (Kornberg and Gefter, 1970). Kornberg and Gefter purified the supernatant activity approximately twenty fold and found that the purified system exhibited a linear rate of synthesis for at least thirty minutes over a ten-fold range in concentration and was no longer stimulated by ATP.

iii) DNA Polymerase II

An enzyme capable of polymerising deoxribonucleotide-5'-

triphosphates has been extracted from pol A1 cells and purified to homogeneity (Knippers, 1970; Moses and Richardson, 1970b; Kornberg and Gefter, 1971). This enzyme has also been purified from pol$^+$ cells (Moses and Richardson, 1970c). The purified enzyme, designated polymerase II, is completely dependent on added primer and is not stimulated by ATP. High molecular weight DNA, single-stranded DNA, and single- or double-stranded closed circles are poor primers. Nicks introduced by pancreatic DN'ase do not increase the priming efficiency of double-stranded DNA. DNA sonicated to pieces of approximately one million molecular weight or high molecular weight DNA partially digested with exonuclease III are the most efficient primers (Knippers, 1970). Polymerase II is unable to commence synthesis at nicks and appears only to fill in single-stranded gaps in double-stranded DNA. The product is covalently linked to the primer (Knippers, 1970). More detailed study of the properties of polymerase II has shown an absolute requirement for a primer with a free 3'-hydroxyl group and that synthesis occurs in the 5'-to-3' direction only (Gefter, Molineux, Kornberg, and Khorana, pers. comm.).

DNA polymerase II is stimulated up to ten fold by the DNA-binding protein isolated from E. coli (Segal and Alberts, pers. comm., Kornberg and Gefter, pers. comm.).

It is probable that polymerase II is responsible for replicative DNA synthesis in vivo, however the properties of the pure enzyme do not clarify the mechanism of DNA replication. Investigation of the other components of the replicating complex such as those responsible for the ATP and K^+ dependence observed in toluene-treated cells may lead to a better understanding of

replicative DNA synthesis.

A third enzyme capable of DNA synthesis has been purified from pol A1 cells. This enzyme (DNA polymerase III) is distinct from polymerase II (Kornberg and Gefter, 1971). The properties of polymerase III have not been studied in detail.

C. Genetic Approach to the Mechanism of DNA Replication

One approach to the problem of DNA replication is to isolate mutants defective in DNA replication in vivo and attempt to identify the defect by in vitro studies. Two classes of mutants in which DNA replication is affected are known: conditional lethal mutants and mutants with an altered fidelity of DNA synthesis.

i) Conditional lethal mutants

Temperature-sensitive, salt-dependent and streptomycin-dependent mutants, unable to synthesise DNA under restrictive conditions, have been isolated in bacteria. These mutants may have a defect in the mechanism of initiation of rounds of replication, in precursor synthesis or in a process required for progression of the replicating fork.

Mutants with a defect in initiation are recognised by their ability to complete rounds of replication in progress at the time of transfer to restrictive conditions. Mutants with a defect in precursor synthesis or replication per se may show little or no residual synthesis on transfer to restrictive conditions but if inactivation of the defective component is slow residual synthesis is observed.

ii) Mutator mutants

Mutants with an altered fidelity in DNA synthesis can be

recognised by an increased or decreased rate of spontaneous mutation. Mutants with an increased spontaneous mutation rate (mutator mutants) have been found in a number of organsims. Several mechanisms for mutator activity have been proposed: a) A defect in the repair of normally occuring DNA damage (Hanawalt and Haynes, 1965). The E. coli excision and repair mechanism has been shown to repair both lethal and mutative damage induced by UV. (Hill, 1965; Witkin, 1966). Mutators of this type would also exhibit an increased sensitivity to DNA damage. b) The insufficiency of a base or the production of an abnormal base analogue. Insufficiency of a base could be mutagenic in the manner that thymine starvation is mutagenic (Coughlin and Adelberg, 1956; Weinberg and Latham, 1956), and an abnormal base analogue which was incorporated into DNA could lead to errors in subsequent rounds of replication. An abnormal base analogue has been reported to be present in a mutator strain of Salmonella typhimurium in which the mutator gene is linked to the purine locus (Kirchner and Rudden, 1966). c) A defect in the mechanism responsible for base selection or error recognition during replicative DNA synthesis. The base-paired structure of DNA and the semi-conservative mode of replication suggest that the selection of bases is determined by the stereochemistry of base pairing; however mutations in T4 DNA polymerase alter the rate of spontaneous mutation, implying that the polymerase functions in base selection (Speyer, 1965; Speyer et. al., 1966; Drake and Allen, 1968). Mutations in gene 32 and in a number of other genes involved in phage T4 DNA replication also influence spontaneous mutation rates (Bernstein, 1971; Drake, pers. comm.).

iii) Genetic analysis of phage T4

The genetic analysis of phage T4 using conditional lethal (amber and ts) mutants has led to the construction of a genetic map for this phage, containing approximately 70 genes of which 20 are believed to function in DNA synthesis (Epstein et. al., 1963; Edgar et. al., 1964; Edgar and Lielausis, 1964). The proteins coded for by eight of the DNA genes have been identified in in vitro studies. Five of the gene products identified are concerned with the production of deoxyhydroxymethyl-cytosine-5'-triphosphate; gene 1, deoxynucleotide kinase (Duckworth and Bessman, 1967); gene 42, deoxycytidylate hydroxymethylase (Dirksen et. al., 1963; Wiberg and Buchanan, 1964); genes 46 and 47, host specific DN'ase (Wiberg, 1966) and gene 56, dCTP'ase (Wiberg, 1967). Gene 30 codes for the phage polynucleotide ligase (Fareed and Richardson, 1967), gene 43 codes for T4 DNA polymerase (de Waard et. al., 1965; Warner and Barnes, 1966) and gene 32 is the structural gene for a protein which binds co-operatively to single-stranded DNA (Alberts et. al., 1968; Alberts and Frey, 1970).

In the context of understanding the mechanism of DNA replication, the properties of gene 43 and gene 32 are most interesting. T4-specific DNA polymerase has been purified. The pure enzyme requires a partially single-stranded template with a free 3'-hydroxyl priming terminus. The DNA synthesised is covalently linked to the 3'-OH of the primer (Goulian et. al., 1968). The properties of the T4-induced polymerase produced by five temperature-sensitive mutants of T4 with a lesion in gene 43 were examined by de Waard et. al., (1965). Two of the mutants produced

active DNA polymerase at permissive temperatures only, although this polymerase had the same heat stability as wild type enzyme. One mutant produced equivalent levels of polymerase at permissive and non-permissive temperatures, but the enzyme exhibited greater heat lability than wild type enzyme. The properties of the polymerase produced by the remaining two mutants did not differ significantly from wild type. These results show that gene 43 codes for T4 DNA polymerase; they also show that temperature-sensitivity observed *in vivo* is not necessarily detectable *in vitro*.

A number of ts mutants of gene 43 have been found to exhibit mutator or antimutator activity at permissive temperatures. (Speyer, 1965; Speyer *et. al.*, 1966; Drake and Allen, 1968). The mutator and antimutator activities have been shown to be associated with the ts lesion (Speyer *et. al.*, 1966; Drake *et. al.*, 1969). Purified T4 DNA polymerase from a mutator mutant has been shown to incorporate thymine in place of guanine during the replication of poly dC approximately four times more frequently than wild type enzyme (Hall and Lehman, 1968).

T4 DNA polymerase has not been proven to be responsible for phage DNA replication. Speyer and Rosenberg (1968) have argued that the rate of mutation observed in one gene 43 mutator mutant cannot be accounted for by mutations arising during recombination and repair, and infer that T4 DNA polymerase functions in replication.

Whether T4 DNA polymerase is responsible for replicative DNA synthesis or not, the mutagenicity of defective T4 polymerases demonstrates that this enzyme functions in base selection.

The properties of the gene 32 product and its possible role in DNA replication have been discussed (see p. 5). A number of T4 ts mutants with a lesion in gene 32 have been shown to exhibit mutator activity (Bernstein, 1971; Drake, pers. comm.).

iv) Genetic analysis of B. subtilis

Temperature sensitive mutants of B. subtilis defective in DNA synthesis have been isolated. The mutations are distributed in 9 genetic linkage groups (Gross et. al., 1968; Karamata and Gross, 1970). Mutants in two groups were initiation defective. Mutants in group A are unable to reduce ribonucleotides at restrictive temperatures, presumably because they have a defective ribonucleotide reductase, (Bazill and Karamata, pers. comm.). One mutant, designated mut-1, with an increased rate of spontaneous mutation at permissive temperatures was isolated by selection for mutator activity. The mutator activity and temperature sensitivity have been shown to result from the same mutation, and it is suggested that the function affected is directly involved in DNA replication (Gross et. al., 1968).

v) Genetic analysis of E. coli

Temperature sensitive mutants of E. coli defective in DNA synthesis have been isolated and characterised by a number of groups of workers (Bonhoeffer, 1966; Fangman and Novick, 1968; Hirota et. al., 1968; Kohiyama et. al., 1966; Inouye 1969; Wechsler and Gross, pers. comm.; Carl, 1970; for review see Gross, 1971).

The mutations are located in seven distinct genetic linkage groups (Groups A-G) (Fig. 2) (Hirota et. al., 1968; Ricard and Hirota, 1969; Carl, 1970; Hirota et. al., 1970; Wechsler and

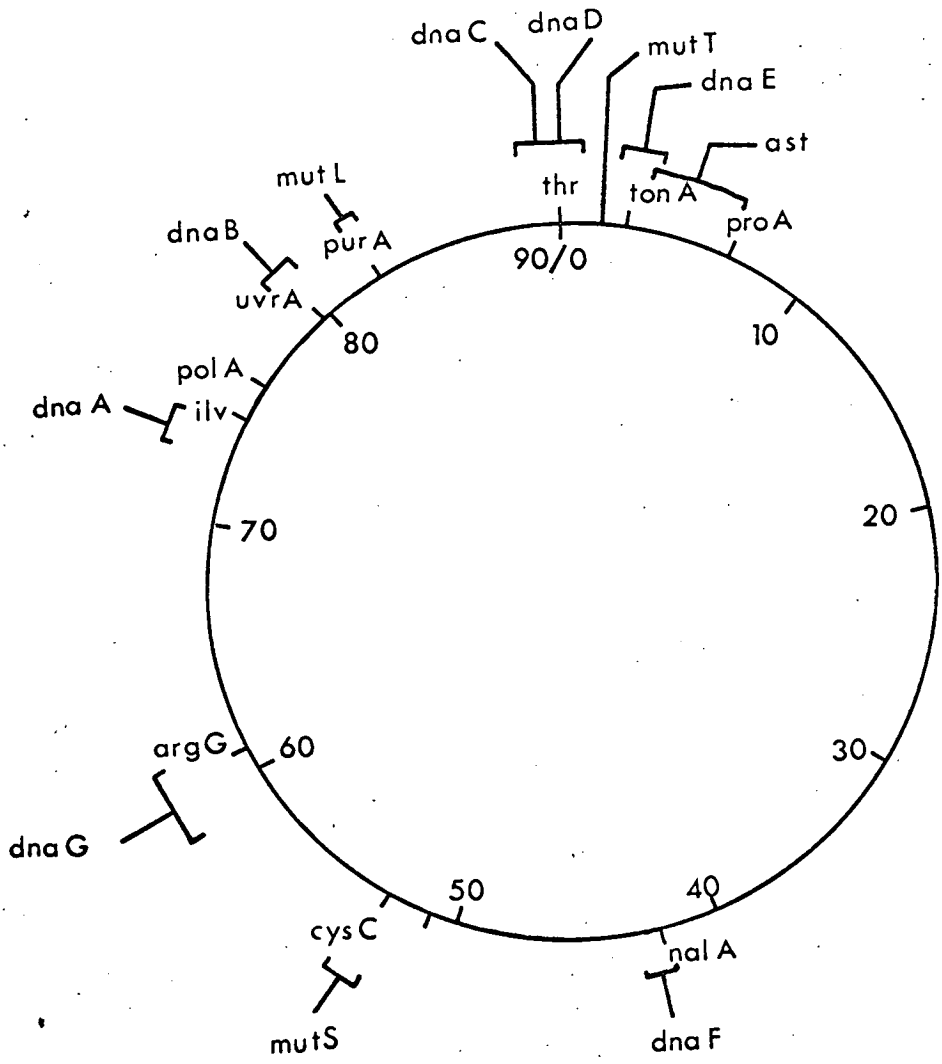

Figure 2. Map locations of *dna* and mutator mutations on the *E.coli* chromosome.

The locations of the *dna* mutants are after Wechsler and Gross (in preparation). The locations of the mutator mutants are summarised by Liberfarb and Bryson (1970).

Gross, pers. comm.; Marinus and Adelberg, 1970). The number of genes in each linkage group has not been determined.

Mutants in groups A and C appear to be defectives in initiation. Mutants in the groups B, D, E, F and G are presumed to be defective in precursor synthesis or replication *per se*, however, the function of the genes concerned remains unknown although the properties of these mutants have been studied *in vivo* and *in vitro*. As mentioned previously *in vitro* temperature sensitivity of ts DNA mutants has been demonstrated in the toluene-treated cell system.

A number of *dnaB* mutants were found to be defective in DNA synthesis when assayed at high temperature (Moses and Richardson, 1970a; Kohiyama and Kolber, 1970 and Mordoh *et. al.*, 1970) and one initiation-defective mutant, *dna-46A*, was unable to synthesise DNA at high temperature when incubated at the restrictive temperature to allow completion of rounds, prior to toluenisation. (Mordoh *et. al.*, 1970). It appears that mutants behave in this system as they do *in vivo*, and no information about the nature of the defective component is obtained.

Moses and Richardson (1970c) have purified DNA polymerase II from two B group mutants (*dna*-43B and *dna*-266B). The pure enzymes had the same heat stability as wild-type enzyme. Mutants in other groups have not been tested for a defect in polymerase II. Mutator mutants of *E. coli* have also been isolated by a number of groups of workers (Treffers *et. al.*, 1954; Goldstein and Smoot, 1955; Siegel and Bryson, 1963; Mohn and Kaplan, 1967; Jyssum, 1960; Hill, 1968; Liberfarb and Bryson, 1970). The lesions conferring mutator activity map at three genetic loci (fig. 2)

(Cox and Yanofsky, 1969; Zamenhof, 1966; Mohn, 1968; Helling, 1968; Siegel and Bryson, 1967; Hill, 1970; Liberfarb and Bryson, 1970). Complementation analysis has not been performed and the number of genes involved is not known. The properties of one mutant have been studied in detail. This mutant, isolated by Treffers et. al., (1964), and designated mut T1, has been shown to induce AT-to-GC transversions preferentially (Yanofsky et. al., 1966a). The mut T1 mutation is closely linked to the azi locus. Mut T1 is recessive in merodiploids but acts in trans on F'-factors and a number of phage (Cox and Yanofsky, 1969). Mut T1 induced mutations do not occur in the absence of DNA synthesis (Cox, 1970), and it was suggested that the mutator activity is due to a faulty error-recognition system associated with DNA replication.

The purpose of the work to be described in this thesis was to attempt to clarify the mechanism of replicative DNA synthesis in E. coli. The first approach was to attempt to isolate mutants with a defect in the DNA polymerase responsible for replicative DNA synthesis. Mutator mutants and dna mutants may be defective in the polymerase; however mutants defective in a number of other processes also exhibit these phenotypes. In order to increase the probability that a mutant had an altered polymerase it was assumed that such a mutant could exhibit both temperature sensitivity for DNA synthesis and mutator activity, as has been shown for T4 DNA polymerase (see section C iii). Mutants of this type were sought amongst mutator and amongst temperature-sensitive mutants.

The second approach was to study DNA synthesis *in vitro*. It was hoped to test purified preparations of DNA polymerase II and DNA polymerase III from temperature-sensitive mutator mutants for defective DNA synthesis. A crude *in vitro* DNA synthesising system was developed. However attempts to purify the DNA synthetic activity from the crude system proved unsuccessful. The DNA synthesis observed in the crude system was found to be completely dependent on ATP. In this way the system differs from the crude lysate systems studied by other workers (see section B ii). Since the DNA synthesis observed in toluene-treated cells, which appears to correspond to replicative DNA synthesis, is completely ATP-dependent (see section B i), and purified DNA polymerases II and III are not ATP-dependent (Moses and Richardson, 1970; Kornberg and Gefter, 1971), it seemed possible that the study of the role of ATP in *in vitro* DNA synthesis might lead to the identification of another component of the replicating complex. The nature of the ATP-dependence observed was therefore further investigated.

Since it seemed probable that the polymerase responsible for replicative DNA synthesis contributes to the DNA synthesis observed in crude lysates and that at least one other component of the replicating complex may be involved, preliminary tests for temperature-sensitive DNA synthesis in this system were performed on *pol*$^-$ derivatives of a *dna* mutant which exhibited mutator activity and two *dna* mutants belonging to other *dna* groups.

MATERIALS AND METHODS

A. Strains

The bacterial strains used are listed in Table 1.

B. Media

The concentration of amino acid supplements refers to a final concentration of the L-isomer.

Minimal buffer: 0.3% KH_2PO_4, 0.7% Na_2HPO_4, 0.4% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$.

M9 Minimal medium: 0.7% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.025% $MgSO_4$, 0.2% carbon source (D-glucose unless otherwise indicated) supplemented with 5ug/ml vitamin B_1 and 20 ug/ml thymine and amino acids when required.

Enriched M9 minimal medium: M9 minimal medium supplemented with 0.5% Difco casamino acids, and with 20ug/ml tryptophane, and 20 ug/ml thymine when required.

Synthetic enriched minimal medium: M9 minimal medium supplemented with 0.05% tryptone, 0.05% NaCl, 20 ug/ml alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, and tryptophane, and 10 ug/ml thymine.

Minimal agar: M9 minimal medium solidified with 1.5% Davis N.Z. agar.

TABLE 1: Bacterial strains

Strain	Relevant characteristics	Derivation	Source
CA7026	HfrH: gal ep^-_{PL2}, str^s		K.Ippen
RH14	HfrH: gal ep^-_{PL2}, trpA23, thy^-, str^s	see text	
RH15	HfrH: gal ep^-_{PL2}, trpA21, thy^-, str^s	see text	
RH16	HfrH: gal ep^-_{PL2}, trpA23, thy^-, str^s, gal^R	spontaneous gal^R of RH14	
	W3110: trpD(AE8), T_1^R		B.Brammar
	W3110: trpA23, his^-, cys^-		C.Yanofsky
	W3110: trpA21		via B.Brammar
CR34	F^-: thr^-, leu^-, B_1^-, λ^s, thy^-	see Wechsler and Gross,	
JW100	CR34: dna-101F	(in preparation)	
JW101	CR34: dna-107B	"	
JW102	CR34: dna-125B	"	
JW104	CR34: dna-173B	"	
JW105	CR34: dna-177A	"	
JW107	CR34: dna-279B	"	
JW108	CR34: dna-293E	"	
JW109	CR34: dna-386B	"	
JW111	CR34: dna-486E	"	

JW112	CR34: dna-508A	"	
JW113	CR34: dna-511E	"	
JW114	CR34: dna-517A	"	
JG28	HrfH: B_1^-, thy$^-$, str^R, dna-70B		F.Bonhoeffer
JG32	CR34: dna-308G		"
JG34	CR34: dna-399G		"
JG148	W1485: thy$^-$, leu$^-$, str^R, dna-1C	see Carl (1970)	P.Carl
JG149	W1485: thy$^-$, leu$^-$, str^R, dna-2C	"	"
JG151	W1485: thy$^-$, leu$^-$, str^R, dna-5A	"	"
JG153	W1485: thy$^-$, leu$^-$, str^R, dna-7D	"	"
JG155	MX74: F$^-$, lac$^-$, thy$^-$, str^s, dna-27B	see Inouye (1969)	F.Bonhoeffer
JG157	K12SH-28: F$^-$, pho$^-$, dna-22B	see Fangman and Novick (1968)	"
JB8	F$^-$: B_1^-, polA1, endoI$^-$		J.Brockes
	W3110: metE$^-$, $recB_{21}$, pol-4*	see Bazill et.al. (1971)	
	W3110: metE$^-$, recB$^+$, pol-4*	"	
JG212	CR34: dna-486E, pol-4*	JW111 x JG80 (HfrR: metE$^-$, pol-4*, rif^R), selecting for rif^R	
JG213	CR34: dna$^+$, pol-4*	dna$^+$ transductant of JG212	
JG186	W3110: thy$^-$, rha$^-$, polA1, str^R, dna-70B	JG114 x P$_1$(JG28) selecting mal$^+$	
JG114	W3110: thy$^-$, rha$^-$, malB$^-$, polA1, str^R		
JG204	CR34: dna-308G, pol-4*	JG32 x JG80 as for JG212	

L- broth: 1% Difco tryptone, 0.5% yeast extract, 1% NaCl, pH adjusted to 6.8 supplemented with 20 ug/ml thymine when required.

L-agar: L-broth solidified with 1.5% Difco agar.

Nutrient Broth: 2.5% Oxoid No. 2 nutrient broth, supplemented with 20 ug/ml thymine when required.

Nutrient agar: Nutrient broth solidified with 1.5% Difco agar.

LC-agar: 1% Difco tryptone, 0.5% yeast extract, 0.5% NaCl solidified with 1% agar (bottom layer) or 0.7% agar (top layer).

Difco stab agar: 1% Oxoid tryptone, 0.7% NaCl, 0.3% glucose, 1% Difco Bactogar.

C. Chemicals and Enzymes

(^{3}H-methyl)-thymine (14-20 C/mM), 1-14C -leucine (62 C/mM), and (^{3}H-methyl)-thymidine (5.2 C/mM) were obtained from the Radiochemical Centre, Amersham. The following chemicals were used; N-methyl-N'-nitro-N-nitrosoguanidine (Aldrich Chemical Co.), trimethoprin (Burroughs Welcome), indole (BDH Ltd.), 5-methyl tryptophane (Koch Light), DL norleucine (Nutritional Biochemicals Corporation), 5-flurouracil (Roche Products Ltd.), 6-azauracil (Sigma), rifampicin (Gruppo Lepittit), naladixic acid (Calbiochem), methylmethane sulphonate (Eastman Organic Chemicals). Lysozyme, calf thymus DNA, soluble RNA, were obtained from Sigma Chemical Co., and deoxynucleotide triphosphates and ATP from Calbiochem.

D. Other materials

Millipore filters: type HAWP, pore size 0.45u, 25mm. diameter (Millipore Filter Corp.)

Dialysis tubing: Visking tubing cut into appropriate lengths was boiled in 1% Na_2CO_3 for 15 minutes, rinsed with distilled water, filled with 0.01M EDTA and dialysed overnight against

distilled water. Washed tubing was stored in 10^{-4}M EDTA at 4^{o}.

DEAE cellulose: DE 11 (Whatman) was washed and equilibrated with buffer as recommended by the makers.

E. Coli DNA: Prepared according to the method of Kelly and Pritchard (1965).

E. General methods

i) Growth and maintainence of bacterial strains

Strains in use were maintained on Oxoid nutrient agar or L-agar plates at 4^{o}. Stationary phase liquid cultures were prepared by innoculating 5-10 ml of medium with a single colony and incubating overnight. Log phase cultures were prepared by diluting a stationary phase culture 1/50 or 1/100 into fresh medium and incubating with aeration. Growth of cultures was followed using a Klett-Summerson Photoelectric Colorimeter (Arthur Thomas Co.). A Klett reading of 100 corresponds to a cell density of 5×10^{8}/ml for broth cultures. All strains were maintained on Difco agar stabs at 4^{o}.

ii) Viable count

The number of colony-forming units in a culture was determined by spreading 0.1ml samples of an appropriate dilution on nutrient agar plates and incubating overnight.

iii) Radioactive counting

All radioactive samples were counted in a Packard Tricarb liquid scintillation spectrometer. Organic scintillant contained 0.3% 2,5-diphenyloxazole and 0.03% 1,3-bis-2-(4-methyl-5-phenyloxazole)-benzene in toluene. Samples containing one radioactive isotope were counted under conditions giving maximum efficiency of counting. For samples containing two isotopes (^{14}C and ^{3}H)

conditions for counting were determined using pure samples of the two isotopes. The isotope of higher energy (^{14}C) was counted under conditions where less than 0.1% of the low energy isotope counts were detected. For the isotope of lower energy the percentage of the high energy isotope counts detected under the conditions used was determined and the appropriate correction made.

iv) Preparation of radioactive samples for counting

0.05ml samples were pipetted onto 2.5 cm Whatman 3MM filter discs. The discs were dropped into ice-cold 5% TCA containing 1% $Na_2P_4O_7.7H_2O$. At the completion of an experiment the filters were washed 3 times for 10 minutes in fresh TCA then washed with distilled water, absolute alcohol and dried at 60°.

F. Growth of Galactose epimerase$^-$ strains

Gal ep$^-$ strains grow poorly in nutrient medium. These strains were grown in nutrient medium supplemented with 0.02% glucose, and 2 ug/ml tryptophane when required, dilutions were made into the same medium as used for growth. Gal ep$^-$ strains are sensitive to galactose (H. Nikaido, 1961), gal^R revertants were selected by plating on minimal medium with 0.2% galactose and 0.2% glycerol as carbon source.

G. Preparation of P_1v transducing lysate

An overnight L-broth culture containing approximately 10^9 cells/ml was diluted with an equal volume of MC (0.1M $MgCl_2$, 0.005M $CaCl_2$) and incubated with 10^6 P_1v phage/ml at 37° for 20 min. and plated in LC-soft agar on LC-hard agar plates supplemented with 0.1% glucose and 0.0025 M $CaCl_2$. Phage were harvested after 8 hours incubation at 37°.

H. P_1 transduction

2×10^8 cells in L-broth containing 0.0025M $CaCl_2$ were incubated with 10^9 P_1 phage at 37° for 20 min. The cells were spun down, resuspended in 0.1M sodium citrate pH 7 and plated on selective medium; for selection of T_1^R cells were respun and resuspended in nutrient broth, grown overnight and plated on nutrient agar with a loopful of T1 phage.

I. Isolation of thymine$^-$ mutants

An overnight culture was diluted 1/50 into minimal medium containing 50 ug/ml thymine and 10 ug/ml trimethoprin and grown to stationary phase. This culture was diluted 1/50 into minimal medium containing 10 ug/ml thymine and 10 ug/ml trimethoprin, grown to stationary phase and plated for single colonies on nutrient agar supplemented with 10 ug/ml thymine. Thy$^-$ mutants were selected by replica plating to nutrient agar. Low level thymine requirers were selected by testing for growth on minimal agar supplemented with 2 ug/ml thymine.

J. Mutagenesis

N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was dissolved in water, at a concentration of 500 ug/ml, less than 30 min. prior to use. Mutagenesis was performed by incubating a log-phase culture ($2-5 \times 10^8$ cells/ml) with 20 ug/ml NTG at 30° for 15 min. with aeration. The mutagenised culture was filtered through Millipore and resuspended in nutrient medium. This treatment gave a survival of 10-20%. To determine the number of auxotrophs induced, the culture was diluted 1/10, grown overnight, and plated on nutrient agar. Auxotrophs were detected by replica plating to minimal agar. Approximately 10% of the survivors were auxotrophs.

K. Selection of mutator mutants

10^4 - 10^6 cells of a mutagenised culture of RH14 (gal ep$^-$, tryp A23, thy$^-$) or RH15 (gal ep$^-$ tryp-A21) were plated on nutrient agar and incubated overnight. The resulting lawns were replicated to minimal plates with galactose and glycerol as carbon source. The gal^R colonies which arose after 3 days incubation were replicated to three different selective plates with galactose and glycerol as carbon source. Plates of the following composition were used: enriched minimal containing 100 ug/ml 6-azauracil, enriched minimal containing 50 ug/ml naladixic acid, minimal containing 50 ug/ml norleucine, minimal containing 25 ug/ml L-valine, and minimal with the tryptophane supplement reduced to 0.2 ug/ml. Colonies giving rise to growth on all plates were selected from the master plate or from the azauracil or norleucine selective plate and further tested.

L. Tests for mutator activity

Four single colonies of a mutant were streaked on minimal medium, and replicated to selective minimal plates. Selection for resistance to the following drugs, naladixic acid (50 ug/ml), L-valine (25 ug/ml), 5-flurouracil (5 ug/ml) and reversion of tryp$^-$ were used. Non-mutator strains give rise to 0-5 resistant colonies per streak in this test. Mutants which give rise to tryp$^+$ nal^R, val^R, and 5-FU^R at high frequency were scored as mutator. The tsDNA mutants were screened for mutator activity using resistance to 6-Azauracil (100 ug/ml), rifampicin (100 ug/ml) and valine (25 ug/ml).

M. Determination of mutation frequencies

1 ml of nutrient medium was innoculated with approximately

1,000 cells of a thick log culture, and grown overnight with aeration. Resistance to valine, and naladixic acid 6-azauracil and rifamicin was determined by plating appropriate dilutions on minimal plates containing 25 ug/ml valine, nutrient plates containing 50 ug/ml naladixic acid enriched minimal plates containing 100 ug/ml 6-azauracil and nutrient plates containing 100 ug/ml rifampicin. Viable counts were determined by plating appropriate dilutions on nutrient agar. Reversion of tryp$^-$ to prototrophy was determined by plating approximately 10^7 cells on minimal plates supplemented with 0.2, 0.5, and 1 ug/ml tryptophane. Plates were incubated for 2-3 days.

N. Indoleglycerol accumulation in tryp A$^+$ revertants

A number of tryp$^+$ revertants were picked and pruified. Single colonies were innoculated into 1 ml nutrient medium and grown overnight. A drop of the overnight culture was diluted into 2 ml minimal medium supplemented with 4 ug/ml tryptophane and grown overnight. 0.75 ml $FeCl_3$ reagent (Yanofsky, 1956) was added to 0.5 ml minimal culture. Within a few minutes after mixing a red-purple colour develops with indoleglycerol.

O. Isolation of temperature-sensitive mutants after ^{3}H-thymine suicide

A log phase culture of RH14 (gal ep$^-$, tryp A23, thy$^-$) in enriched minimal medium containing 5 ug/ml thymine was mutagenised with NTG at 30°, diluted 1/100 into the same medium and grown overnight at room temperature without aeration. This culture was diluted 1/20 into the same medium and grown at 30° to a cell density of 10^8/ml, and transfered to 41°. After 5 min. incubation at 41° an equal volume of high specific activity

^{3}H-thymine in supplemented minimal medium was added. After a further 30 min. incubation at 41° cold thymine was added for 2 min. and the culture cooled to 4°, filtered through Millipore, washed with minimal medium containing 200 ug/ml thymine and resuspended in supplemented minimal medium containing 20 ug/ml thymine and 7% dimethylsulphoxide. 0.2 ml samples were distributed in small tubes and frozen at -60°. Viable count was determined during storage at -60°. After 36 days (viable count 1% original value) and 42 days (viable count 0.6%) a number of tubes were thawed diluted, plated on nutrient agar and incubated at 30°. Temperature-sensitive colonies were identified by replica plating to nutrient agar and incubating at 41°. Temperature-sensitive colonies were tested for mutator activity.

P. Identification of temperature-sensitive DNA mutants by double labelling

An overnight culture in synthetic enriched minimal medium was diluted 1/50 into 2 ml of the same medium containing ^{3}H-thymine and ^{14}C-leucine at a specific activity of 0.03 mC/ml and 1 uC/ml, and grown at 30°. When the cell density had reached 10^{8}/ml, 1 ml was transferred to 42° and incubated for 90 minutes. 0.05 ml samples were taken at 15 minute intervals during growth at 30° and 42° and prepared for counting as described in section E. Growth was followed using a parallel culture in cold medium. A wild type strain (RH 14 gal^{R}) and a known DNA mutant (JG 28) were included as controls.

Q. Preparation of extracts for assay of DNA polymerase activity

i) Cells grown in L-broth were harvested in log phase. 1 gm of cells was washed twice with 20 ml of ice-cold 0.025M tris HCl pH

7.6, and once with 20 ml of ice-cold 0.025 M tris HCl, 0.005 M EGTA pH 7.6, and resuspended in 10 ml of 0.025 M tris HCl 0.005 M EGTA pH 7.6 containing 500 ug/ml lysozyme at 4^{o}. This suspension was frozen rapidly and thawed at 30^{o} one to three times. When lysis was complete, as determined by microscopic examination, $MgCl_2$ was added to a final concentration of 0.005 M. The lysate was sheared for 1 minute at full speed in a Waring blender and spun at 30,000 g for 30 min. The supernatant fraction was stored frozen at -60^{o}, and used for assays.

R. Treatment of cells with toluene

0.01 ml of toluene was added to 10^{10} cells in 1 ml tris 0.025 M pH 7.6 and the suspension agitated (vortex mixer) for two minutes at room temperature, then left on ice for 20 minutes. Toluene was removed by blowing air over the surface of the suspension. 0.2 ml samples were used in the assay for DNA synthesis.

S. Assay of DNA polymerase activity

0.2 ml samples in a final volume of 0.25 ml were used in the assay for DNA synthesis. The composition of the reaction mixture (final concentrations) was; dATP, dGTP, dCTP, 2.5×10^{-5} M; ^{3}H-TTP, 10^{-5}M; ATP, when present, 4×10^{-3}M; $MgCl_2$, 0.006 M; KCl, 0.1 M; tris HCl pH 7.6, 0.025 M; mercaptoethanol 0.005 M. The specific activity of ^{3}H-TTP was 125 C/M. Sodium p-hydroxymercuribenzoate, when added, was 1.5×10^{-4}M and mercaptoethanol was omitted. DNA when added was at 40 ug/ml. Incubation was at 28^{o}. At intervals 0.05 ml samples were taken and precipitated and prepared for counting as described in section E.

T. Preparation of primer DNA

1) Calf thymus DNA (1 mg/ml) in 0.025M tris HCl pH 7.6 was sonic-

ated for 1 minute using an MSE Ultrasonic Disintegrator.

ii) Equal volumes of sonicated and unsheared E. coli DNA (1 mg/ml) in 0.4M NaCl were heated together at 90° for 5 minutes and cooled slowly to room temperature.

RESULTS

Introduction

The results are presented in five parts. Parts I and II are concerned with attempts to isolate mutants of *E. coli* with a single mutation resulting in temperature-sensitive DNA synthesis and an increased frequency of spontaneous mutation. Since it is possible that mutants of this type occur only rarely and are not represented amongst the *dna* mutants isolated to date, a selection proceedure for the isolation of mutator mutants similar to that used by Gross *et. al.* (1968) to isolate mutator mutants of *B. subtilis* was devised for *E. coli*. This proceedure facilitates the screening of a large number of cells for mutator activity. Part I describes the selection proceedure for the isolation of mutator mutants and the properties of a number of mutants isolated by this proceedure. Part II describes the isolation of temperature sensitive mutator mutants by isolating mutator mutants and screening them for temperature sensitivity as well as by isolating temperature-sensitive mutants and screening them for mutator activity. The properties of the mutants isolated are described. This part also contains the results of tests for mutator activity performed on the *dna* mutants previously isolated by other workers.

Parts III, IV and V are concerned with the study of DNA synthesis *in vitro*. Part III contains the results of an analysis of the properties of DNA synthetic activity in lysates of *pol*$^-$ *E. coli* cells, and describes an attempt to purify the activity.

Part IV contains the results of further investigations into the nature of the ATP-dependence of the DNA synthetic activity observed in lysates. Part V contains the results of tests for

temperature-sensitive DNA synthesis in lysates of pol⁻ derivatives of a number of temperature-sensitive dna mutants, including one mutant which exhibits mutator activity and may be defective in the polymerase responsible for DNA replication.

PART I: Isolation and Characterisation of Mutator Mutants

Introduction

A clone of a mutator mutant has a high probability of containing a number of different types of mutant cells. Mutator mutants can therefore be detected by screening colonies arising from single mutagenised cells for the presence of different mutant types. To facilitate the screening of a large number of mutagenised cells for mutator activity Gross et. al. (1968) working with B. subtilis selected for mutator mutants in two steps: 10^5 mutagenised cells were plated on nutrient agar and grown to form a confluent lawn; 8-azaguanine resistant cells present in the lawn were selected by replica plating onto plates containing 8-azaguanine and the resulting single colonies were then screened for the presence of mutant cells of a number of different types. The principle of the selection proceedure is that each mutagenised cell gives rise to a small clone within the lawn and cells resistant to 8-azaguanine arise spontaneously at high frequency to that there is a high probability that a small clone arising from a mutator mutant will contain 8-azaguanine resistant cells. The 8-azaguanine resistant colonies arising from a mutator mutant can be detected by screening for the presence of mutant cells within the clone.

For the present work a similar selection proceedure was devised for E. coli. Mutator mutants of E. coli have been shown to preferentially induce a limited number of specific mutational events (Yanofsky et. al., 1966; Siegel and Bryson, 1967). In order not to select against mutators with any particular specificity

it is necessary to select for an altered phenotype which can arise from any one of a number of different mutational events.

A. Isolation of Mutator Mutants

The strain RH14 (Hfr H gal ep$^-_{PL2}$ trp A23 thy$^-$ str^s) was constructed and used as the parent for the isolation of mutator mutants. Strains lacking functional galactose-4-epimerase are sensitive to galactose; any mutation leading to a loss of functional galactokinase gives rise to galactose resistant derivatives (Nickaido, 1961). Galactose resistant cells arise spontaneously at a high frequency. Selection for galactose resistance is therefore suitable for the first step of the isolation proceedure. The trp A23 mutation is revertible by a number of transitional and transversional base substitutions (Yanofsky et. al., 1966b), hence selection for trp$^+$ revertants is suitable for use in the second step of the isolation proceedure. Reversion of trp A23 was also used to study the specificity of the mutators isolated. The thymine requirement was introduced to enable testing of any temperature-sensitive mutators isolated for a defect in DNA synthesis. The mutations were introduced into an Hfr strain, to facilitate mapping of mutator mutants since it is not possible to select for the mutator phenotype directly.

i) Construction of the parent strain

A trp∇(AB) deletion was introduced into CA7026 (gal ep$^-_{PL2}$ str^s) by transduction with P_1kc (W3110 trp ∇ AE8) (obtained from W. Brammar) selecting for T_1^R. A T_1^R trp$^-$ transductant which was unable to grow on indole was selected (RH12). RH 12 was transduced with P_1v (W3110 his$^-$ cys$^-$ trp A23) selecting for growth on indole (RH13). A low thymine requiring mutant of RH13 was

isolated by trimethoprin selection (RH14) (Stacey and Simons, 1965).

ii) Isolation of mutants

Mutator mutants were isolated as described in Methods section K. 10^4 - 10^6 NTG mutagenised cells of RH 14 were plated on nutrient agar and grown to form a confluent lawn. Each cell plated gives rise to a small clone within the lawn. The galactose resistant cells present in the lawn result from galactose resistant cells induced during mutagensis and from spontaneous or mutator induced mutational events which occur during growth of the lawn. Clones arising from mutator mutants have a high probability of containing galactose resistant cells and should be selected in the first step. The galactose resistant colonies selected were screened for the presence of valine resistant, 6-azauracil resistant, norleucine resistant and *trp*$^+$ revertant cells. Colonies which gave rise to growth on all selective plates were purified and further tested for mutator activity.

A number of mutator mutants of RH14 were isolated using this proceedure (Table 2).

TABLE 2

Mutator mutants of RH14 (*gal ep*$^-_{PL2}$, *trp A23*, *thy*$^-$, *str*^{s})

Strain	Positive in mutator test selecting for	Characteristics
M 10 - M 12	nal^R, val^R trp$^+$	*gal*^{R}, *trp A23*, *thy*$^-$, *str*^{s}, NL^R, *mut*
M 30 - M 46	val^R, trp$^+$	*gal*^{R}, *trp A23*, *thy*$^-$, *str*^{s}, 6-AU^R, *mut*
M 50 - M 58	val^R, trp$^+$	*gal*^{R}, *trp A23* *thy*$^-$ *str*^{s}, 6AU^R *mut*

Mutants in the series M10 - M12 were picked from norleucine selective plates, M30 - M46 and M50 - M58 were picked from 6-aza-uracil plates as described in Methods section K.

B. Properties of the Mutator Mutants Isolated

The mutation frequency and the mutational specificity of a number of the mutants isolated was determined.

i) Mutation frequency

The frequency of mutation to valine resistance and to nalad-ixic acid resistance was determined. A measure of the frequency of reversion of the trp A23 mutation was obtained by plating cells on minimal plates containing limiting amounts of tryptophane supp-lement, The amount of growth which can occur and the number of trp$^+$ revertants which arise are proportional to the concentration of the tryptophane supplement (Fig. 3). Mutation frequencies were compared to those of a spontaneous galactose resistant deriv-ative of the parent strain (RH16). The results are listed in Table 3. All the mutator mutants show a significant increase in mutation frequency for all three mutant types.

ii) Mutator specificity

The nature of the base substitutions induced by a number of the mutator mutants was investigated by studying the reversion of the trp A23 mutation. The reversion of trp A23 has been studied in detail by Yanofsky et. al. (1966b). Three classes of trp$^+$ revertants can be distinguished by measuring the degree of indole-glycerol accumulation and of 5-methyltryptophane sensitivity. The amino acid replacements which give rise to each revertant type are known and the base substitutions which have occured can be deduced. The properties of the revertants are listed in Table 4.

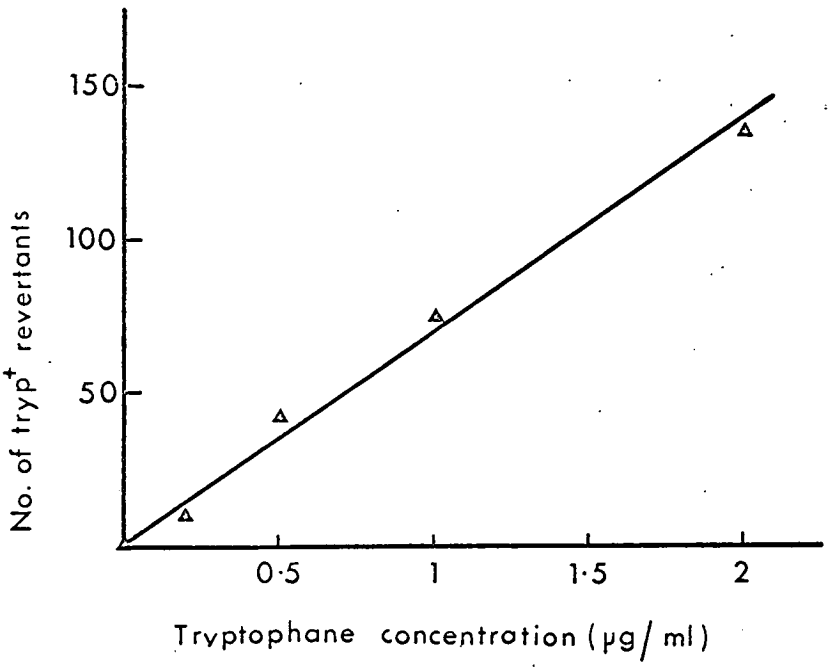

Figure 3. Reversion of the trypA23 mutation. Approximately 10^7 cells of an overnight culture of M 56 were plated on minimal plates containing limiting concentration of the tryptophane supplement. Tryp$^+$ revertants were scored after 3 days incubation at 37°.

Table 3. Mutation frequencies of derivatives of R14

Strain	Nature of Mutation					
	Naladixic Acid Resistance (mutants/cell)	Mutator/ Non-mutator	Valine Resistance (mutants/cell)	Mutator/ Non-mutator	Reversion of *trpA23* (no. of *trp*$^+$)	Mutator/ Non-mutator
RH16	7×10^{-10}	-	2×10^{-8}	-	8.5	-
M40	2×10^{-7}	300	6×10^{-6}	300	80	10
M41	5.5×10^{-7}	800	4×10^{-6}	200	130	15
M42	4×10^{-7}	600	2.5×10^{-6}	125	1,200	140
M43	3×10^{-7}	400	3×10^{-6}	150	32	4
M44	6×10^{-7}	850	9×10^{-6}	450	100	12
M50	1.3×10^{-6}	2,000	1×10^{-5}	500	80	10
M51	2.5×10^{-7}	350	3×10^{-6}	150	115	13
M52	1×10^{-7}	150	2×10^{-6}	100	125	15
M53	2.5×10^{-7}	350	2×10^{-6}	100	100	12
M54	5×10^{-7}	700	3.5×10^{-6}	175	60	7
M55	4.5×10^{-7}	650	3.5×10^{-6}	175	95	11
M56	3.5×10^{-7}	500	3.5×10^{-6}	175	140	16

Frequencies are the average of three determinations

Table 4. Properties of revertants of trpA23 (Yanofsky et al., 1966b).

Mutant type	Amino Acid	Codon	Base substitution	Indole glycerol Accumulation	5-methyl-tryptophan sensitivity
trpA23	Arginine	AGA			
Full Revertant Wild type	Glycine	GGA	$A/_T$ to $G/_C$ Transition	-	Resistant
Full Revertant	Serine	AGC	$A/_T$ to $C/_G$ Transversion	-	Resistant
		AGU	$A/_T$ to $T/_A$ Transversion		
Partial revertant	Isoleucine	AUA	$G/_C$ to $T/_A$ Transversion	+	Sensitive
Partial revertant	Threonine	ACA	$G/_C$ to $C/_G$ Transversion	feint	Slightly sensitive

Trp$^+$ revertants of the mutator mutants M40 - M44 and M50 - M56 were selected, purified and tested for indoleglycerol accumulation. Strains which accumulate indoleglycerol show a dark pink colour on mixing with $FeCl_3$ reagent; strains which accumulate small amounts of indoleglycerol show a pale pink colour. The results are listed in Table 5. RH16 gave rise to all three types of revertants. The spontaneous reversion pattern of this strain differs from that observed by Yanofsky et. al. (1966b) for the trp A23 mutation in a K-12 Ymel strain of E. coli which gives rise to full revertants and strong indoleglycerol accumulators only.

A number of the spontaneous trp$^+$ revertants of RH16 were tested for sensitivity to 5-methyltryptophane using the qualitative test described by Allen and Yanofsky (1963). Partial revertants which are strong indoleglycerol accumulators are sensitive to 5-methyltryptophane, weak accumulators are only slightly more sensitive than the wild type. The full revertants showed a zone of inhibition of 2.8 - 3.0 cm, partial revertants which showed strong indoleglycerol accumulation gave a zone of inhibition of 5.8 - 6.0 cm and weak accumulators 3.2 - 3.8 cm. These results indicate that the revertants had been correctly classified on the basis of the test for accumulation of indoleglycerol.

The majority of the revertants isolated from mutator strains are mutator induced since the frequency of reversion is at least ten-fold greater than the spontaneous reversion frequency in the non-mutator parent, except in the case of the mutants M43 and M54 (see Table 3). The pattern of reversion differs from that of the parent strain in all cases.

Mutator mutants M40 and M51-M55 gave rise to full revertants

TABLE 5

Distribution of *trp*$^{+}$ revertants of RH 16 and mutator mutants

Strain	Number of revertants tested	Number of full revertants	Number of strong IG accumulators	Number of weak IG accumulators
RH 16	50	15	13	22
M 40	30	29	0	1
M 41	30	24	2	4
M 42	23	15	0	8
M 43	29	24	0	5
M 44	30	26	0	4
M 50	29	17	1	11
M 51	30	28	0	2
M 52	30	27	0	3
M 53	29	28	0	1
M 54	25	25	0	0
M 55	30	28	0	2
M 56	27	19	1	7

only, indicating that the preferred mutator-induced base substitutions were one or more of the transitions and transversions A/T to G/C, A/T to C/G and A/T to T/A. The mutants M41-M44, M50 and M56 gave rise to full revertants and a significant number of partial revertants which were weak indoleglycerol accumulators, indicating that these mutators can also induce G/C to T/A transversions.

These results indicate that the mutants tested exhibit specificity for the mutational events induced.

C. Isolation of Mutator Mutants with an Increased Frequency of Induction of Frameshift Mutations

The proceedure for isolation of mutator mutants was modified to enable the selection of mutators which induce frameshift mutations by constructing a strain with a frameshift mutation in the tryptophane A gene.

The trp A21 mutation is a frameshift mutation which arose by deletion of one or two base pairs. This mutation is strongly reverted by ICR 191-A (3-chloro-7-methoxy-9-(3-(chloroethyl)-amino propylamino)-acridine dihydrochloride) which induces base additions and deletions but does not respond to other chemical mutagens (Berger et. al., 1968).

The trp A21 mutation was introduced into RH12 by transduction with P_1v (W3110 trp A21) (obtained from W. Brammar), selecting for growth on indole (RH15).

Mutator mutants of RH15 (gal ep^-_{PL2} trp A21 str^S) were isolated as described in Methods section K. Gal^R colonies were screened for the presence of valine resistant, norleucine resistant and trp^+ revertant cells. Colonies giving rise to growth on all three selective plates were picked from the norleucine

selective plate, purified and checked for an increased frequency of mutation to val^R, $5\text{-}FU^R$ and trp^+. Four mutants M 17 - M 20 were isolated by this proceedure.

The frequency of reversion of the trp A21 mutation was determined for each of the mutants and a spontaneous gal^R derivative of the parent strain. The results are shown in Table 6.

TABLE 6

Relative reversion frequencies of the trp A21 mutation in derivatives of RH15

Strain	Number of trp^+ revertants * (0.2 ug trp/ml)	Mutator/ Non-Mutator
RH15 gal^R	15	-
M 17	90	6
M 18	32	2
M 19	80	5
M 20	70	5

* Average of 2 independent determinations

The mutants all exhibit a small increase in the frequency of induction of frameshift mutations. These mutants were not studied further.

PART II: Search for Mutants with a Defect in the DNA Polymerase Responsible for DNA Replication

Introduction

Having developed methods for the selection and identification of mutator mutants it was possible to attempt to isolated mutants exhibiting both temperature-sensitive DNA synthesis and mutator activity. To establish that such mutants are of the type sought it is necessary to show that both characteristics result from the same mutation. One way to demonstrate this is to show that the two characteristics revert together.

A. Isolation of Temperature-Sensitive Mutator Mutants.

Temperature-sensitive mutator mutants were isolated in two ways: (1) by selecting for mutator mutants at low temperature and screening for temperature-sensitivity and (2) by isolating temperature-sensitive mutants and screening for mutator activity.

i) Isolation of mutator mutants at low temperature

a) RH 14 was mutagenised with NTG at 30° and galactose resistant colonies selected as described in Methods section K. These colonies were replicated to two selective plates containing valine which were incubated at 30° and 41°. Colonies which gave rise to growth on valine at 30° but not at 41° were purified and tested for an increased frequency of reversion of trp A23 and mutation to valine resistance at 30° and 41°, and for growth at 41°.

The properties of three mutator mutants isolated in this way are listed in Table 7. One of the mutants, M14 was temperature-sensitive for growth. M14 was tested for a defect in DNA synthesis at the restrictive temperature (see below).

TABLE 7

Properties of mutator mutants M13, M14 and M16

Strain	Reversion of * trp A23		Mutation to * valine resistance		Growth at 41°
	30°	41°	30°	41°	
M 13	-	-	+	-	+
M 14	+	-	+	-	-
M 16	+	+	+	-	+

* + high frequency of mutation
\- low frequency of mutation

see Methods section L.

b) RH 14 was mutagenised with NTG and mutator mutants were selected at 30°. 27 mutator mutants were isolated. These mutants were tested for growth at 41°. One of the mutants, MTS2, was found to be temperature sensitive.

ii) Isolation of temperature-sensitive mutants using ^{3}H-thymine suicide enrichment

It is possible to enrich for temperature-sensitive mutants defective in DNA synthesis by exposing a culture to high specific activity ^{3}H-thymine at 41° and storing the cells at -60°. On exposure to ^{3}H-thymine at high temperature only those cells which are able to synthesise DNA will incorporate the radioactive thymine. During storage of the cells at -60°, the decay of tritium results in lethal damage to cells which contain ^{3}H-thymine, leading to preferential loss of these cells (Lubin, 1959).

A culture of RH 14 was mutagenised at 30°, grown overnight to allow expression of the induced mutations, exposed to high specific activity ^{3}H-thymine at 41°, frozen and stored at -60°. The viability of this culture and of a control culture, which was not exposed to ^{3}H-thymine was determined at various times during storage at -60°. The kinetics of loss in viability for the two cultures are shown in Figure 4. During the first twelve days of storage the viability of both cultures decreased exponentially at similar rates. On continued storage the ^{3}H-thymine treated culture continued to die at the same rate whilst the viability of the control culture decreased at a significantly slower rate. The reason for the initial rapid loss in viability of the control culture is not known, but may result from a high sensitivity to damage during freezing and thawing due to the abnormal structure of the cell-wall observed in gal ep^{-} mutants (Fukasawa and Nick-aido, 1961).

After 36 days (0.9% survivial, 30 fold enrichment) and 42 days (0.5% survival, 50 fold enrichment) a batch of cells was thawed and plated on nutrient agar at 30°. The single colonies which arose were replicated to nutrient plates and incubated at 41°. Approximately 1% of the colonies were temperature sensitive. These mutants were screened for an increased frequency of mutation to valine resistance and tryptophane prototrophy as described in Methods section L.

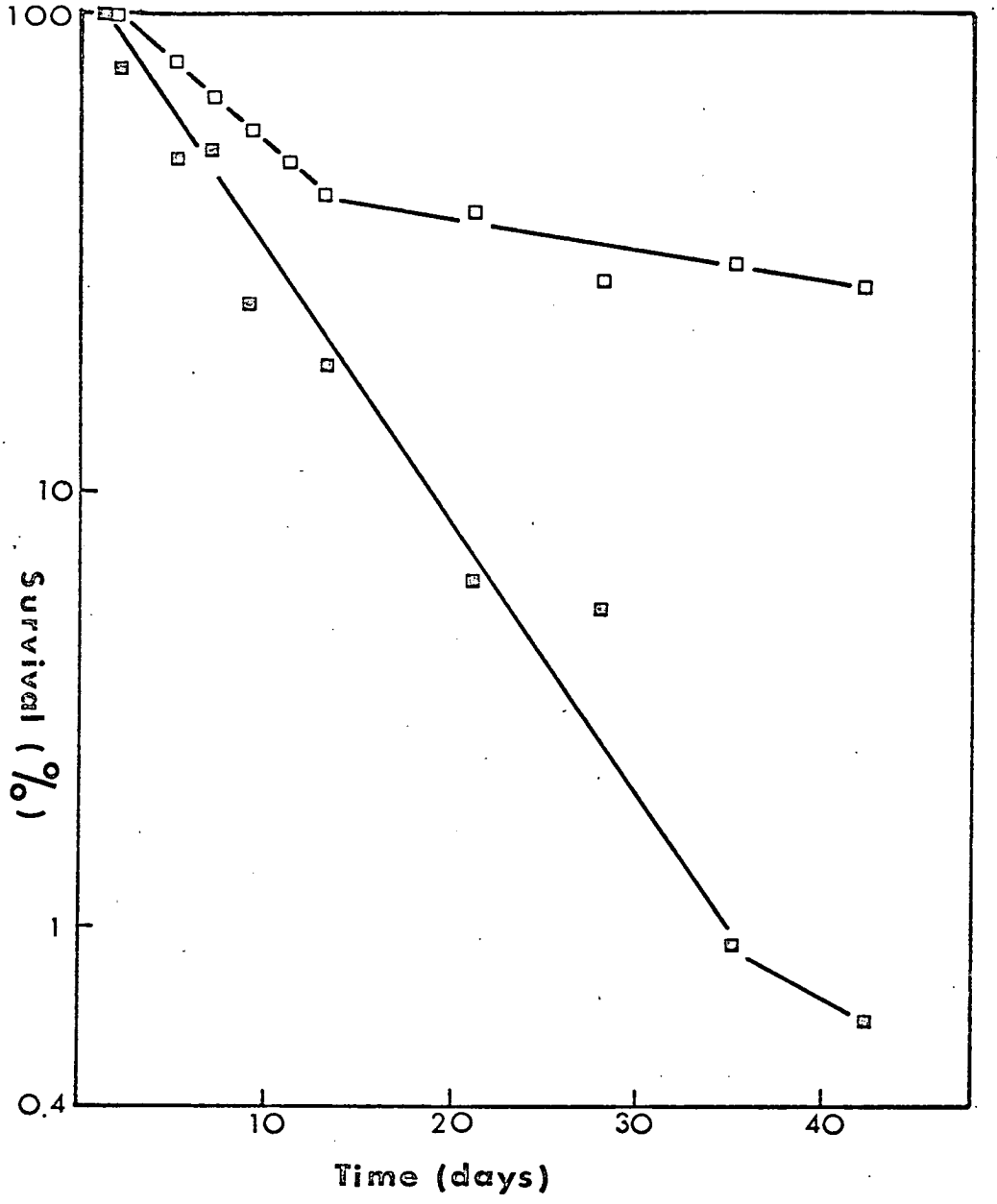

Figure 4. Vability of RH 14 cells on storage at -60°.

— ▣ — Exposed to ^{3}H-thymine at 41°

— □ — Control culture

Two mutants were found which exhibited mutator activity. These mutants were designated MTS 3 and MTS 4.

B. Properties of the Temperature-sensitive Mutator Mutants Isolated

The four temperature-sensitive mutator mutants, M 14, MTS 2, MTS 3 and MTS 4, obtained by the proceedures described above were tested further to determine whether the temperature-sensitivity and mutator activity resulted from a single mutation in a gene whose product is required for DNA replication.

M 14 was tested for a primary defect in DNA synthesis at high temperature by following the incorporation of ^{3}H-thymine into DNA and ^{14}C-leucine into protein after transfer to 42°. The kinetics of incorporation of ^{3}H and ^{14}C into TCA insoluble material are shown in Figure 5a. Figure 5b shows the results of a parallel experiment with MH 16 (wild type control). For 105 minutes after transfer of M 14 to 42° no difference between the rates of DNA and protein synthesis was observed. This shows that the temperature sensitivity of M 14 does not result from a primary defect in DNA synthesis.

Temperature-resistant revertants of MTS 2 were isolated by plating 0.1 ml of an overnight culture on nutrient agar at 41°. A number of revertants were purified and tested for mutator activity at 30°. All of the 12 revertants of MTS 2 tested exhibited mutator activity. This suggests that the mutator activity and temperature sensitivity do not result from the same mutation.

MTS 3 and MTS 4 were found to be leaky temperature-sensitive mutants and were not studied further.

C. Mutator Activity of Temperature-sensitive E. coli dna Mutants

Temperature-sensitive mutants of E. coli with a primary defect

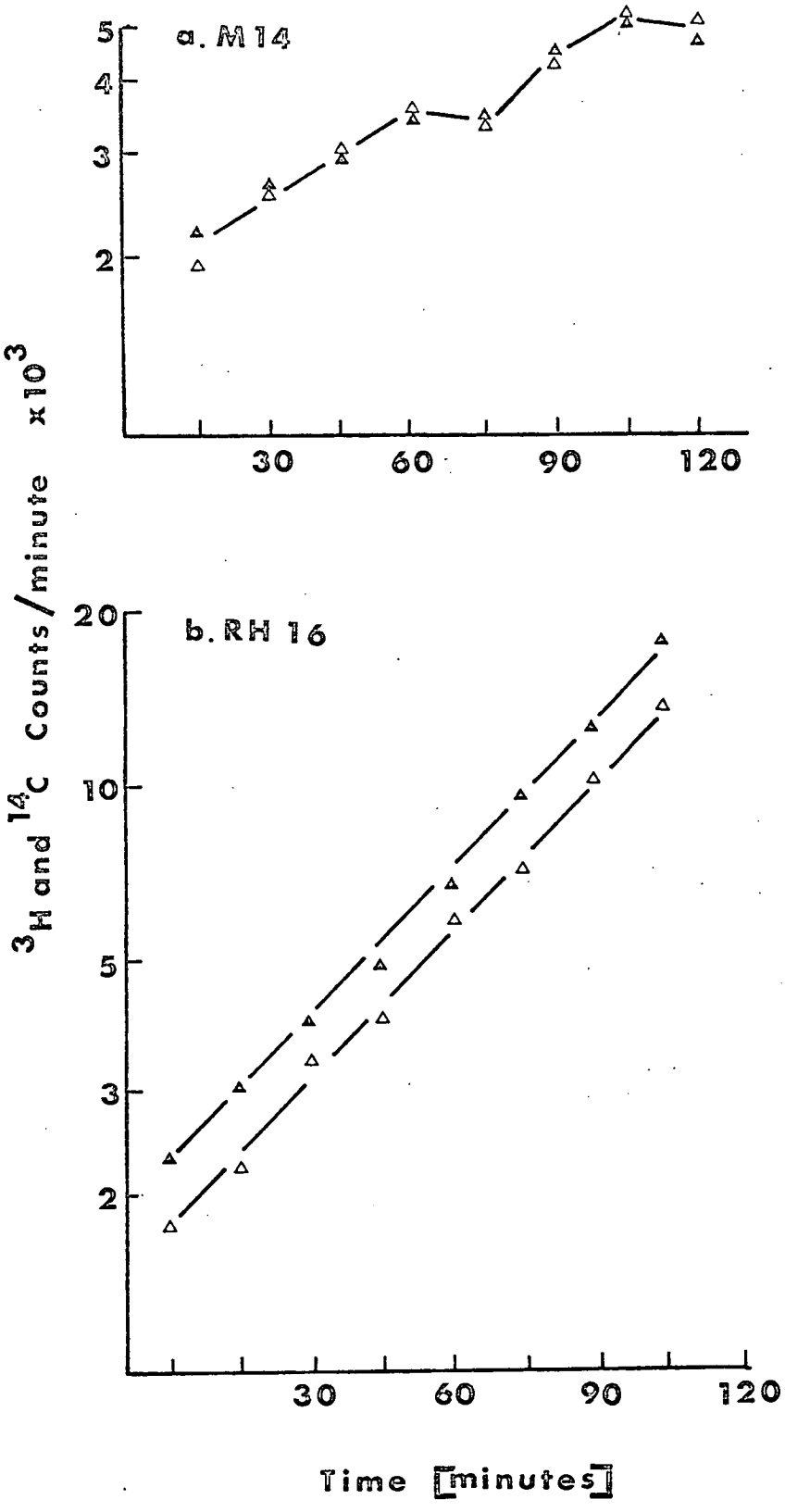
a. M14
5
4
3
2
30
60
90
120
b. RH 16
20
10
5
3
2
30
60
90
120
^3H and ^{14}C Counts / minute x 10^3
Time [minutes]

Figure 5. Kinetics of DNA and protein synthesis at 42^{o} in the temperature-sensitive mutant M 14 and in the control RH 16.

Cultures of M14 and RH 16 were grown at 30^{o} in enriched minimal medium containing ^{3}H-thymine and ^{14}C-leucine. At a cell density of approximately 10^{8}/ml the cultures were transferred to 42^{o}. The incorporation of ^{3}H-thymine into DNA and ^{14}C-leucine into protein was followed as described in the Methods section P.

—▲— ^{3}H-thymine incorporated

—△— ^{14}C-leucine incorporated

in DNA synthesis have been isolated by a number of groups of workers. The mutants have been assigned to seven genetic linkage groups on the basis of map position (see Introduction, section C v). Mutants in groups A and C are defective in the initiation of rounds of replication (Kohiyama, 1968; Hirota et. al., 1970; Carl, 1970). Mutants in groups B,D,E,F and G are defective in precursor synthesis or in replication per se. Reduced levels of deoxynucleotide triphosphates were not detected at the restrictive temperature in a number of mutants belonging to groups B and G (Bonhoeffer, 1966; Fangman and Novick, 1968; Carl, 1970), and mutants in these groups are therefore not defective in precursor synthesis. Mutants belonging to groups B,E, and F have been tested for a defect in ribonucleotide reductase with negative results (Karlstrom, see Gross in press). It is possible that mutants in one of the five groups B,D,E,F and G have a defect in the DNA polymerase responsible for DNA replication.

i) Screening of dna mutants for mutator activity

A number of dna mutants, representing each of the seven groups recognised to date, were tested for mutator activity at permissive temperatures. The mutants were tested for an increased frequency of mutation to valine resistance and to 6-azauracil resistance at 30°. Mutants belonging to the Edinburgh collection were also tested at 34°. The results are shown in Table 8.

One out of four group A mutants tested, (dna-177A), appeared to exhibit an increased frequency of mutation to resistance to both drugs tested. Since mutants belonging to group A are defective in initiation it seemed unlikely that the mutator activity is associated with the dna-177A lesion.

TABLE 8

Mutator activity of *E. coli* *dna* mutants

Strain	dna Mutation	Valine Resistance		6-Azauracil Resistance	
		30°	34°	30°	34°
JG 151	*dna*-5A	-		-	
JW 105	*dna*-177A	+	+	+	+
JW 112	*dna*-508A	-	-	-	-
JW 114	*dna*-517A	-	-	-	-
JG 155	*dna*-27B	+		R	
JG 157	*dna*-22B	+		±	
JW 101	*dna*-107B	+	R	-	-
JW 102	*dna*-125B	-	-	-	-
JW 104	*dna*-173B	-	-	R	-
JW 107	*dna*-279B	-	-	-	-
JW 109	*dna*-368B	+	+	-	-
JG 148	*dna*-1C	-		-	
JG 149	*dna*-2C	-		-	
JG 153	*dna*-7D	-		-	
JW 111	*dna*-486E			+	
JW 113	*dna*-511E	+	R	+	+
JW 108	*dna*-293E	±	±	±	±
JW 100	*dna*-101F	-	±	-	-
JG 32	*dna*-308G	-		-	
JG 34	*dna*-399G	-		R	

The designations of the dna mutations are those assigned by Gross (in press).

Qualitative tests for mutator activity were performed as described in Methods section L.

-	5 resistant colonies/streak
±	5, 20 resistant colonies/streak
+	20 resistant colonies/streak
R	resistant

Three group B mutants (dna-22B, dna-27B and dna-368B), out of 9 tested appeared to show an increased frequency of mutation to valine resistance only. The DNA polymerase II from two group B mutants (dna-43B and dna-266B) has been purified and found to have the same heat stability as the wild type enzyme (Moses and Richardson, 1970c). This strongly suggests that dnaB mutants do not produce a defective DNA polymerase II. These mutants have not been tested for a defective DNA polymerase III.

All three group E mutants tested (dna-486E, dna-511E and dna-293E) exhibited mutator activity at 30° and 34°. Since mutator activity appeared to be associated particularly with group E mutants, these mutants were studied further.

ii) Mutation frequency of dna E mutants

The frequency of mutation to resistance to rifampicin, 6-azauracil and naladixic acid at 30° was determined for the mutant strains JW 111 (dna-486E), JW 113 (dna-511E) and JW 108 (dna-293E) and for the parent strain CR 34. The results are shown in Table 9.

The mutation frequency of JW 113 was significantly greater than that of CR 34 for the three characters tested. JW 108 showed a slight increase in the frequency of mutation to rifampicin and naladixic acid resistance and JW 111 showed an increase in the frequency of mutation to rifampacin resistance only. Since all three group E mutants exhibit some degree of mutator activity it seemed probable that the mutator activity of these strains is associated with the dnaE mutation.

iii) Reversion of temperature-sensitive and mutator characteristics

In order to determine whether the mutator activity resulted

Table 9. Mutation frequencies of dna-E mutants (mutants/cell)

Strain	Nature of Mutation					
	Rifampicin Resistance	Mutator/ Non-mutator	Naladixic Acid Resistance	Mutator/ Non-mutator	6-Azauracil Resistance	Mutator/ Non-mutator
CR34	1.1×10^{-9}	-	6.6×10^{-10}	-	1.6×10^{-7}	-
JW 108(dna-293E)	3.7×10^{-9}	3	2×10^{-9}	3	2.3×10^{-7}	1.4
JW 111(dna-486E)	9.6×10^{-9}	9	7.7×10^{-10}	1.2	2.1×10^{-7}	1.3
JW 113(dna-511E)	1.6×10^{-7}	150	6.2×10^{-9}	9	3.1×10^{-6}	20

Frequencies are the average of four independent determinations

from the dnaE mutation, temperature-resistant revertants of JW 113 were selected by plating 0.1 ml of an overnight culture on nutrient plates at 41°. A number of revertants were purified and tested for mutator activity at 30°. 12 of the 16 temperature-resistant revertants tested had lost the mutator characteristic, showing that the mutator activity and temperature sensitivity result from a single mutation.

These results provide strong evidence that the product of the dnaE gene is involved in base selection and may in fact be a DNA polymerase involved in DNA replication.

PART III DNA Synthesis in Extracts of pol⁻ E. coli Cells

Introduction

In vitro DNA synthesis was studied in crude lysates of a strain lacking DNA polymerase I and endonuclease I. Endonuclease I is responsible for the majority of the nucleolytic activity detectable in crude extracts (Durwald and Hoffman-Berling, 1968), and may be unmasked during the preparation of the lysate and interfere with the detection of DNA synthetic activity.

Partial purification of the DNA synthetic activity by DEAE batch chromatography was attempted.

A. Preparation of Crude Extract

The DNA synthetic activity observed in extracts of *pol⁻ E. coli* cells has been found to be located in the membrane fraction of extracts prepared by gentle lysis of spheroplasts, and in the supernatant fraction of extracts prepared by breakage in a pressure cell. The location of the synthetic activity appears to result from its association with the DNA (see Introduction section B ii).

Lysates of JB 8 (*pol A1*, *endo I⁻*) cells were prepared by treatment with lysozyme followed by cycles of freezing and thawing. The lysate and the membrane and supernatant fractions of the lysate were assayed for DNA synthetic activity.

i) Lysis of cells

To eliminate the possibility that DNA synthesis in unlysed cells made permeable to precursors during the lysis treatment might contribute to the DNA synthetic activity observed in a crude lysate, it was necessary to ensure that the majority of cells had lysed completely.

Log-phase cells of JB 8 were harvested, washed once with 0.025M tris HCl (pH 7.6) and resuspended in tris EGTA (0.025M, 0.005M, pH 7.6) containing lysozyme 100 ug/ml at 4^{o}. After a number of cycles of freezing to -60^{o} and rapid thawing a large fraction of the cells remained intact. However, when cells were washed once with tris HCl (0.025M, pH 7.6) and once with the same buffer containing 0.005M EGTA before treatment with lysozyme, complete lysis occured after one or two cycles of freezing and thawing. $MgCl_2$ was added to a final concentration of 0.005M immediately after lysis was seen to be complete by microscopic examination.

ii) DNA synthetic activity in lysates

JB 8 cells lysed as described above were spun at 30,000 g for 30 minutes. The majority of the DNA remained in the supernatant. The pellet (membrane fraction) was resuspended in 0.025M tris buffer (pH 7.6) containing 0.005M $MgCl_2$.

The uptake of ^{3}H-TTP into cold TCA insoluble material in the presence of ATP (4×10^{-3}M) and calf thymus DNA primer was determined for the lysate and the membrane and supernatant fractions. The results are shown in Figure 6.

The lysate incorporated ^{3}H-TTP into acid-insoluble material during the first five minutes of incubation.

85% of the activity observed in the lysate was recovered in the supernatant, which also contained most of the DNA. Approximately 5% of the activity was found in the membrane fraction.

In subsequent experiments varying amounts of DNA were found in the pellet fraction. In the preparation of supernatants for future experiments the lysate was sheared in a microblender before centrifugation. This proceedure also reduced the viscosity of

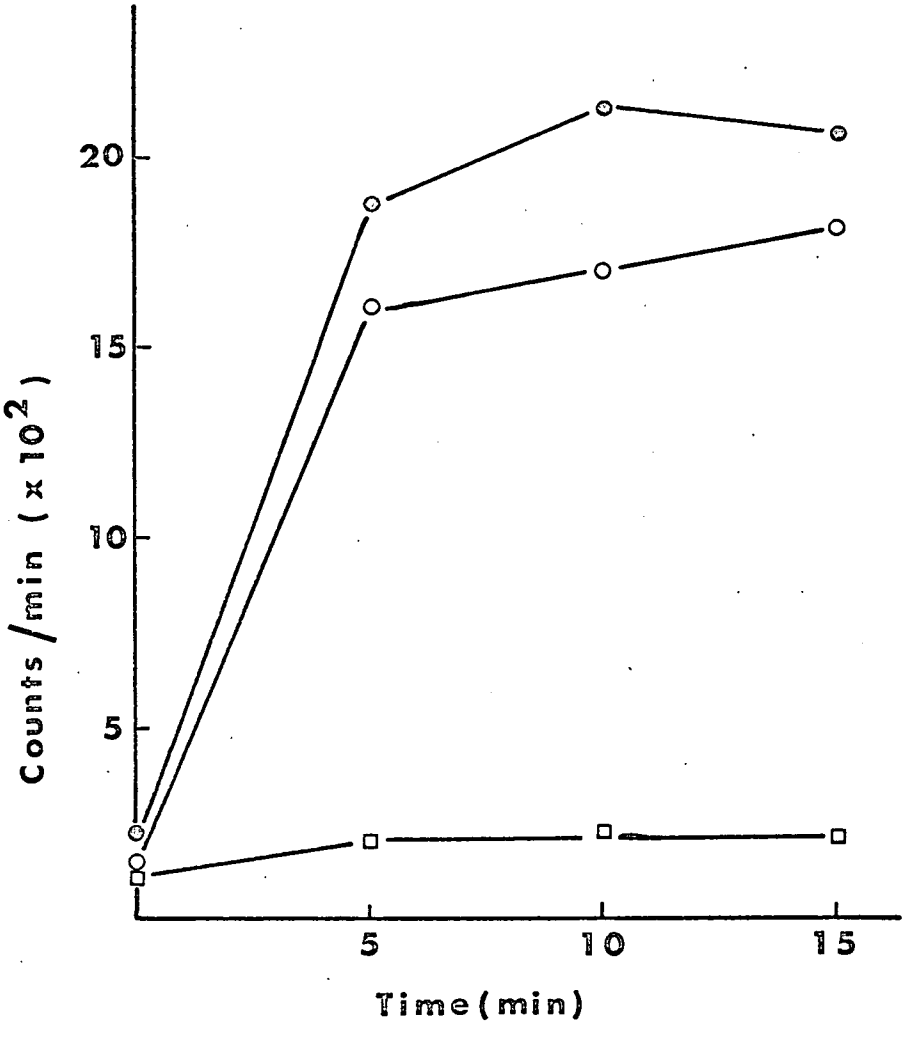

Figure 6. DNA synthesis in crude extracts of JB 8. The lysate and the supernatant and membrane fractions of the lysate were prepared as described in the text. DNA synthetic activity was assayed at 28° in the presence of ATP (4×10^{-3}M), and sonicated calf thymus DNA (40 ug/ml).

— o — Lysate

— o — Supernatant fraction

— □ — Membrane fraction

the supernatant and facilitated sampling.

The kinetics of ^{3}H-TTP incorporation in the lysate and supernatant fraction were similar to the kinetics of DNA synthesis in crude systems observed by other workers (Smith et. al., 1970; Knippers and Stratling, 1970; Okazaki et. al., 1970). The reason for the rapid cessation of synthesis is not known.

The product of the reaction was shown to be sensitive to DN'ase. Addition of 50 ug of pancreatic DN'ase after 5 minutes incubation at 28^{o} led to a gradual loss of ^{3}H counts from the acid precipitable material (Table 10).

TABLE 10

Effect of DN'ase on acid precipitable ^{3}H counts incorporated by the supernatant. Synthesis was allowed to proceed for 5 minutes at which time pancreatic DN'ase was added to one tube.

Time at 28^{o}	^{3}H counts/minute in TCA precipitable material	
	No DN'ase	+ DN'ase
5	1820	1725
15	1890	1205
30	1816	470

B. Properties of the DNA synthetic activity in the Supernatant

The DNA synthetic activity which has been observed in in vitro studies of pol^{-} E. coli cells is distinguished from DNA polymerase I-dependent activity by its sensitivity to sulphydryl-blocking agents and insensitivity to antiserum prepared against polymerase I.

Replicative DNA synthesis in toluene-treated cells is dependent on ATP (Moses and Richardson, 1970a) and on potassium ions (Mordoh et. al., 1970). The activity of purified DNA polymerases II and III is not dependent on ATP (Moses and Richardson, 1970b; Knippers, 1970; Kornberg and Gefter, 1971). DNA synthetic activity in crude extracts prepared by other workers has been found to be stimulated 1.5-3 fold by the addition of ATP (Smith et. al., 1970; Knippers and Stratling, 1970; Kornberg and Gefter, 1970).

The effect of the sulphydryl blocking agent pHMB (para-hydroxymercuribenzoate), ATP, KCl and DNA primer on the DNA synthetic activity observed in the supernatant fraction of lysed JB 8 cells was studied.

i) Effect of pHMB and primer DNA

Supernatant fraction was prepared as described in Methods section Q. DNA synthetic activity was assayed as described in Methods section S. The results are shown in Figure 7.

The reaction was inhibited completely by the addition of pHMB (1.5×10^{-4}M). It is possible that DNA polymerase II and DNA polymerase III both contribute to the DNA synthetic activity observed in lysates of pol$^-$ E. coli cells. Since both polymerases are pHMB sensitive and no reliable way of distinguishing between the two enzymes is known, it is not possible to assess the contribution of each to the total activity observed.

The addition of sonicated calf thymus DNA (40 ug/ml) depressed the total amount of ^{3}H-TTP incorporated by approximately 40%. This effect was also observed by Okazaki et. al., (1970). All further assays were carried out in the absence of exogenous primer DNA.

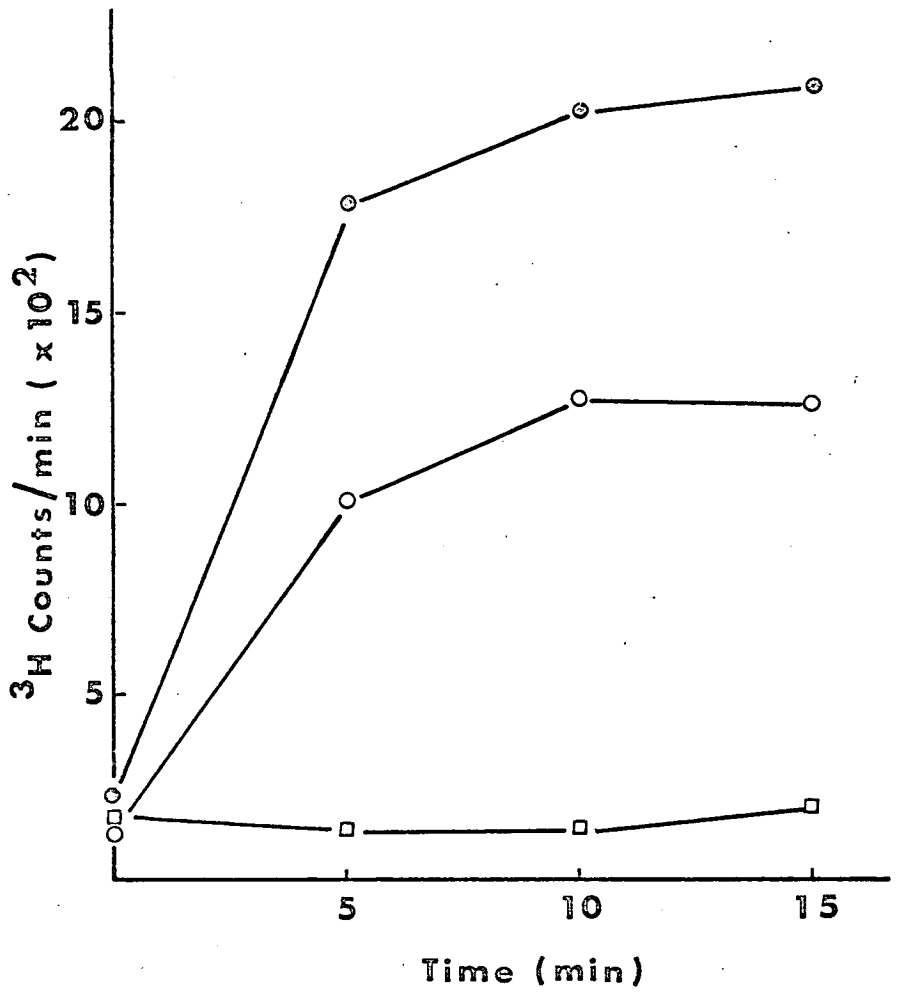

Figure 7. DNA synthesis in the supernatant fraction of lysates of JB 8 in the presence and absence of DNA primer and pHMB. Assays were performed at 28° in the presence of ATP (4×10^{-3}M). Sonicated calf thymus DNA was at 40 ug/ml, pHMB was at 1.5×10^{-4}M.

—o— + DNA

—⊙— - DNA

—□— + DNA, + pHMB

ii) Effect of ATP and KCl

The DNA synthetic activity of the supernatant was assayed in the presence and absence of ATP (4×10^{-3}M) and of KCl (0.1M). The results are shown in Figure 8.

The activity was completely dependent on the addition of ATP and stimulated approximately two fold by the addition of KCl.

It seemed possible that ATP is required to maintain adequate levels of the deoxynucleotide triphosphate precursors. If this were so polymerase I added to the assay should also be dependent on added ATP. 0.04 ml of a sonicated extract of *pol*$^+$ cells was added to 0.16 ml of the supernatant and assays carried out in the presence and absence of ATP (4×10^{-3}M). ^{3}H-TTP was incorporated into acid precipitable material in the absence of ATP, the addition of ATP did not increase the amount of ^{3}H-TTP incorporated (Figure 9). No synthesis was observed in the absence of ATP when a sonicated extract of *pol*$^-$ cells was added to the supernatant. This indicates that the requirement of ATP for synthesis in *pol*$^-$ lysates was not due to limiting levels of deoxynucleotide precursors.

The possibility that the ATP dependence results from a requirement for the ATP-dependent *rec B* nuclease (Barbour and Clark, 1970; Oishi, 1969) was also investigated. The results of this investigation are presented in Part IV.

C. Partial Purification of the DNA Synthetic Activity from the Supernatant

Partial purification of the DNA synthetic activity in the supernatant was attempted following the proceedure of Moses and Richardson, (1970b).

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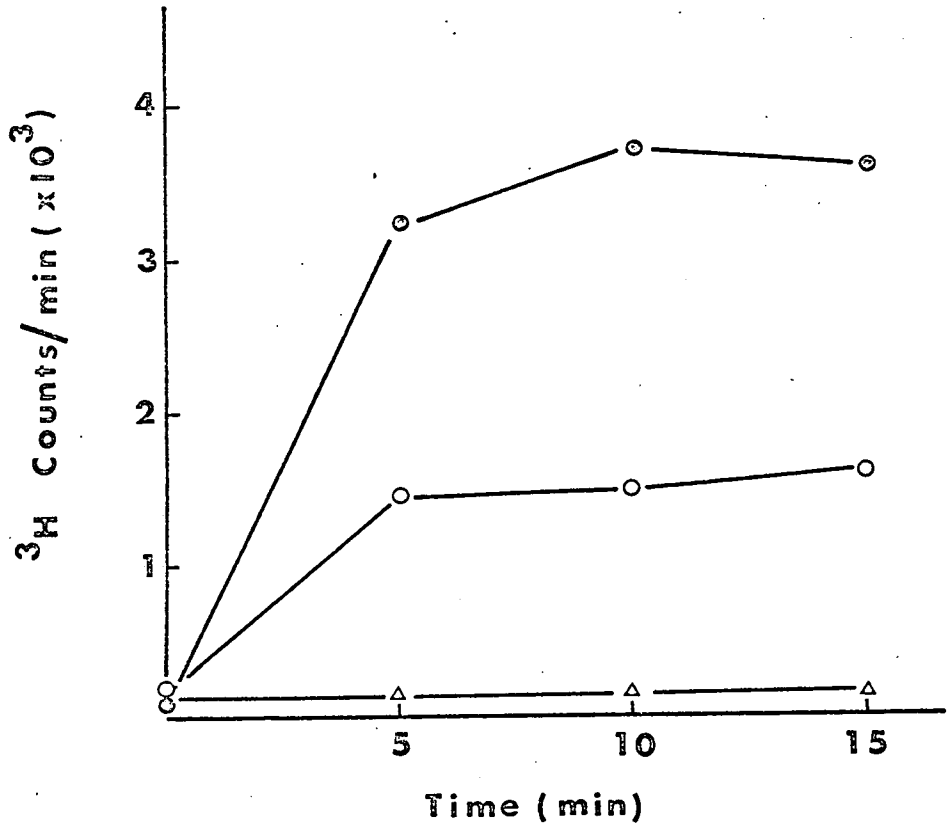

Figure 8. DNA synthesis in the supernatant fraction of lysates of JB 8 in the presence and absence of ATP and KCl. Assays were performed at 28°. ATP was at 4 x 10^{-3}M, KCl at 0.1M.

—⊙— + ATP, + KCl

—△— - ATP, + KCl

—○— + ATP, - KCl

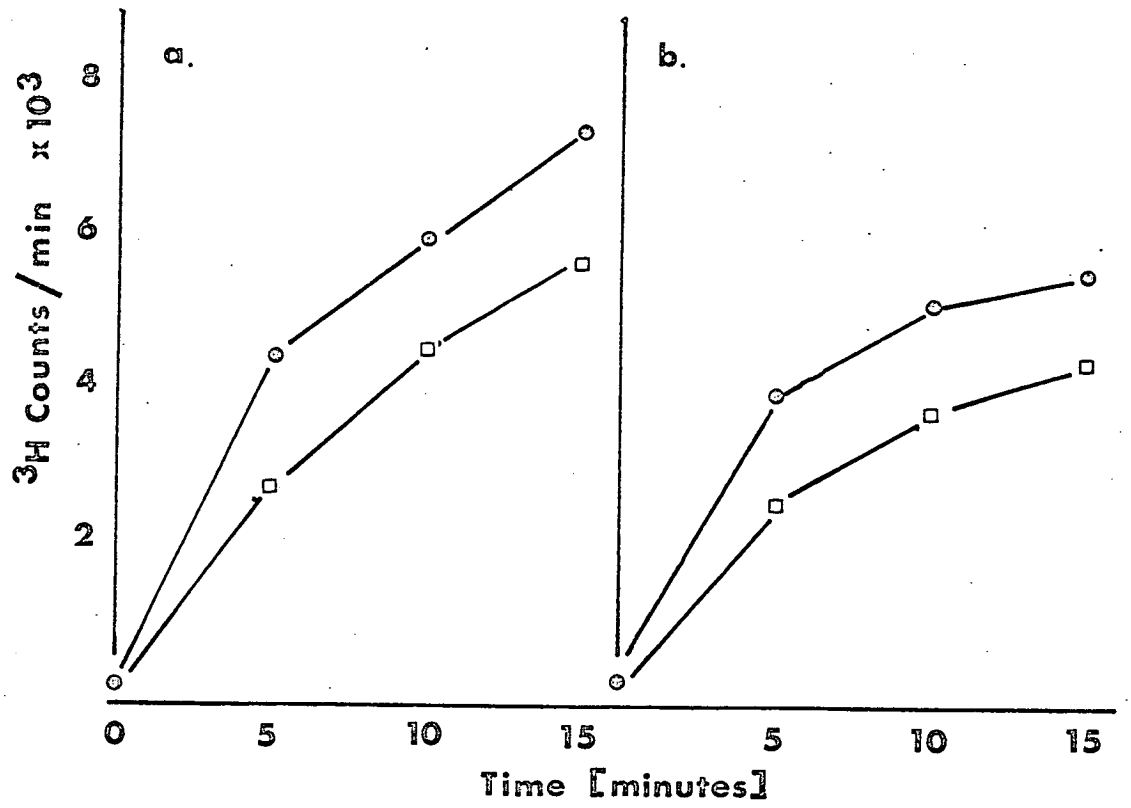

Figure 9. The effect of ATP on the activity of DNA polymerase I added to the supernatant fraction of a JB 8 lysate.

0.04 ml of a sonicated extract of *pol*$^+$ cells was added to (a) 0.16 ml 0.025M tris HCl (pH 7.6) and (b) 0.16 ml of the supernatant fraction of a lysate of JB 8 cells prepared as described in the Methods section Q.

DNA synthesis was assayed in the presence and absence of added ATP (4×10^{-3}M), as described in Methods section S.

-□- + ATP

-○- - ATP

i) DEAE chromatography

A DEAE cellulose column (DE-11, Whatman, 8 cm^2 x 12.5 cm) was equilibrated with 0.2M potassium phosphate buffer pH 7.0. 100 ml of a supernatant fraction prepared from 10 gm of JB 8 cells to which potassium phosphate buffer (pH 7.0), 2-mercaptoethanol and EDTA had been added to give a final concentration of 0.2M, 0.001M and 0.001M respectively, was applied to the column followed by 0.2M potassium phosphate buffer (pH 7.0) containing 1mM 2-mercaptoethanol and 1mM EDTA. A single fraction of 120 ml containing the major protein peak was collected. A portion of this fraction was dialysed for 2 hours at 4^o against 0.025M tris buffer (pH 7.6) containing 0.005M $MgCl_2$ and 0.001M 2-mercaptoethanol, and assayed for DNA synthetic activity as described in Methods section S.

ii) Properties of DNA synthetic activity in the eluate

Figure 10 shows the kinetics of incorporation of ^{3}H-TTP into acid precipitable material at 26^o in the presence of sonicated calf thymus DNA and ATP. In contrast with the synthesis in the crude extract, ^{3}H-TTP incorporation was linear for 15 minutes. The total amount of ^{3}H-TTP incorporated during this time was approximately 10% of the ^{3}H-TTP incorporated by a sample of the supernatant which had been dialysed for two hours under the same conditions as the column eluate. Though the two assays are not directly comparable, due to the non-linearity of synthesis in the supernatant, the recovery of activity after DEAE chromatography appears to be low.

The activity in the eluate was dependent on the addition of ATP and unlike synthesis in the crude extract also required sonic-

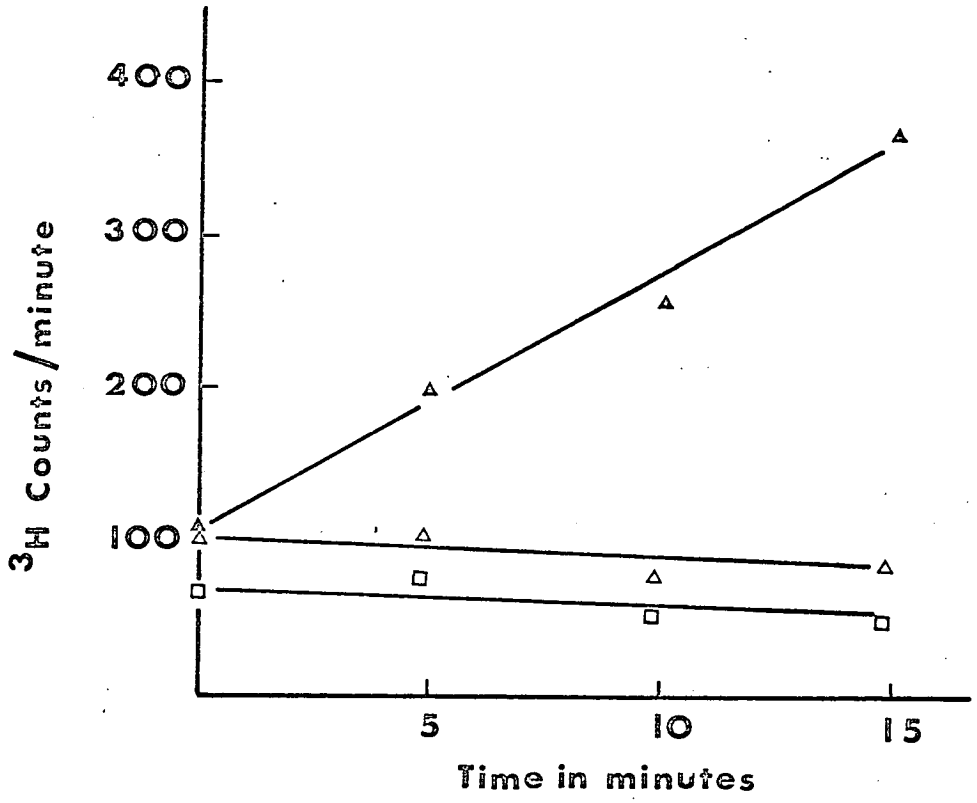

Figure 10. DNA synthesis after partial purification.

The DNA synthetic activity in the supernatant fraction of a lysate of JB 8 was partially purified by DEAE chromatography as described in the text. The DNA synthetic activity in the eluate was assayed at 26° in the presence and absence of ATP (4×10^{-3}M) and sonicated DNA primer (40 ug/ml).

— △ — + ATP, + DNA

— △ — - ATP, + DNA

— □ — + ATP, - DNA

ated calf thymus primer DNA (Figure 10). Omission of KCl from the assay was without effect. The addition of higher concentrations of primer DNA did not increase the rate of incorporation of ^{3}H-TTP indicating that the rate of the reaction was not limited by the concentration of primer. Further purification was not attempted.

The complete ATP dependence of the DNA synthetic activity in both the crude and partially purified system is note worthy since the DNA synthetic activity observed by other workers in crude systems was stimulated only slightly by ATP, (Knippers and Stratling, 1970; Kornberg and Gefter, 1970) and in partially purified systems was independent of ATP, (Moses and Richardson, 1970b; Kornberg and Gefter, 1970).

PART IV: The Role of ATP in DNA Synthesis in Crude Extracts

Introduction

A striking feature of the DNA synthesis observed in crude extracts was its ATP-dependence. It has already been shown that the ATP is not required to maintain adequate levels of deoxynucleoside precursors (see Part III). Since DNA synthesis in toluene-treated cells, which appears to correspond to true replication, is ATP-dependent (see Introduction section Bi), the role of ATP in the crude extract was further studied in the hope that the role of ATP in DNA synthesis might be clarified.

The activity of DNA polymerases II and III is not ATP dependent (Knippers, 1970; Moses and Richardson, 1970b; Kornberg and Gefter, 1971). Purified DNA polymerase II and DNA polymerase III are able to fill in single-stranded gaps in double-stranded DNA but unlike DNA polymerase I are unable to act at nicks in double-stranded DNA (Knippers, 1970; Kornberg and Gefter, 1971).

The DNA synthesis observed in crude extracts has not been shown to be a limited continuation of replication and it is possible that it in fact results from the action of polymerases II and III at primer sites produced by the action of a nuclease on endogenous DNA.

An ATP-dependent nuclease with exo- and endonucleolytic activity has been shown to be present in extracts of wild type *E. coli* cells but absent from *recB*$^-$ and *recC*$^-$ mutants (Buttin and Wright, 1968; Oishi, 1969; Barbour and Clark, 1970; Goldmark and Linn, 1970). To investigate the possibility that this nuclease (the *recB* nuclease) is required for DNA synthesis in crude extracts a *pol*$^-$ *recB*$^-$ strain was required.

The combination of recB or recC with polA1 has been shown to be lethal (see Gross, in press). However, a recB strain which lacks polymerase I activity *in vitro* but appears to have substantial polymerase I activity *in vivo* has been constructed (Bazill *et. al.*, 1971). This strain was used in the present study.

A. The recB21 pol-4* double mutant

The recB21 pol-4* double mutant was constructed by introducing the recB21 mutation into a pol-4* strain. The pol-4* mutant is a revertant of the cold-sensitive pol-4 mutation, (Gross *et. al.*, 1971) which has regained virtually normal radiation resistance, yet has negligible DNA polymerase I activity *in vitro*. The reversion does not appear to involve an external suppressor and presumably results from a second mutation in the polA gene which compensates for the pol-4 defect *in vivo* but not under *in vitro* conditions (Bazill *et. al.*, 1971).

B. DNA Synthesis in the Supernatant Fraction of recB21 pol-4* and recB+ pol-4* Cells

Supernatant fractions of recB21 pol-4* and recB+ pol-4* cells were prepared as described in Methods section Q. A portion of cells was retained after washing with tris and treated with toluene as described in Methods section R. The supernatant fractions and the toluene-treated cells were assayed for DNA synthesis at 28°. The results are shown in Figure 11.

ATP-dependent DNA synthesis was observed in toluene-treated recB- and recB+ cells. The level of activity was low probably as a result of the treatment prior to toluenisation.

The amount of synthesis observed in the supernatant fractions

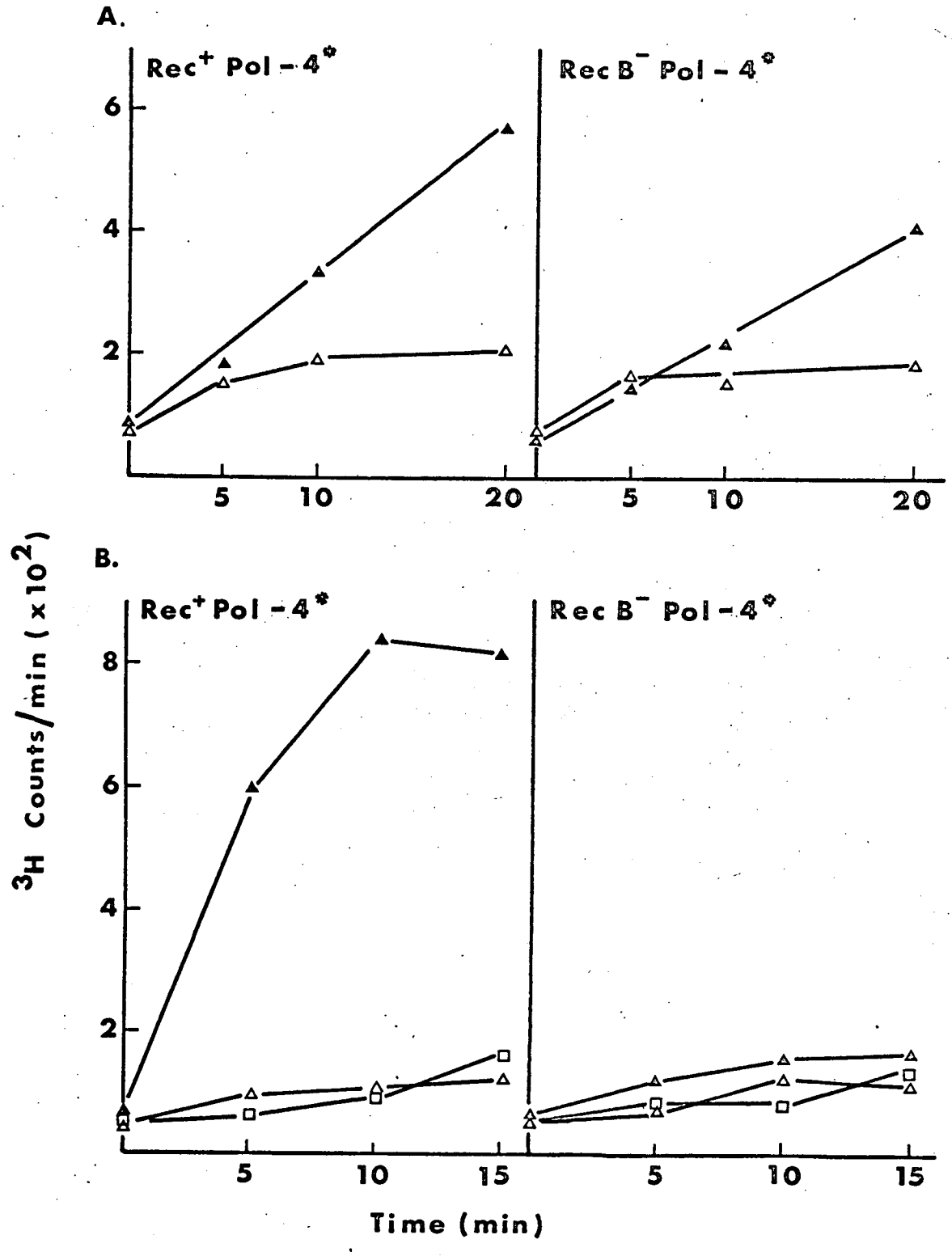
A.
Rec+ Pol - 4*
Rec B- Pol - 4*
6
4
2
5
10
20
B.
Rec+ Pol - 4*
Rec B- Pol - 4*
8
6
4
2
5
10
15
3H Counts/min (x10^2)
Time (min)

Figure 11. DNA synthesis in toluene-treated cells and in supernatant fractions of *recB*$_{21}$ *pol-4** and *rec*$^{+}$ *pol-4**.

Toluene-treated cells and supernatant fractions were prepared as described in the text.

A. Toluene-treated cells were assayed for DNA synthetic activity as described in Methods section S. Assays were performed at 28°. ATP when present was 4×10^{-3}M.

—▲— + ATP

—△— - ATP

B. The supernatant fractions were assayed for DNA synthetic activity at 28°. ATP when present was 4×10^{-3}M. p-HMB when present was 1.5×10^{-4}M.

—▲— + ATP

—△— - ATP

—□— + ATP, + p-HMB

in the presence of ATP was about fifteen-fold lower in the recB$^-$ than in the recB$^+$. A fraction of the ^{3}H-TTP incorporation observed in the recB$^-$ supernatant is ATP-dependent and inhibited by p-HMB. Virtually all of the synthesis in the recB$^+$ supernatant is ATP-dependent and sensitive to p-HMB.

These results indicate that the recB nuclease is required for DNA synthesis in crude extracts, and provide strong support for the suggestion that the synthesis observed in crude extracts takes place at primer sites produced by the action of nucleases on endogenous DNA.

The supernatant fractions were reassayed after 48 hours storage at 4^o. The results are shown in Figure 12.

DNA synthesis is now observed in both recB$^-$ and recB$^+$ supernatants. Approximately 75% of the activity in the recB$^+$ and 85% of the activity in the recB$^-$ is ATP-dependent. The supernatants were considerably less viscous after storage at 4^o and it seems probable that the action of nucleases other than the recB nuclease, has created primer sites for DNA synthesis.

The fact that the majority of the DNA synthesis detectable in the recB$^-$ supernatant after storage, is ATP-dependent suggests that the ATP-dependence of DNA synthesis in crude extracts does not simply reflect the requirement for the recB nuclease.

C. DNA Synthesis in recB21 pol-4* Supernatants in the Presence of Added Primer DNA

If the recB nuclease is required to convert endogenous DNA into a suitable primer for the DNA polymerase it seemed possible that DNA synthesis could take place in the recB$^-$ supernatant provided a suitable DNA primer was added. If this is so it should

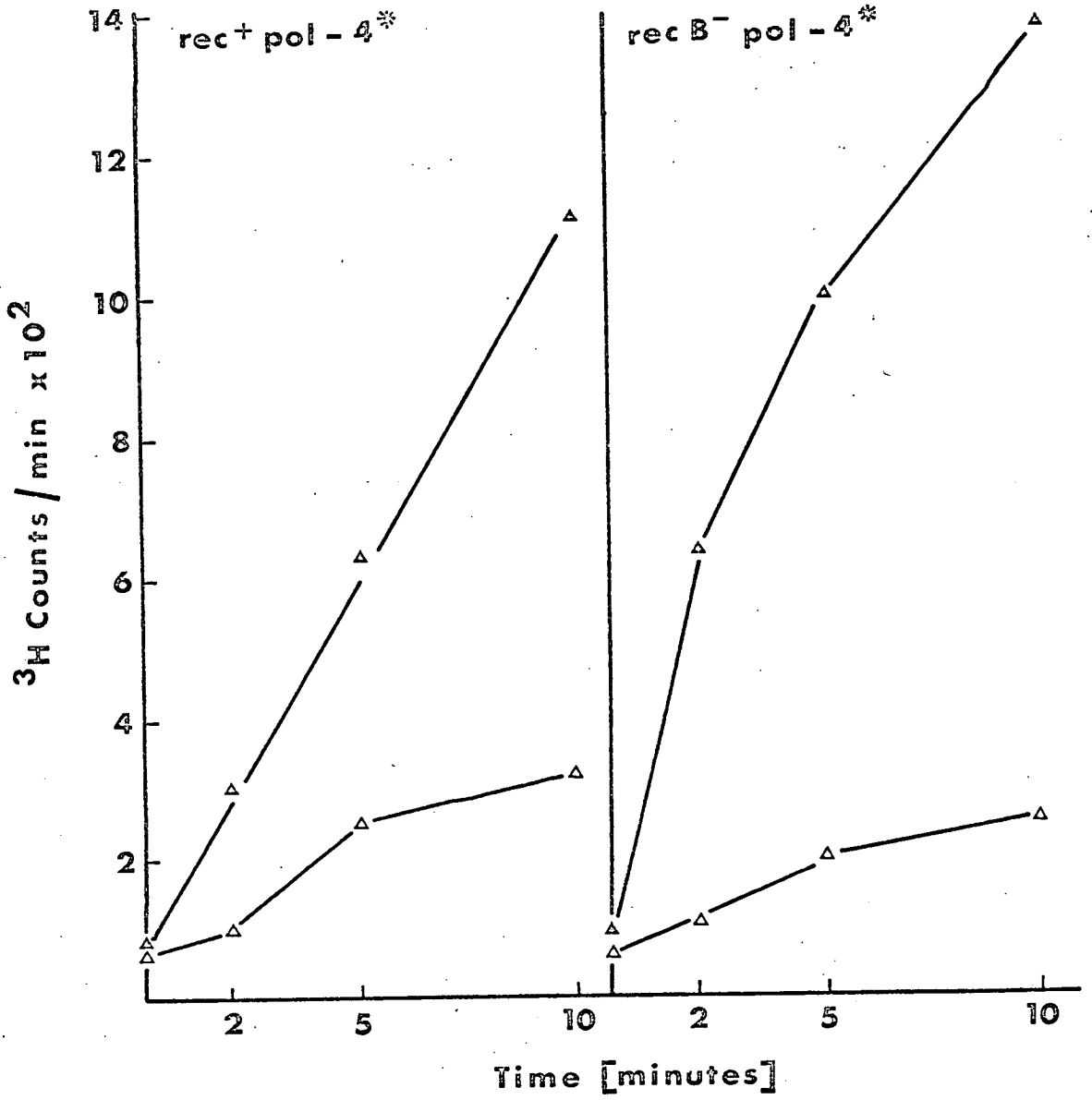

Figure 12 DNA synthesis in supernatant fractions of recB21 pol-4* and recB+ pol-4* after 48 hours storage at 4°. Assays were performed at 28°. ATP when present was 4×10^{-3}M.

—△— + ATP

—△— - ATP

be possible to test whether DNA synthesis in the absence of the recB nuclease is indeed ATP-dependent.

A number of DNA primers were tested. The results are shown in Table 11. Sonicated calf thymus DNA, renatured sonicated E. coli DNA and a reannealled mixture of high molecular weight and sonicated E. coli DNA increased the level of DNA synthesis in the recB supernatant.

TABLE 11

DNA synthesis in the supernatant fraction of recB21 pol-4* cells in the presence of added DNA primers

DNA primer	^{3}H-TTP counts/minute incorporated in 15 min.
- DNA	100
Sonicated calf thymus DNA	200
Sonicated E. coli DNA	130
Reannealed sonicated E. coli DNA	225
Reannealed 1:1 mixture of high molecular weight and sonicated E. coli DNA	350

Sonicated calf thymus DNA and the reannealed mixture of high molecular weight and sonicated DNA were retested in the presence and absence of ATP. The results are shown in Figure 13.

The DNA synthesis which occurs in the recB^{-} supernatant in the presence of both primers is ATP-dependent. Sonicated DNA

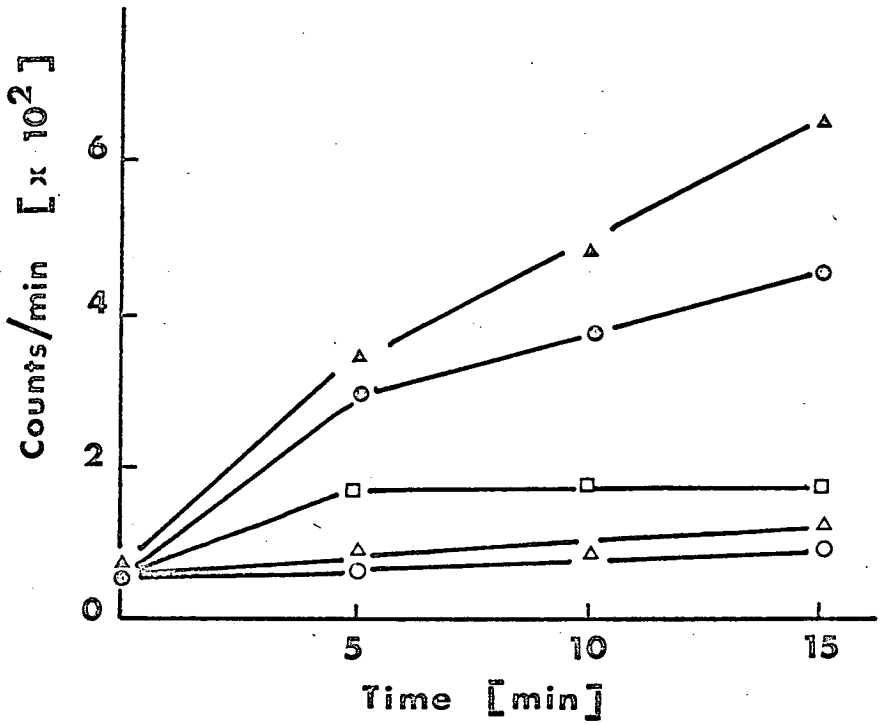

Figure 13 DNA synthesis in the supernatant fraction of recB21 pol-4* in the presence of added primer DNA. Assays were performed at 28°. DNA primer when present was 40 ug/ml. ATP when present was 4 x 10^{-3}M.

—□— - DNA, + ATP

—⊙— + sonicated calf thymus DNA, + ATP

—○— + sonicated calf thymus DNA, - ATP

—▲— + reannealed mixture of sonicated and high molecular weight E. coli DNA, + ATP

—△— + E. coli primer, - ATP

has short stretches of single-stranded DNA at the ends of double-stranded DNA and the primer made by reannealing a mixture of high molecular weight and sonicated DNA would be expected to consist of partially double-stranded DNA with single-stranded gaps. Both primers would therefore satisfy the requirements of DNA polymerases II and III.

Since the synthesis observed in the recB$^-$ pol-4$^+$ lysate in the presence of primer DNA is ATP-dependent it may be concluded that the ATP dependence of synthesis in lysates does not simply result from the requirement for the ATP-dependent recB nuclease and may indicate the involvement of the ATP-dependent component of the replicating complex.

In order to test whether the DNA synthesis observed in crude systems by other workers might also result from polymerase action at artificial primer sites, a system more analogous to the systems used by these workers was developed by G. Bazill. Lysates were prepared by gentle lysis of spheroplasts using Brij-58. The synthesis observed in this system was also dependent on the recB nuclease. The results of this work have been published and are contained in this thesis as an Appendix.

PART V: DNA Synthesis in Crude Extracts of Temperature-Sensitive Mutants Defective in DNA Synthesis

Introduction

Preliminary tests for temperature-sensitive DNA synthesis were performed in crude extracts of a number of *dna* mutants. Mutants with a defect in the relevant polymerase should exhibit temperature-sensitive DNA synthesis in crude extracts. However, it is possible that other mutants might also exhibit temperature-sensitive DNA synthesis in this system.

Since the DNA synthetic activity in the supernatant is short-lived it was probable that *in vitro* temperature-sensitivity would be detectable only in mutants which cease to synthesise DNA immediately on transfer to high temperature *in vivo*. Mutants representative of groups E,B and G which exhibited this property and had been shown to be defective in the toluene-treated cell system were studied.

DNA synthesis in toluene-treated cells has been studied for mutants belonging to groups B,D,E,F and G. A number of *dnaB* mutants (*dna*-42B, *dna*-43B, *dna*-266B and *dna*-70B) have been shown to be defective in DNA synthesis at high temperature (Moses and Richardson, 1970a; Kohiyama and Kolber, 1970; Mordoh *et. al.*, 1970; Peacey and Gross, pers. comm.). Peacey and Gross have also studied DNA synthesis in *pol*$^-$ derivatives of mutants belonging to groups D,E,F and G. The *dnaE* mutant studied (*dna*-486E) was defective in DNA synthesis at both 26^o and 40^o. One group G mutant (*dna*-308G) was also defective in DNA synthesis at low temperature, whilst another, *dna*-399G, was active at low temperature and defective at 40^o.

Synthesis in the mutants dna-7D and dna-101F which are the only known representatives of the groups D and F was not found to be defective in toluene-treated cells. This is probably because these mutants show residual DNA synthesis in vivo for at least 30 minutes after transfer to high temperature (Peacey, pers. comm., Wechsler and Gross, in preparation). These mutants were therefore not tested in the crude extracts.

A. DNA Synthesis in the Supernatant Fraction of dna-486E

In vivo DNA synthesis in the three known group E mutants has been examined by Wechsler and Gross (in preparation). DNA synthesis stops immediately on transfer to 42.5° in dna-486E only.

JG 212 (dna-486E, pol-4*) a derivative of JW 111 which has negligable polymerase I activity in vitro and JG 213 a dna+ transductant of JG 212 were obtained from J. Gross. Supernatant fractions of JG 212 and JG 213 were prepared as described in Methods section Q. DNA synthetic activity was assayed at 30° and 40° after 1 minute preincubation at the assay temperature in the presence of s-RNA (20 ug/ml), which was added to inhibit endonuclease I. The results are shown in Figure 14.

The total amount of ^{3}H-TTP incorporated by the dna-486E supernatant at 30° was approximately one third that incorporated by the dna+ control. The amount of synthesis in both the dna-486E and dna+ supernatants was reduced by approximately 50% when assayed at 40°.

Though the activity of dna-486E does not appear to be appreciably more temperature-sensitive than that of the dna+ control, the dna-486E mutant appears to be defective in DNA synthesis at 30°. This behaviour resembles that observed in toluene-treated

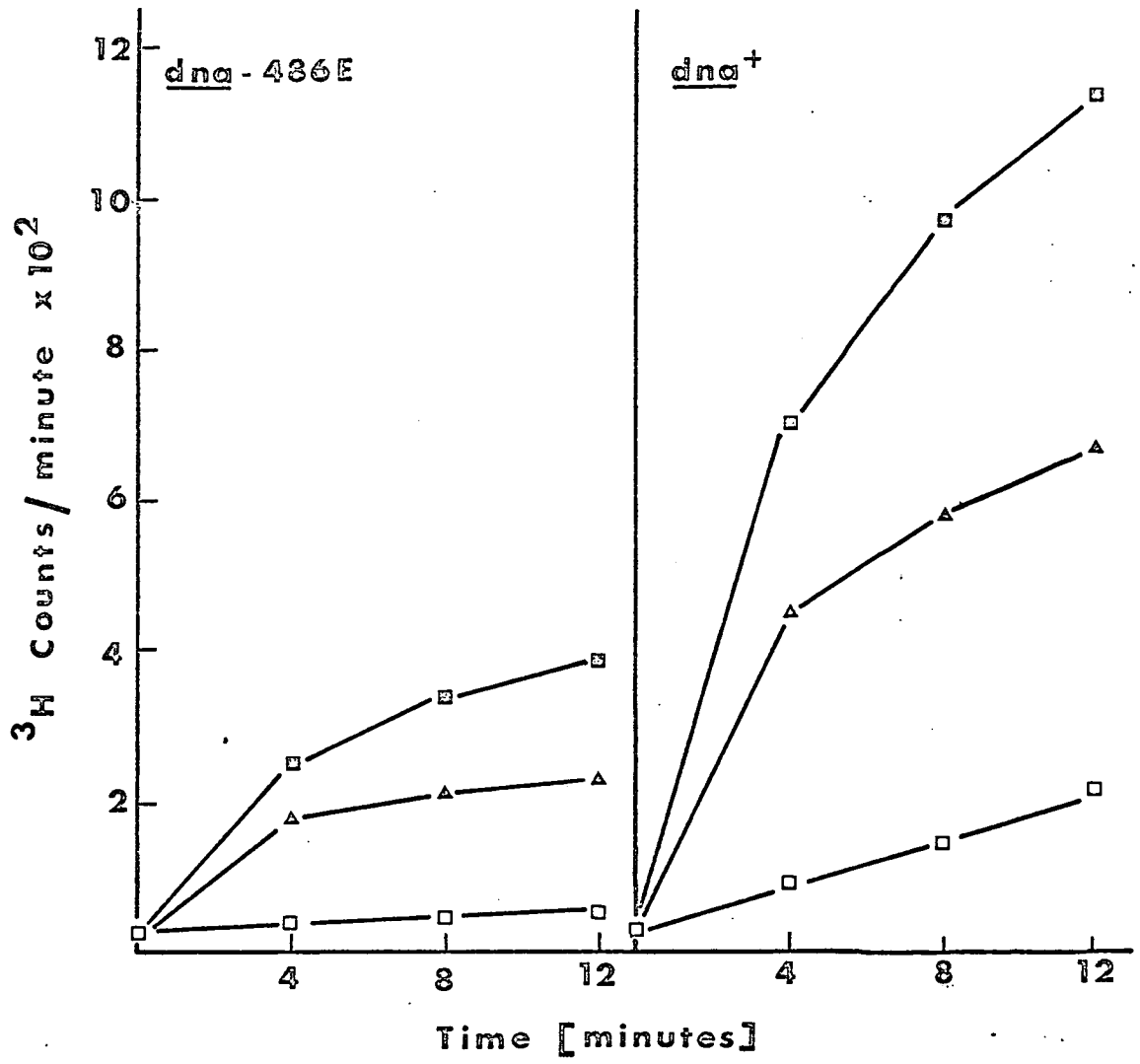

Figure 14 DNA synthesis in supernatant fractions of dna-486E pol-4* and dna+ pol-4* at 30° and 40°. The supernatants were preincubated for 1 minute at the assay temperature in the presence of s-RNA (40 ug/ml). ATP when present was 4 x 10^{-3}M.

—▫— 30°, + ATP

—□— 30°, - ATP

—△— 40°, + ATP

dna-486E cells (Peacey and Gross). The results are consistent with the possibility that more than one DNA polymerase contributes to the DNA synthesis observed in lysates of wild type cells and that one polymerase is defective in lysates of dna-486E.

B. DNA Synthesis in the Supernatant Fraction of dna-70B

A number of mutants in group B cease to synthesise DNA in vivo immediately on transfer to high temperature (see eg. Wechsler and Gross, in preparation). A pol$^-$ derivative of one such mutant, dna-70B, was used. JG 186 (dna-70B pol A1) constructed by introducing the dna-70B mutation into JG 114 (pol$^-$ A1 malB$^-$) and the parent JG 114 were obtained from J. Gross. DNA synthetic activity in supernatant fractions of JG 186 and JG 114 was assayed at 30° and 40° after one minute preincubation in the presence of s-RNA. The results are shown in Figure 15.

The activity of dna-70B and the dna$^+$ control were approximately the same at both temperatures. This indicates that the dnaB gene product is probably not required for DNA synthesis in the crude extract.

C. DNA Synthesis in the Supernatant Fraction of dna-308G

In vivo DNA synthesis has been shown to cease immediately on transfer of a dna-308G mutant to high temperature (Wechsler and Gross, in preparation; Peacey pers. comm.). JG 204 (dna-308G, pol-4*) a pol$^-$ derivative of JG 32 (dna-308G) and a spontaneous dna$^+$ revertant of JG 204 were obtained from J. Gross. DNA synthetic activity in the supernatant fraction of the dna-308G and dna$^+$ strains was assayed at 30° and 40° after 1 minute preincubation in the presence of s-RNA. The results are shown in Figure 16.

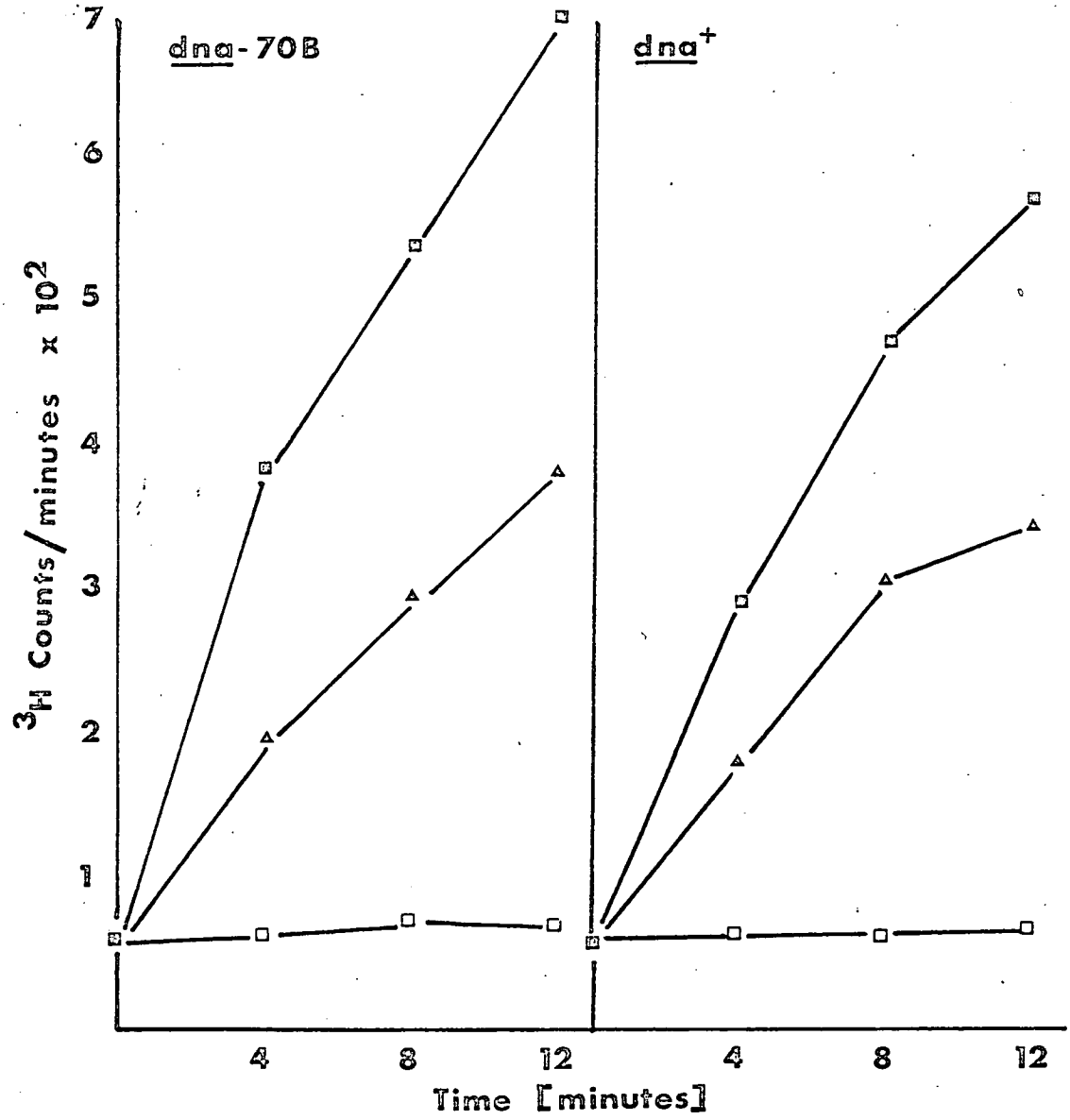

Figure 15 DNA synthesis in supernatant fractions of dna-70B polA1 and dna$^+$ polA1 at 30° and 40°. The supernatants were preincubated for 1 minute in the presence of s-RNA (40 ug/ml). ATP when present was 4 x 10^{-3}M.

—▣— 30°, + ATP

—□— 30°, - ATP

—△— 40°, + ATP

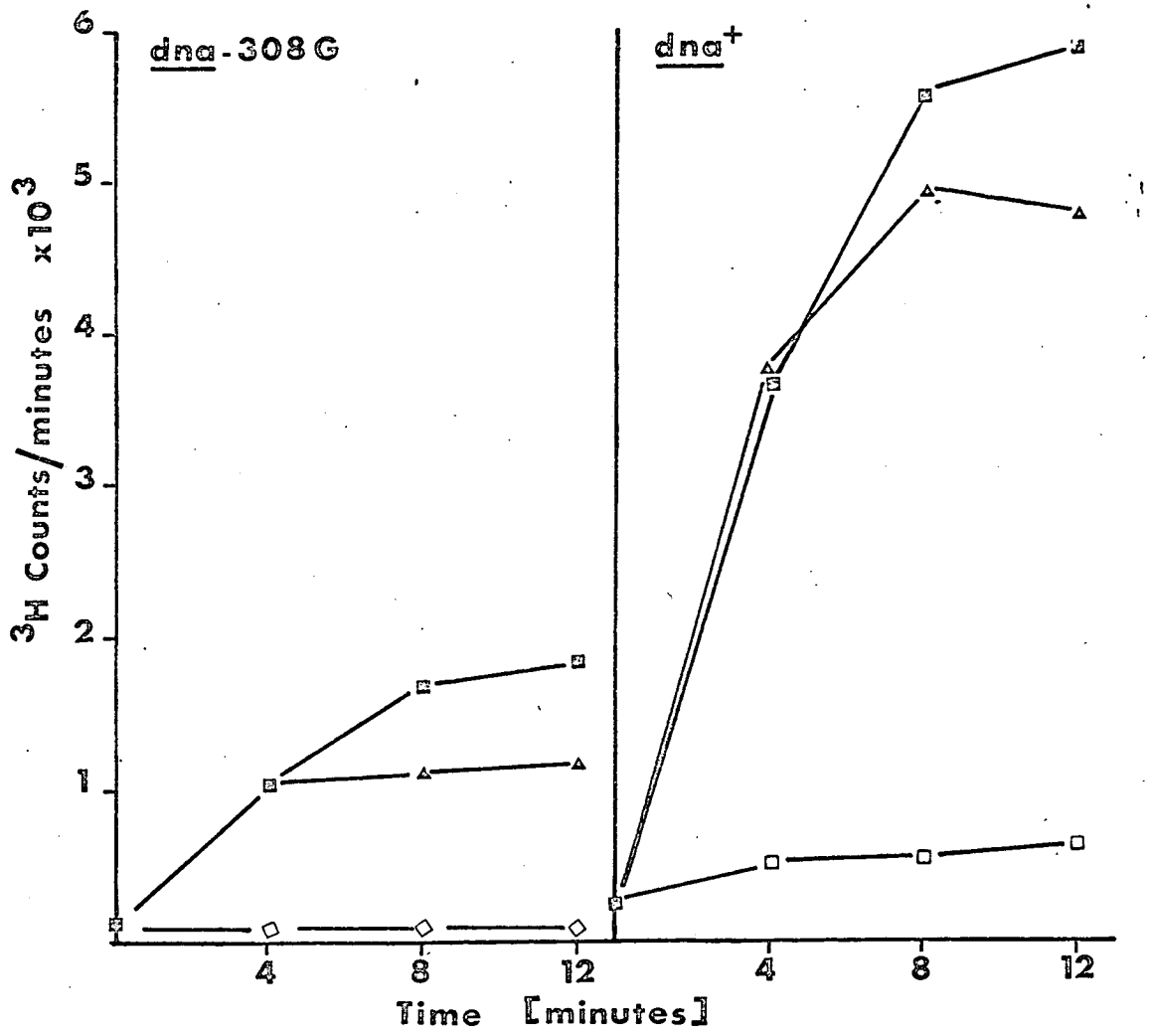

Figure 16 DNA synthesis in the supernatant fractions of dna-308G pol-4* and dna+ pol-4* at 30° and 40°. The supernatants were preincubated for 1 minute in the presence of s-RNA (40 ug/ml). ATP, when present, was 4 x 10^{-3}M.

—▣— 30°, + ATP

—□— 30°, - ATP

—△— 40°, + ATP

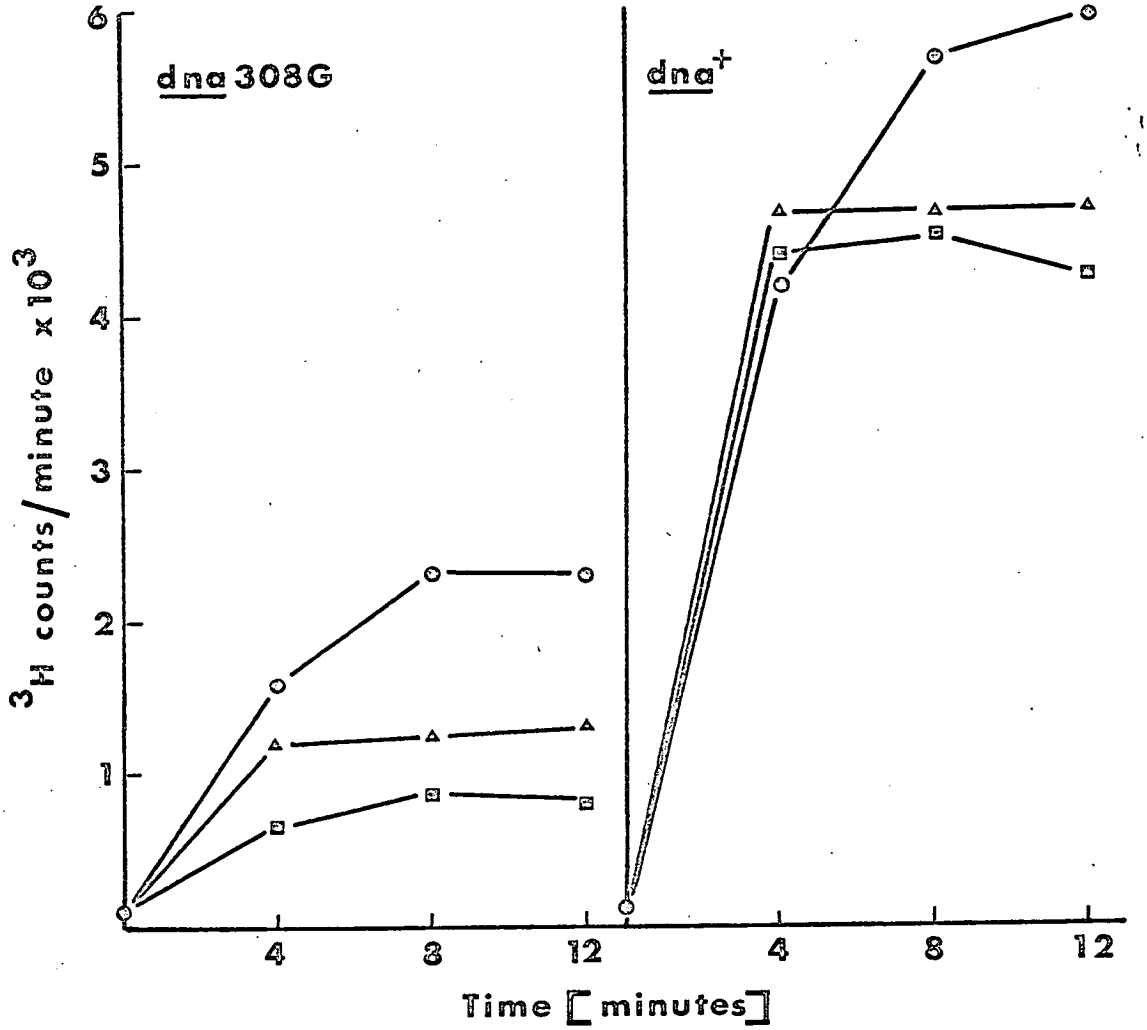

Figure 17 DNA synthesis in supernatant fractions of *dna*-308G *pol-4** and *dna*$^+$ *pol-4** at 30°, 40° and 42°. The supernatants were preincubated for 5 minutes at the assay temperature in the presence of s-RNA (40 ug/ml). ATP, when present, was 4 x 10^{-3}M.

—o— 30°, + ATP

—▲— 40°, + ATP

—▣— 42°, + ATP

The total amount of ^{3}H-TTP incorporated by the *dna*$^{+}$ strain was abnormally high, at 30° and was reduced only slightly at 40°. The behaviour of this strain differs in this way from the behaviour of the *dna*$^{+}$ *pol*$^{-}$ controls JG 213 and JG 114. (see sections A and B). In contrast the DNA synthetic activity in *dna*-308G is reduced by almost 50% when assayed at 40°.

The DNA synthetic activity in the supernatant fractions of *dna*-308G and *dna*$^{+}$ was reassayed at 30°, 40° and 42° after 5 minutes preincubation in the presence of s-RNA. The results are shown in Figure 17.

The *dna*$^{+}$ control exhibits approximately the same level of ^{3}H-TTP incorporation at all three temperatures. The activity in the *dna*-308G supernatant when assayed at 40° and 42° is reduced to approximately 60% and 30% respectively of the activity observed at 30°.

Though the DNA synthetic activity in the *dna*-308G lysate appears to be appreciably more thermolabile than that observed in lysates of the *dna*$^{+}$ strain used as control, its behaviour is similar to that observed with the two *dna*$^{+}$ strains JG 213 and JG 114.

Until the behaviour of the *dna*$^{+}$ revertant of *dna*-308G is better understood no conclusions can be drawn from these results.

DISCUSSION

A. Identification of DNA Polymerase Mutants by their Mutator Activity

Attempts to isolate mutants with a defect in the DNA polymerase responsible for DNA replication by isolating mutator mutants and screening them for temperature-sensitivity and vice versa were unsuccesful. However, three temperature-sensitive dna mutants belonging to group E (Wechsler and Gross, in preparation) have been found to exhibit an increased frequency of spontaneous mutation at permissive temperatures. The mutator activity was shown to result from the dnaE mutation in the case of at least one mutant, dna-511E. The increase in the frequency of spontaneous mutation in the three dnaE mutants tested was substantially lower than that observed in the mutator mutants isolated by the selection proceedure described in Part I. This may explain the failure to isolate dnaE mutants by selection for mutator activity.

The fact that a single mutation in the dnaE gene results in temperature-sensitive DNA synthesis and mutator activity strongly suggests that the dnaE gene product is involved in base selection during replicative DNA synthesis. Mutator and antimutator activity has been shown to be associated with ts lesions in T4 gene 43 which is known to code for T4 DNA polymerase (Speyer et. al., 1966; Drake et. al., 1969; de Waard et. al., 1965), showing that T4 DNA polymerase functions in base selection. Mutations in a number of other genes concerned with DNA synthesis in T4 have also been shown to cause an increase in the spontaneous mutation frequency (Drake, pers. comm.; Bernstein, 1971); however the increases observed in these mutants are, in general, small compared to those

observed in gene-43 mutants. Since significant mutator activity was found to be associated specifically with dnaE mutants, and generally not with mutants belonging to the other groups, it seemed possible that the dnaE gene product is a DNA polymerase required for replicative DNA synthesis.

The results of tests for temperature-sensitive DNA synthesis in crude extracts were consistent with this hypothesis. The level of DNA synthetic activity observed in lysates of dna-486E cells was threefold lower at both 30° and 40° than that observed in lysates of a dna$^+$ transductant of this strain. Furthermore Peacey and Gross (pers. comm.) were unable to detect DNA synthetic activity in toluene-treated dna-486E cells at 30° or 40°. During the course of this work the isolation of two distinct DNA polymerase activities from pol$^-$ E. coli cells was reported (Kornberg and Gefter, 1971). It seemed therefore possible that more than one polymerase contributes to the synthetic activity observed in the crude lysate system described in Part III and that one polymerase activity (possibly DNA polymerase II or III) was absent from lysates of dna-486E, whilst the activity of the other polymerase(s) was normal. Since attempts to purify DNA polymerase II and III were unsuccessful it was not possible to test this hypothesis. The possibility that the dnaE gene codes for a component of the replicating complex other than a polymerase, which is able to influence the spontaneous mutation frequency and is required for synthesis in crude extracts could not be excluded on the basis of the results presented.

Since the completion of this work the dnaE gene has been shown to code for DNA polymerase III (Gefter, Hirota, Kornberg and

Wechsler, in preparation). These workers have purified DNA polymerases II and III from a number of dna mutants representing all seven dna groups. The activity of purified DNA polymerase II was not found to be thermolabile in any of the mutants tested. The activity of purified DNA polymerase III was found to be defective at 45° in each of three dnaE mutants, including dna-511E. In the case of dna-486E no polymerase III activity was detectable at 45° or at 30°.

This result supports the hypothesis that more than one polymerase activity contributes to the DNA synthetic activity observed in the lysate system described, and that one polymerase (in fact polymerase III) is inactive in lysates of dna-486E. The results of Gefter et. al., show that DNA polymerase II is required for replicative DNA synthesis.

The fact that DNA polymerase III is required for DNA replication and is involved in base selection constitutes strong evidence that this polymerase is responsible for the polymerisation of bases during replicative DNA synthesis. The alternative possibility that the function of polymerase III is to monitor errors arising during replication is rendered unlikely by the observation that DNA synthesis ceases immediately on transfer of dna-486E cells to 40°, since a defective monitoring system would be expected to affect the continuation of synthesis only after some delay. However a further possibility, namely that DNA polymerase III functions in the charging of a hypothetical DNA precursor molecule (analagous to t-RNA) cannot be excluded. In this case polymerisation would have to be an integral step in the charging process in order to account for the ability of polymerase III to act as a conventional

DNA polymerase *in vitro*.

The *dnaE* locus is transductionally linked to *tonA* (Wechsler and Gross, in preparation). A number of mutator mutants have been mapped in this region of the chromosome. The mutant designated *ast* (Zamenhof, 1966) and one mutant isolated by Liberfarb and Bryson (1970) have not been accurately mapped and may be located in the *dnaE* gene. The mutator mutant, *mutT1*, which has been shown to be unable to induce mutations in the absence of DNA synthesis (Cox, 1970) has been shown to be closely linked to the *azi* locus (Cox and Yanofsky, 1969). *MutT1* and *mut2* which is closely linked to *mutT1* (Helling, 1968) are therefore not located in the *dnaE* gene

The mutator activity of *dnaE* mutants may be useful in studying the role of polymerase III in base selection and in the replication of various phage and episomes; if the mutagenic polymerase induces mutations in a phage or in an episome this would suggest that the host DNA polymerase plays a role in the replication of that element. In addition it should be possible to study the mutational specificity of different mutant polymerases by examining the reversion of known *trpA* mutants, as described in Part I.

B. DNA Synthesis in vitro

The study of DNA synthesis *in vitro* is of interest mainly for the purpose of identifying the various components of the replicating complex. For this purpose a system is required in which the synthesis observed is related to true replicative DNA synthesis. In such a system temperature-sensitive *dna* mutants should behave as *in vivo*.

The properties of a crude *in vitro* system were studied (Parts III and IV). The most notable feature of the synthesis

observed in the system described in Part III was its complete ATP-dependence. Investigation of the possible involvement of the ATP-dependent recB nuclease led to the discovery that DNA synthesis is not detectable in freshly prepared extracts of recB⁻ cells, indicating that synthesis is completely dependent on the recB nuclease. Since purified DNA polymerase II and DNA polymerase III require primer DNA with single-stranded gaps, the simplest explanation of the requirement for the recB nuclease is that the DNA synthesis observed is not a continuation of replication at the fork which pre-existed *in vivo*, but takes place at artificial primer sites created by the action of the recB nuclease on endogenous DNA.

Since the DNA synthetic activity in crude systems studied by other workers is not markedly ATP-dependent, synthesis in these systems cannot have an absolute requirement for the recB nuclease. It seems probable, nonetheless, that synthesis in these systems also occurs at artificial primer sites, created by the action of some other nuclease, possibly exonuclease III. Exonuclease III treated DNA is known to be a suitable primer for DNA polymerase II and III (Kornberg and Gefter, 1971). The reason for the differences observed between the crude systems previously described and that used in the present study is unclear, but may result from slight differences in the method of preparation of the extract.

Though DNA synthesis is not detectable in freshly prepared lysates of recB⁻ cells, synthesis was observed in recB⁻ lysates after storage at 4°. It seems probable that this synthesis takes place at primer sites created by limited digestion of the DNA by nucleases other than the recB nuclease during storage. DNA synth-

esis was also observed in the absence of the recB nuclease when partially single-stranded DNA was added to freshly prepared recB$^-$ lysates. This result supports the conclusion that the recB nuclease is required for the creation of suitable primer sites in freshly prepared lysates.

In both cases where synthesis was observed in the absence of the recB nuclease the synthesis was ATP-dependent; this implies that the ATP-dependence of synthesis in rec$^+$ lysates does not simply reflect the requirement for the recB nuclease. It is not clear whether this additional ATP-dependence indicates that the component which is responsible for the ATP-dependence of true DNA replication is required for synthesis in the system studied or whether it results from some additional artefact.

C. DNA Synthesis in Lysates of dna Mutants

Three dna mutants were tested for defective DNA synthesis at 40^o in crude lysates.

The results of such tests on lysates of dna-486E have been discussed above.

No difference was observed between the behaviour of the group B mutant, dna-70B, tested and the dna$^+$ control, indicating that the dnaB gene product is probably not required for synthesis in the lysate.

Though the synthesis observed in lysates of dna-308G appeared to be appreciably more thermolabile than synthesis in lysates of the dna$^+$ control, it is not possible to draw conclusions from these results since the behaviour of the control strain appears to be abnormal.

An in vitro DNA synthesising system the properties of which

closely resemble those of replicative DNA synthesis has been reported by Schaller *et. al.*, (in press). A large number of temperature-sensitive mutants, in which DNA synthesis is arrested immediately on transfer to high temperature, have been shown to exhibit a significantly reduced level of DNA synthetic activity at non-permissive temperatures in this system. DNA synthetic activity at high temperature was found to be restored by the addition of soluble factors in the case of *dnaE* mutants.

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APPENDIX

DNA SYNTHESIS IN LYSATES OF *REC B*$^-$ AND *REC*$^+$ *E.COLI* CELLS

G.W. BAZILL, R. HALL AND J.D. GROSS

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DNA Synthesis in Lysates of *RecB*$^-$ and *Rec*$^+$ *E. coli* Cells

THE isolation by De Lucia and Cairns[2] of a mutant (*pol*$^-$)[1] of *E. coli* that lacks DNA polymerase I has led to the demonstration of *in vitro* DNA synthesis that is independent of this enzyme. Moses and Richardson[2] have observed prolonged and extensive DNA synthesis after treatment of *pol*$^-$ or *pol*$^+$ cells with toluene. This synthesis is strongly dependent on ATP as well as on deoxynucleoside triphosphates. Several groups of workers have observed synthesis in lysates of penicillin or lysozyme-induced *pol*$^+$ spheroplasts[3-5]. The synthesis was short lived but was reported to proceed at a rate of chain growth comparable to the rate *in vivo*, and was interpreted as a limited continuation of real replication at the original growth point. An enzyme, DNA polymerase II, which may be responsible for DNA replication has been extracted and purified from both *pol*$^-$ and *pol*$^+$ cells[6-9]. In purified form, this enzyme does not require ATP. It can repair gaps in DNA which has been made partly single stranded by treatment with exonuclease III, but unlike polymerase I it is not able to act on a nicked, endonuclease-treated DNA primer. Sulphydryl blocking agents inhibit polymerase II, but not polymerase I.

There is good evidence that the DNA synthesis observed in toluene-treated cells corresponds to true *in vivo* DNA replication as it is thermosensitive in several genetically-distinct classes of *E. coli* mutants in which *in vivo* DNA synthesis is blocked at high temperature[2,10,11]. However, similar evidence has not been reported for the short lived DNA synthesis in spheroplast lysates and we now present data indicating that this synthesis does not represent true replication.

Our work was prompted by the observation (Fig. 1*a*) that DNA synthesis in *pol*$^-$ lysates resembles synthesis in toluene-treated cells in that it is almost completely ATP-dependent. We therefore decided to examine more closely the role played by ATP in spheroplast lysates. The synthesis observed by us, as by others, does not require the addition of exogenous DNA (in fact it is somewhat depressed by it), and the product of the synthesis is sensitive to DNAase treatment. We were able to show that the ATP is not required simply to regenerate deoxy-

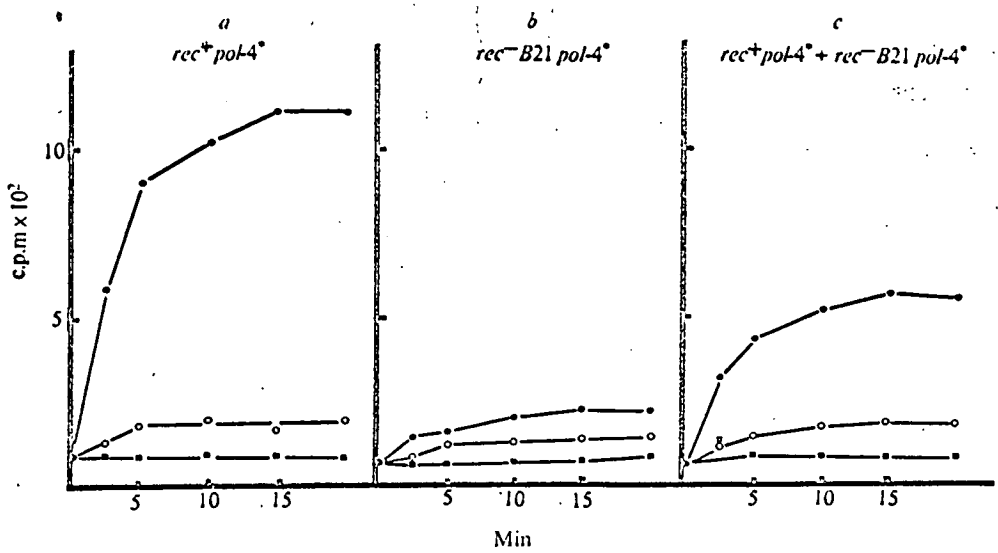

Fig. 1 Log phase cultures (80 ml.) of *E. coli pol-4** and *E. coli pol-4* recB21* were grown at 37° C in Difco L broth to a density of 3×10^8/ml. The bacteria were collected by centrifugation and suspended in 40 ml. of an ice cold solution containing sucrose 0.5 M, KCl 0.1 M, NaCl 0.025 M, Tris-HCl *p*H 8.0, 0.05 M and EDTA 0.001 M. The suspension was centrifuged at 8,000*g* for 10 min and the washed cells were suspended in 2 ml. of 0.5 M sucrose solution containing 0.1 M KCl, 0.025 M NaCl and 0.05 M Tris-HCl, *p*H 8, without EDTA. After 5 min at 37° C the suspension was added rapidly to an equal volume of cold 0.05 M Tris-HCl, *p*H 8.0, containing 100 μg/ml. lysozyme. The mixture was kept at room temperature for 5 min, then at 4° C for 10 min. Microscopic examination showed complete conversion to spheroplasts. Magnesium chloride was added to a concentration of 0.005 M, then 'Brij 58' (0.5%) was added. After 5 min at room temperature the lysate was returned to an ice bath and 0.4 ml. portions in a final volume of 0.5 ml. were used in the assay for DNA synthesis. The composition of the reaction mixture (final concentrations) was: dATP, dGTP, dCTP, 2.5×10^{-5} M; ^{3}HTTP, 10^{-5} M; ATP, when present, 4×10^{-3} M; $MgCl_2$, 0.01 M; KCl, 0.08 M; Tris HCl, *p*H 8.0, 0.05 M. The specific activity of the ^{3}HTTP was 125 Ci/mol. Sodium *p*-hydroxymercuribenzoate, when added, was 1.5×10^{-4} M and incubation was at 30° C. At intervals, 0.05 ml. samples were taken on to paper disks ('Whatman 3MM', 2.5 cm) and immersed immediately in 5% trichloroacetic acid containing 0.01 M sodium pyrophosphate. The disks were washed five times with TCA, twice with ethanol, dried and counted. ●, +ATP; ○, −ATP; ■, +ATP +PHMB.

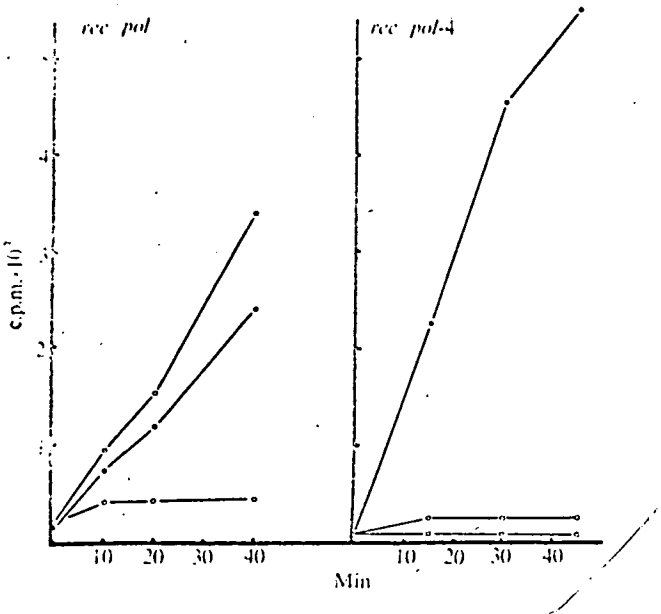

Fig. 2 Log phase cultures (20 ml.) of *E. coli pol-4** and the *pol*⁺ parent strain were grown at 37° C in Difco L broth to a density of 3×10^8/ml. The bacteria were collected by centrifugation and suspended in 1.5 ml. 0.1 M KCl buffered with 0.025 M Tris HCl, pH 7.6. Toluene (0.015 ml.) was added; the suspension was agitated (vortex mixer) for 2 min at room temperature, then left in ice for 20 min; 0.2 ml. portions (in a final volume of 0.25 ml.) were used in the assay for DNA synthesis. The composition of the reaction mixture was as given in Fig. 1, except that $MgCl_2$ concentration was 0.006 M instead of 0.01 M. Pancreatic deoxyribonuclease, when required, was added at 0.4 μg/ml. Incubation was at 30° C. At intervals 0.05 ml. samples were taken on to paper disks and treated as described in Fig. 1. ●, +ATP; ○, −ATP; □, −ATP+PHMB+deoxyribonuclease.

nucleoside triphosphates, because the addition of a small quantity of DNA polymerase I to such a lysate caused extensive synthesis in the absence of ATP (data not shown). An ATP-dependent nuclease which has both exonuclease and endonuclease activity has been shown to be present in lysates of wild type *E. coli* but absent from *recB* and *recC* mutants[12–15]. It seems possible that this nuclease might be acting on endogenous DNA to produce primer sites for DNA polymerase II or for some other enzyme that has more stringent primer requirements than DNA polymerase I. To test this hypothesis, we constructed a strain that lacked the nuclease (which we shall refer to as the *recB* nuclease) as well as DNA polymerase I.

Several workers (see ref. 16) have observed that the combination of *pol-1* with *recB* or *recC* mutations is lethal. The same is true of *pol-4*[17]. We therefore attempted to prepare a *recB*⁻ strain which would lack DNA polymerase I *in vitro* but would have substantial polymerase I activity *in vivo*.

Signer, Cairns and Gross (unpublished results) have observed that many revertants of *pol-4* that regain virtually normal radiation resistance still have negligible DNA polymerase I activity *in vitro*. The characteristics of radiation resistance and lack of polymerase activity *in vitro* are transduced together by P1 lysates grown on such revertant strains, and are linked to *metE*, as are all *pol* mutations. Such revertants do not, therefore, involve an external suppressor mutation, and presumably result from a second mutation within the *polA* gene which compensates for the *pol-4* defect *in vivo*, but not *in vitro*.

To construct the double mutant, we mated a *thy*⁻ strain carrying such a *pol-4* reversion (selected on the basis of resistance to methyl methane sulphonate and designated *pol-4**) with an appropriate *recB*⁻ Hfr strain (JC5412; ref. 21) and selected for transfer of *thy*⁺ to which the *recB* locus is linked. The recombinants that appeared were of two sizes; the small ones proved to be *recB*⁻, the large ones *rec*⁺. One recombinant of each type was retained.

Figs. 1*a* and 1*b* show typical results obtained with lysates of spheroplasts of *recB*⁺ and *recB*⁻ derivatives of the *pol-4** strain. The amount of synthesis observed in the presence of ATP is consistently about ten times lower in the *recB*⁻ derivative (Fig. 1*b*) than in the *recB*⁺ (Fig. 1*a*) and in each case all incorporation is abolished by the sulphydryl blocking agent *p*-hydroxymercuribenzoate (PHMB) indicating that there is no contribution from DNA polymerase I. The small amount of residual synthesis in the *recB*⁻ lysate is partially ATP-dependent while the synthesis in the *recB*⁺ lysate is almost completely ATP-dependent. The addition of 1 part in 15 of the *rec*⁺ lysate (itself contributing negligible DNA synthetic activity) to a *recB*⁻ lysate permitted about half as much synthesis as occurred in the undiluted *recB*⁺ lysate (Fig. 1*c*). This result indicates that a small amount of the nuclease from the *recB*⁺ lysate is sufficient to restore almost full DNA synthetic activity to the *recB*⁻ lysate.

The dependence of DNA synthesis in spheroplast lysates on the *recB* nuclease may be explained in two ways. (1) The synthesis observed in lysates is a true continuation of the process of DNA synthesis *in vivo*, and *recB* nuclease is required for this process. (2) The synthesis in lysates is not a true continuation of *in vivo* replication, but derives from artificial primer sites generated by the *recB* nuclease.

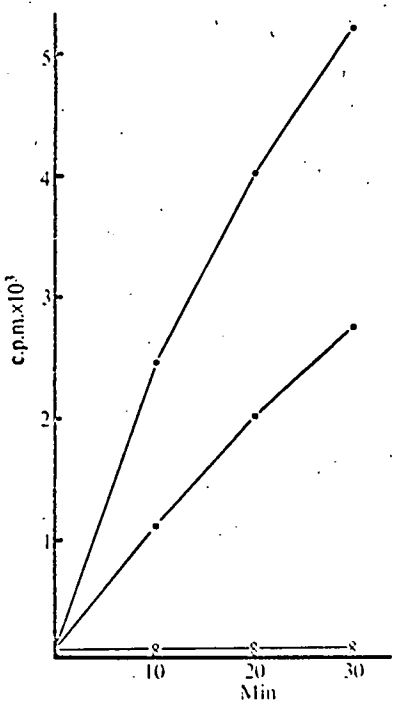

Fig. 3 Cultures of *E. coli pol-4** and *E. coli pol-4* recB21* were treated with toluene and assayed for DNA synthesis as described in Fig. 2. ●, *rec*⁺*pol-4**; ○, *rec*⁺*pol-4**, −ATP; ■, *recB21 pol-4**; □, *recB21 pol-4**, −ATP.

The first hypothesis implies that *recB* nuclease should be required for DNA synthesis *in vivo*. This is clearly not the case, since *recB* and *recC* mutants exist and a strain with a deletion of both genes has been isolated (Karlström, O., personal communication). The inviability of RecB⁻PolA⁻ mutants, however, suggests the further possibility that polymerase I, which has exonuclease activity[19,20], might be able to compensate for the absence of *recB* nuclease. In that case, DNA replication would be possible in PolA⁺RecB⁻ and PolA⁻RecB⁺ bacteria but would not take place in the absence of both polymerase I and the *recB* nuclease. We cannot rule out this hypothesis at present because we have no evidence that the *pol-4** mutation affects the exonuclease activity of polymerase I, but some evidence against it is provided by observations on DNA synthesis in toluene-treated cells. The *pol-4** mutation abolishes detectable synthesis by DNA polymerase I in toluene-treated cells, as well as in cell extracts, but substantial ATP-dependent DNA synthesis nevertheless occurs in toluene-

treated *recB21 pol-4** cells. The absence of polymerase I activity is established by the data in Fig. 2, which shows that toluene-treated *pol*$^+$ cells carry out extensive ATP-independent synthesis in the presence of pancreatic deoxyribonuclease and PHMB, whereas no such synthesis is detected in *pol-4** cells. DNAase treatment has been shown previously to stimulate DNA polymerase I activity in toluene-treated *pol*$^+$ cells, but not in *pol*$^-$ cells[2]. The ability of toluene-treated *pol-4* recB21* cells to carry out ATP-dependent synthesis is shown in Fig. 3. The reduced rate of synthesis is consistently observed and is probably adequately accounted for by the slower growth rate and lower viability of *recB*$^-$ compared with *recB*$^+$ cells. We conclude that DNA chain elongation can occur in the absence of both DNA polymerase I and the *recB* nuclease. It may be noted that even if *pol-4** cells retain polymerase I-associated exonuclease activity, the fact remains that DNA synthesis is observed in the toluene-treated *recB* derivative but not in lysates of this derivative. As the *recB* nuclease is not required for *in vivo* replication but is necessary in lysates, we infer that little if any of the DNA synthesis that we have observed in such lysates corresponds to true *in vivo* replication. It remains to be determined to what extent this is also true of the synthesis observed by other workers.

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