DNA POLYMERASES AND THEIR ROLE IN ISOLATED MOUSE NUCLEI

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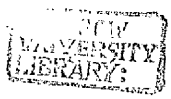
Thesis presented for the degree of

Doctor of Philosophy, University of Glasgow, August, 1974.



ACKNOWLEDGEMENTS

I thank Professor R.M.S. Smellie for providing the excellent facilities for this research, and I am grateful to the Medical Research Council for the provision of a Research Studentship for the duration of the project.

I am especially indebted to Dr. R.L.P. Adams for guidance and advice, always gladly given.

I should like to thank other members of the Institute of Biochemistry for stimulating and helpful discussion and also the staff of the Wellcome Tissue Culture Unit for their assistance throughout the work.

I am also grateful to my wife, Sally for her support and help with the preparation of this thesis, and to Mrs. J.M. Moyes for typing it.

ABBREVIATIONS

These are as laid down in the Biochemical Journal Instructions to Authors (revised, 1972), with the following additions:-

araCTP	-	1-B-D-arabinofuranosylcytoside 5' triphosphate
BHK	-	baby hampster kidney
bisMSB	-	p-Bis (o - methyl styryl) benzene
BSA	-	bovine serum albumin
BSS	-	basic salt solution
DAB	-	N, N-dimethyl-p- (m-tosylazo)-aniline
DNase	-	deoxyribonuclease
DOC	-	sodium 7-deoxycholate
EGTA	-	ethylene glycol-bis-(2-aminoethyl ether) -N,N'-tetracetate
FdU	-	5-fluorodeoxyuridine
log.	-	logarithmically growing
NDPK	-	nucleoside diphosphokinase
NEM	-	N-ethylmaleimide
PAS	-	P-aminosalicylate
PCA	-	perchloric acid
PHA	-	phytohaemagglutinin

PPi	-	pyrophosphate
PPLO	-	pleuro-pneumonia-like organisms
PPO	-	2, 5 diphenyloxazole
RNase	-	ribonuclease
SDS	-	sodium dodecyl sulphate
SV 40	-	Simian Virus 40
TCA	-	trichloroacetic acid

Footnotes:

Unless otherwise stated, all points on figures are an average of duplicate values.

Half shaded symbols represent coincidence of points e.g.

◐ - coincidence of ○ and ◉ .

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I INTRODUCTION

1. DNA REPLICATION

1.1 General

DNA is the genetic material of all known cellular organisms. Some viruses contain only RNA, as well as protein, but it has come to light recently that they may go through a DNA intermediate in order to replicate themselves (Baltimore, 1970; Temin & Mizutani, 1970). In general, for species integrity, daughter cells have been shown to contain a full complement of the parent DNA; whether or not it is necessary for the cells future phenotype is as yet unknown. Therefore prior to division the parent cell must have duplicated its DNA, one copy of which is destined for each daughter cell.

1.2 Gross Structure of Replicating DNA

Meselson & Stahl (1958) were first to present evidence for semi-conservative and sequential replication of DNA in Escherichia coli. This semi-conservative mechanism was later shown for plant, (Taylor et al., 1957) and animal cells (Djordjevic & Szybalski, 1960; Simon, 1961; Prescott & Bender, 1963). Lark et al., (1963) demonstrated that a piece of DNA is replicated at the same time during subsequent DNA synthetic phases confirming the sequential nature.

From autoradiographic experiments, Cairns (1963 a,b) revealed the structure of replicating E. coli DNA. Pulses of less than one generation gave rise to Y-shaped conformations with two legs of equal length generally shorter or longer than the third leg. Longer pulses permitted visualisation of the entire chromosome, which proved to be a closed circle 1100 μm. in length. The distribution of label around the Y-conformation confirmed semi-conservative replication and suggested that it occurred uni-directionally. Early models of

chromosomal growth differed concerning the symmetry of the process (Gilbert & Dressler, 1968; Sueoka & Quinn, 1968). Both assymetric i.e. the two strands behave differently during partition to the daughter molecules, and symmetric replication are found at different developmental stages of the same bacteriophage, or under different conditions in the same bacterium. (Klein & Bonhoeffer, 1972). In all cases investigated there seems to be one defined origin of replication for each replicon (Replicon Hypothesis: Jacob, Brenner & Cuzin, 1963; Dove, Inokuuchi & Stevens, 1971; Caro & Berg, 1968, 1969; Masters & Broda, 1971). The direction of replication from the origin has proved difficult to clarify. Cairns' (1963a) original Y-shaped autoradiographs immediately suggested a uni-directional mechanism, but more recent genetical studies in E.coli, using density labels, (Caro & Berg, 1968), and transducing phage, (Masters & Broda, 1971) to calculate genetic marker frequencies, have provided stronger evidence in favour of general bi-directional replication. Cairns' autoradiographic results can be reconciled, if an assymetric (i.e. the terminus not diametrically opposed to the origin in the chromosome) model is assumed which may be the case. Similar autoradiographic experiments have shown that the DNA of mammalian chromosomes is arranged in the form of long fibres (Cairns, 1966; Huberman & Riggs, 1968). Pulse labelling studies revealed that the newly replicated regions are present as tandemly joined sections, which replicate separately (each region being similar to the individual replicons of bacteria and phage) (Cairns, 1966). Bi-directional synthesis has also been shown for eukaryotic cells (Huberman & Riggs, 1968; Weintraub, 1972). Furthermore, it was shown that most replicons are less than 30μ long (c.f. 1100μ for E.coli), that

neighbouring replicons can begin replication at different times and that there are 2 - 10 x 10^4 replicons per cell.

1.3 Fine Structure of DNA at the Replicating Point

The enigma of how DNA appears to synthesise both new strands simultaneously in the same direction, and the existence of DNA polymerising activities from a variety of sources which can polymerise nucleotides in one direction only (5' → 3'), has given rise to ingenious models as to how DNA is actually made at the replicating point.

Okazaki and his co-workers (Sakabe & Okazaki, 1966; Okazaki et al., 1967; Okazaki et al., 1968a) have provided evidence for a model of discontinuous DNA replication that allows for synthesis of both strands in a 5' → 3' direction. This is accomplished if one, or both, new strands are synthesised in short segments which are subsequently joined to form bulk DNA. Evidence for this derives from the identification of segments (~10s) of single-stranded DNA in growing E. coli cells given very short pulse labels of ^{3}H-thymidine. Further experiments using variable pulse times and selective nuclease degradation, have revealed that the segments are synthesised in a 5' → 3' direction (Sugino & Okazaki, 1972). Such short fragments have been found in several systems (Sadowski et al., 1968; Okazaki et al., 1968a,b) for E. coli and T4 phage; (Ginsburg & Hurwitz, 1970; Tomizawa & Ogawa, 1968) for replicating phage; (Schandl & Taylor, 1969) for Chinese hamster cells; (Sato, et al., 1970) in Ehrlich ascites tumour cells; (Painter & Schaefer, 1969 a,b) in HeLa cells in culture. Stronger evidence for their intermediate role in replication is their ability to be chased into bulk DNA. Werner (1971) working with

E. coli and ^{3}H-thymine instead of thymidine as precursor has produced data to suggest that the short segments only appear after long pulses of ^{3}H-thymine, and that they are not direct replicating intermediates but arise later as a result of endonuclease action as part of the unwinding requirements for replication.

Evidence for a 'knife and fork' model (Richardson, 1969) i.e. continuous 5' → 3' synthesis on one strand accompanied by periodic strand switching and specific endonucleolytic cuts, arises from the finding that very short labelled pulses produce labelled fragments capable of rapid re-annealment, suggesting intra-molecular sequence complementarity (Pauling & Hamm, 1969).

Most of the evidence supports the assumption that DNA chains are growing discontinuously. This appears to be the case for both or only one of the strands depending on the organism. If discontinuous growth is accepted, the problem still remains as to how the short segments are initiated. The 'knife and fork' model, which requires no separate initiation step is rapidly losing support because kinetic studies of the appearance of self-annealing structures are not confirmed by experimental results (Klein & Bonhoeffer, 1972). Nicked parental DNA as a primer as postulated by the model of Haskell & Davern (1969) also has weak evidence in its favour (Geider & Hoffman-Berling, 1971). The weakening of the previous two hypothesis leaves only *de novo* initiation (as yet unable to be achieved *in vitro* by any isolated DNA polymerase) or the use of short oligonucleotide primers. The latter were originally expected to be DNA primers (Schandl, 1972) but more recently model systems have revealed that RNA may

be able to prime *in vitro* DNA synthesis (Keller, 1972). This has gained overwhelming support by the discovery that Okazaki-type fragments, if analysed on formamide-denaturing equilibrium density gradients, have anomalous bouyant density values suggestive of an RNA-linked DNA molecule. Indeed alkali or ribonuclease reduces the density to that expected of DNA (Sugino & Okazaki, 1973; Sugino et al., 1972).

These fragments have also been isolated from eukaryotic systems, Sato et al. (1972) from Ehrlich ascites tumour cells; Waqar & Huberman (1973) from the slime mould *Physarum polycephalum*; Fox et al., (1973) from cultured human lymphocytes; Magnusson, et al. (1973) from polyoma infected cells. Further evidence comes from the finding that RNA polymerase inhibitors such as rifampicin inhibit double stranded M 13 phage DNA synthesis. This effect is not observed in cells containing rifampicin-resistant RNA polymerase (Brutlag et al., 1971). Lark (1972) demonstrated an RNA requiring step for initiation of DNA synthesis in *E. coli*. This RNA was not used for synthesis of protein, as the requirement for protein synthesis preceded the RNA requiring step. The recent discovery of an activity, RNase H, which specifically degrades RNA from an RNA/DNA hybrid (Keller & Crouch, 1972) adds additional support for a mechanism of removal of RNA priming regions.

The finding that it is topologically impossible to separate the two strands of a closed circle without at least one nick brings into light the need for a nuclease activity (whether specific or not is unknown). However, good indications that initiation of replication can occur without an initial endonucleolytic cut have recently emerged. Jaenisch et al.

(1971) for SV40 viral DNA and Kasamatsu et al. (1971) for mitochondrial DNA have isolated intermediates with short newly synthesised pieces attached to still covalently closed circles. These short pieces are shown to be attached to one strand only, the other being displaced as a loop (D loop). This type of intermediate appears to build up until some event, possibly a specific nick, enables it to continue, with subsequent synthesis of the other strand and completion of the molecule (Kasamatsu et al., 1971). These experiments further weaken the involvement of parental nicks as priming sites.

The work of Kornberg on DNA repair and replication implicated the deoxynucleotide 5' triphosphates as the direct precursors for DNA synthesis. (Kornberg, 1969; Lehman et al., 1958). This has been strengthened by the fact that all DNA polymerases isolated to date use the 5' dNTPs as by far their best substrates. However, Werner (1971) put into doubt this basic assumption by showing that in E. coli, using ^{3}H-thymine as precursor, incorporation of label into DNA had reached a steady state before the levels of thymidine mono-, di-, or triphosphate had reached steady state. Rubinow & Yen (1972) have further substantiated Werner's conclusions by re-interpreting his data quantitatively. No further evidence has been forthcoming and kinetic evidence derived from recent in vitro systems have re-established the 5' dNTPs as immediate precursors for DNA replication (Gefter et al., 1971; Friedman, 1974).

1.4 Cellular Organisation of DNA Replication

A short pulse of ^{3}H-thymidine added to an exponentially

growing culture of Bacillus subtilis appearsfirst in the DNA in a membrane fraction and can subsequently be chased into bulk DNA (Ganesan & Lederberg, 1965; Ganesan, 1968). This supports the theory (Jacob et al., 1963) that newly synthesised DNA at the replication point is specifically membrane bound. Proteases and ionic detergents can release the DNA from this binding giving a clue to the nature of the binding. Similar observations have been shown for E. coli, (Smith & Hanawalt, 1965, 1967) and bacteriophages (Sinsheimer, 1959a,b; Knippers & Sinsheimer, 1968; Knippers et al., 1969). Membrane association in bacteria is likely because the membrane plays an important role in daughter chromosome segregation. This has never been shown in eukaryotic systems where a complex segregational apparatus has evolved. However, many investigators have produced autoradiographic evidence that DNA is replicated at the nuclear membrane in eukaryotes (Comings & Kakefuda, 1968; O'Brien et al., 1972). Other supporting evidence is that newly synthesised DNA, on isolation, behaves as if it attached to membranous material (Ben-Portat et al., 1962; Levis et al., 1967; Friedman & Mueller, 1969; Painter & Schaefer, 1969a; Mizuno et al., 1971; Hatfield, 1972). More recent work, both autoradiographic and on isolated pulse-labelled DNA have disputed the earlier work (Habener et al., 1969; Williams & Ockey, 1970; Ockey, 1972; Comings & Okada, 1973; Fakan et al., 1972). The problem of whether newly synthesised DNA is single or double stranded was cleared up by Habener et al., (1969) who suggested that differences observed were probably due to the method of isolation used, and that the in vivo state was probably neither but a destabilised structure. Fakan et al., (1972) reported that the association of newly synthesised DNA with membranous material

may also be an artefact of isolation. They showed that exogenously added labelled single-stranded DNA was recovered in the interphase of a phenol extraction behaving as if it was membrane attached. They argue that newly synthesised DNA is observed in the interphase because of some single-strandedness. In summary, there is no undisputable evidence for the association of DNA replication and the nuclear membrane.

2. ENZYMATIC SYNTHESIS OF DNA

2.1 General

In order to investigate the enzyme activities involved in any process, the integrity of the cell has to be broken. Two methods can be employed to this end.

i Analytical. Mild treatments can be used, doing as little damage as possible so as to preserve the in vivo situation, yet sufficient to make the system accessible to biochemical analysis.

ii Synthetic. Particular enzyme activites can be purified and their role in the prcess under study can be characterised by trying to reconstitute the in vivo situation.

The two approaches have their drawbacks, no more so than in the field of DNA replication, where the discovery and purification of a DNA polymerase from E. coli held back progress as it was assumed to be the enzyme of DNA replication.

2.2 Similarities between in vitro systems and the in vivo situation in prokaryotes

For the study of a limited number of problems, the development of systems with permeable cells has proven fruitful (Vosberg & Hoffman-Berling, 1971; Moses & Richardson, 1970). By using density shift experiments (Geider & Hoffman-Berling, 1971; Burger, 1971) or

or transformation assays with newly made DNA (Matsushita et al., 1971), it has been demonstrated that, in cells made permeable to nucleotides by either toluene or ether treatment, DNA synthesis continues at the in vivo replication point. The main drawback to these systems is that they are not amenable to macromolecular exchange, which is necessary in the analysis of the replication problem. They also exhibit Okazaki fragment formation with conversion of the fragments to bulk DNA (Geider & Hoffman-Berling, 1971). The fragments have also been shown to be RNA-linked in permeable cells (Sugino & Okazaki, 1973).

Evidence that a specific enzyme activity is needed for replication can best be obtained by finding a mutation that causes temperature-sensitivity in replication owing to alteration in this enzyme. Using an in vitro system in which DNA is replicated properly such mutants can help to detect activities involved. Such a system has been developed by Schaller et al., (1972): it is a crude system and contains practically all the proteins of the cell at very high concentration. It is amenable to macromolecular exchange. DNA synthesis in this system has many characteristics typical of DNA replication. It reflects the temperature-sensitivity of many thermo-sensitive DNA-replication-defective mutants. DNA synthesis at restrictive temperatures in the systems prepared from such mutants can be restored by adding wild-type extracts (Klein et al., 1973). This complementation of systems showing impaired synthesis can be used as an assay for proteins which are required for DNA replication. Complementation assays of this type have been used recently to purify several gene products needed for DNA replication in E. coli (Nusslein et al., 1971; Klein et al., 1972; Wickner et al., 1973 a,b).

2.3 DNA polymerases in prokaryotes

Ever since the discovery by Kornberg and his co-workers in the late 1950s of an activity in crude extracts of *E. coli* capable of synthesising DNA *in vitro*, there has been a tremendous effort to elucidate the properties and roles of similar activities from a variety of sources. *E. coli* DNA polymerase was duly purified and its ability to synthesise infective ØX 174 viral DNA *in vitro* was overwhelming evidence of its role as replicase, despite its inability to replicate native DNA *in vitro*. (Goulian et al., 1967). It was not until 1969 when de Lucia & Cairns produced a mutant with very much reduced levels of DNA polymerase activity, yet able to replicate its DNA essentially normally, that a search for other activities began. A second unrelated candidate was found and named DNA polymerase II (Kornberg & Gefter, 1971). Its presence in membrane fractions was good evidence in favour of its role as replicase but no further evidence has appeared, and recently a mutant lacking the enzyme has been isolated (Campbell et al., 1972). It too replicates its DNA normally. A third enzyme, again unrelated, termed DNA polymerase III has proved the most successful candidate primarily because of its temperature-sensitivity in thermosensitive mutants of the dna E type (Gefter et al., 1971). In fact purification of the dna E gene product by the complementation - assay has shown it to be identical with purified DNA polymerase III (Nusslein et al., 1971).

DNA polymerase I plays a major role in excision repair of damaged DNA (Gross, 1972). It also may have a secondary role in replication with the finding that in mutants with low levels of the enzyme, newly synthesised short pieces are much slower in joining to larger DNA, suggesting a role in gap-filling perhaps after RNA

primer removal (Kuempel & Voemett, 1970; Okazaki et al., 1971). The function of DNA polymerase II is at present unknown.

DNA polymerase III differs from the other two polymerases in its low pH optimum, stimulation by ethanol and sensitivity to salt (Kornberg & Gefter, 1972; Otto et al., 1973). None of the enzymes can achieve the replication of native double stranded DNA, nor can any of them initiate chains de novo on single stranded, circular or linear, DNA (Kornberg, 1969; Gefter et al., 1972; Kornberg & Gefter, 1972; Otto et al., 1973). DNA polymerase I is the only one able to use double-stranded DNA with single-strand scissions by displacement of the other strand. DNA polymerases II and III require a gapped template i.e. E. coli exonuclease III treated nicked native DNA. Some of the properties of the three E. coli DNA polymerases are summarised in Table 1.

A modified version of DNA polymerase III has recently been isolated from E. coli (Wickner et al., 1973c). This species, designated DNA polymerase III star, is similar in properties to DNA polymerase III even in being temperature-sensitive in dna E mutants, but it differs in being able to use long single-stranded DNA templates with the aid of a cofactor, copolymerase III star. Copol. III star is unable to aid any of the other three polymerases. DNA polymerase III star behaves similarly to DNA polymerase III on glycerol gradients, but elutes in front of it on agarose gels suggesting assymmetry. Further studies have shown that ATP and copolymerase III star are only required for initiation on the single-stranded templates. Once initiated the reaction is resistant to antibody to copol. III star (Wickner & Kornberg, 1973).

TABLE 1. PROPERTIES OF DNA POLYMERASES OF E. COLI

	I	II	III
Gene	Pol A	Pol B	dna E
Molecular Weight	109,000	90,000-120,000	140,000
Molecules/cell	400	20	10
Rate of DNA synthesis (dNTPs/min/molecule)	1,000	300	15,000
Direction of synthesis	5'→ 3'	5'→ 3'	5'→ 3'
Associated nuclease	5'→ 3' 3'→ 5'	3'→ 5'	3'→ 5'
Reference	Kornberg (1969)	Wickner et al. (1972b)	Kornberg & Gefter (1972) Otto et al. (1973)

2.4 Other factors involved in prokaryotic replication

Genetic studies have revealed many mutations known to affect DNA replication. In bacteriophage T4, the products of three genes necessary for replication have been identified. Gene 43 has been shown to be DNA polymerase (Speyer et al., 1966; Speyer & Rosenberg, 1968). Gene 30 is a T4-induced ligase (Fareed & Richardson, 1967), and gene 32 is a protein which preferentially binds to single-stranded DNA and may be involved in strand separation at the growing point (Alberts & Frey, 1970). A complex of the products of genes 44 and 62 from T4 has been purified but their function is unknown (Barry & Alberts, 1972). In E. coli several mutants of DNA replication have been described. So far only DNA polymerase III and a ribonucleotide reductase have been characterised (Nusslein et al., 1971; Fuchs et al., 1972). Other proteins have been purified by complement assay, but functions for them are still being sought (Wickner et al., 1973a,b). Using a synthetic approach, several groups have isolated and described protein factors capable of stimulating nucleotide incorporation in a system containing activities purified from crude extracts of E. coli shown to be required for duplex formation of single-stranded fd DNA. The system contained DNA polymerase III, RNA polymerase, DNA unwinding protein, fd DNA, rNTPs and dNTPs (Wickner et al., 1972a; Hurwitz et al., 1973; Hurwitz & Wickner, 1974). An activity similar to T4 gene 32 protein has been isolated from E. coli but it surprisingly stimulates only nucleotide incorporation with DNA polymerase II and not III (Sigal et al., 1972). The role of E. coli ligase in replication is still unclear. Wang (1971) has isolated a protein from E. coli, the ω protein, capable of relaxing supercoiled DNA, an eventual prerequisite

for strand separation. No role for any known E. coli nucleases in the process of chain elongation has been established so far. Newly isolated mutants deficient in exonuclease I (Kushner et al., 1971) or exonuclease III (Milcarek & Weiss, 1971) do not appear to be affected in their viability. In bacteriophage λ, two genetic functions O and P are believed to be involved in initiation of DNA replication (Dove et al., 1971). Earlier indications that these two functions control an endonuclease have been confirmed (Shuster & Weissbach, 1969; Freufelder & Kirschner, 1971).

2.5 Studies on nuclei isolated from eukaryotes

Incorporation of nucleotides into endogenous DNA by isolated nuclei has been reported from many eukaryotic sources including cultured HeLa cells (Friedman & Mueller, 1968; Kidwell & Mueller, 1969; Hershey et al., 1973 a,b.; Bernard & Brent, 1973); cultured mouse fibroblasts (Kemper et al., 1969; Kidwell, 1972); isolated rodent tissues (Thomson & McCarthy, 1968, 1973; Lynch et al., 1970, 1972; Ove et al., 1971; Waqar et al., 1971; Kaufman et al., 1972; Probst et al., 1972; Shimada & Terayama, 1972; Hyodo & Ono, 1970 a,b); Ehrlich ascites tumor cells (Teng et al., 1970); cultured baby hamster kidney cells (Lazarus, 1973); and in polyoma-infected fibroblasts (Winnaker et al., 1972).

2.6 Effect of isolation procedures on correlation between in vivo and in vitro systems in eukaryotes

Hyodo & Ono (1970 a) and Kaufman et al. (1972) stressed the danger of introducing artefactual or unschedueled DNA synthesis into nuclei as a result of maltreatment during isolation. Hyodo & Ono (1970a) showed that preparation of nuclei using hypertonic sucrose solutions (2.2-2.4M) resulted in nuclear DNA synthesis which did not

reflect the *in vivo* state of the cells from which they were isolated, whereas the use of isotonic sucrose (0.25M) did. Also the increased rate of incorporation was more evident after 20 min incubation when the incorporation by isotonically-prepared nuclei from regenerating liver had levelled off. Kaufman et al. (1972) reported autoradiographic evidence for incorporation of labelled triphosphates by 85% of normal and regenerating nuclei isolated by a hypertonic sucrose preparation. In contrast, Lynch et al. (1970) showed only 15% of regenerating liver nuclei incorporated *in vitro* which was similar to the number incorporating *in vivo* before isolation. A control experiment revealed that the same 15% of nuclei were incorporating *in vivo* and *in vitro*. These workers use a hypertonic sucrose isolation technique but under milder conditions. Several groups showed that nuclear DNA synthesis did correlate with the DNA synthetic capacity of the cell of origin: Lynch et al. (1970), in regenerating liver; Friedman & Mueller (1968), in amethopterin-reversed HeLa cells; Bernard & Brent (1973), in mitotic selection synchronised HeLa cells; Lazarus et al. (1973), in BHK cells entering stationary phase of growth; Shimada & Terayama (1972), in developing rat brain. Many workers (Lynch et al., 1970, 1972; Hershey et al., 1973a; Kidwell & Mueller, 1969) present further evidence that isolated nuclei continue *in vivo* replication by the finding that the bulk of ^{3}H-TTP incorporated *in vitro* is associated with a bromodeoxyuridine (BrdUrd) prelabel prior to isolation, on CsCl density gradients. Kaufman et al. (1972) show that only about half of the label is associated with the dense peak confirming their system is not only a continuation of *in vivo* DNA synthesis. In general prolonged subjection to hypertonic sucrose solutions during nuclear isolation tends to

induce unscheduled DNA synthesis which is not a reflection of the DNA replicative ability of the cell of origin. Kidwell (1972) has shown that nuclei isolated during that part of the S phase when mouse fibroblast cells are making satellite DNA preferentially incorporate ^{3}H-TTP into satellite DNA *in vitro*. Similarly those not engaged in satellite synthesis do not. Isolated nuclei incorporate added ^{3}H-TTP preferentially into short segments similar to those found *in vivo* (Friedman, 1973; Lynch et al., 1972; Kidwell & Mueller, 1969).

2.7 Properties of nuclear systems

Most workers have shown an absolute requirement for the 4 deoxynucleoside triphosphates and Mg^{2+}. The role of ATP seems to be variable, the stimulation varying between zero (Kaufman et al., 1972) and an absolute requirement (Probst et al., 1972; Bernard and Brent, 1973) but in general a 2 - 5 fold stimulation is found. Friedman (1973) finds an initial ATP independent step; all label after an *in vivo* BrdUrd prelabel being dense. With ATP present 30 - 50% appears as light material. He interprets this as ATP being necessary for new strand initiation whereas incorporation in the absence of ATP is a continuation of chains already growing *in vivo*. Sulphydryl reagents generally stimulate incorporation as does EDTA. The pH optimum is to the alkaline side of neutrality as in most DNA polymerases characterised. Salt effects are variable, the majority of workers reporting 100 mM NaCl as optimum. Lynch et al., (1972) improved their assay conditions by showing that EGTA, a calcium ion binder, not only stimulates the initial rate but prevents DNA breakdown during incubation (Hyodo and Ono, 1970b), by presumably inhibiting Ca^{2+}-activated nucleases. Also high molecular weight dextrans had a stimulatory effect by preventing nuclear swelling. Ove et al. (1971) showed that sucrose

at a concentration of 0.8M also prevented nuclear swelling but in doing so increased *in vitro* incorporation of normal liver nuclei to the level of regenerating nuclei, which does not reflect their *in vivo* state before isolation. Cadaverine also increased *in vitro* incorporation (Lynch et al., 1972) its action being to preserve the nuclear morphology (McGregor and Mahler, 1967).

The amount and extent of incorporation varies from group to group. Most workers achieve reasonable rates of incorporation with initial rates approaching 10 - 50% of *in vivo* rates under optimal conditions (Hershey et al., 1973a; Lynch et al., 1972). A falling-off in rate after 5 - 30 min. is also observed by the majority of workers although one group (Probst et al., 1972) report a linear rate for 2 hours. This levelling cannot be attributed to exhaustion of some added assay requirement as fresh nuclei can use spent assay mixture as efficiently as fresh mixture (Lynch et al., 1972; Hershey et al., 1973a; Bernard and Brent, 1973). It is also not due to completion of DNA sites available as the rate change is not delayed when differing initial rates are induced by altering the incubation temperature (Lynch et al., 1972). Results of Lazarus (1973), suggest that available DNA sites are exhausted in his nuclei, because inclusion of all four ribonucleotide triphosphates allows a linear rate up to two hours suggesting continued priming of new sites by RNA (see page 6). Fresh assay mixture or cytoplasm did not prolong initial rates after levelling off (Bernard and Brent, 1973), therefore probably due to some limiting nuclear factor.

2.8 Effect of cytoplasm on incorporation by isolated nuclei

The use of cell lysates or the mixing of cytoplasm with nuclei have shown that cytoplasm contains factors capable of

stimulating incorporation by isolated nuclei (Friedman and Mueller, 1968; Mueller, 1969; Kidwell and Mueller, 1969; Bernard and Brent, 1973). Friedman and Mueller (1968) showed that the cytoplasmic factor is sensitive to sulphydryl inhibitors but it does not have a controlling effect as it failed to stimulate incorporation in nuclei not synthesising DNA *in vivo*. Later work by the same group (Hershey et al., 1973a) revealed the factor to be macromolecular (> 100,000) but separable from cytoplasmic DNA polymerase on gel filtration. It is heat labile and susceptible to extremes of pH, all pointing to a protein nature. Purified fractions of DNA polymerase activity failed to stimulate incorporation in their isolated nuclear system. The factor binds strongly to DNA cellulose columns not being eluted until 2M salt. Bernard and Brent (1973) found their cytoplasmic factor to be heat labile and pronase-sensitive but that it could be replaced by purified calf thymus DNA polymerase in contrast to the findings of Hershey et al. (1973a). By prelabeling the DNA with BrdUrd at certain selected times during S-phase, Hershey et al. (1973a) showed that the cytoplasmic factor was not initiating new sites that were not active *in vivo* prior to nuclear isolation. Kidwell (1972) also showed this with L cell satellite DNA. These experiments suggest an ordered replication of DNA *in vitro* as *in vivo* (Barlow, 1972). Other workers have found a low M.W. heat stable molecule capable of stimulating *in vitro* incorporation: Thomson & McCarthy (1968; 1973) in taper mouse tumour cytoplasm; Shimada & Terayama (1972) in infant rat brain cytoplasm; Erhan et al. (1970) in HeLa cells.

2.9 Controlling effect of cytoplasm

Rao & Johnson (1970) showed that DNA synthesis and mitosis are induced in multinucleate HeLa cells formed by fusion between

cells at different phases of the cell cycle. Cells in the G1 phase of growth initiate DNA synthesis earlier when fused to a cell in the DNA synthetic or S-phase. This displays a dosage effect i.e. the G1 nucleus of a tri-nucleate cell with two S-phase nuclei initiates even eariler. This finding prompted workers to look for cytoplasmic controls over initiation and continuation of DNA replication. Thomson & McCarthy (1968, 1973) claim to have found a factor in tumour cytoplasm capable of initiating DNA synthesis in previously dormant nuclei. However most other cytoplasmic factors only stimulate incorporation at sites in nuclei already incorporating in vivo. Kumar & Friedman (1972) provided autoradiographic evidence to suggest that cytoplasm from late G1 or early S is capable of inducing late G1 nuclei to enter S in an in vitro system. The activity is heat labile and partially lost on dialysis, in contrast to that of Thomson & McCarthy (1973).

2.10 DNA Polymerases from eukaryotes

2.10 i Background

Two kinds of deoxynucleotide polymerising activities have been isolated from eukaryotic systems; terminal deoxynucleotidyl transferase and replicative deoxynucleotidyl transferase (DNA polymerase). The terminal transferase from calf thymus glands was characterised by Kato et al. (1967) and purified to homogeneity (Chang & Bollum, 1971a). The terminal transferase is a low M.W. enzyme (32,460), is found only in the thymus gland, and has a developmental cycle in the thymus during embryonic growth (Chang, 1971). The replicative DNA polymerase was first observed in 1957 (Bollum & Potter, 1957) and shown to be present in the cytoplasmic-soluble fraction of regenerating rat liver (Bollum & Potter, 1958). A search for a similar activity from other sources revealed a surprising enigma. In most cases

studied, 80% of the total cellular DNA polymerase was found in the cytoplasmic fraction, whereas the DNA, the enzyme's template and product, was known to lie almost wholly in the nucleus. This finding prompted suggestions of nuclear damage leading to leakage and although alternative preparative techniques including inclusion of calcium ions (Main & Cole, 1964) known to stabilise nuclear membrane, and use of non-aqueous isolation techniques (Keir et al. 1962) improved the nuclear activity yield, the bulk of the activity was still found in the soluble fraction of the cell. (For review of pre- 1965 findings, see Keir, 1965). Another general finding, which was in favour of the lack of involvement of this activity, or at least this form of the enzyme, was its preference for denatured DNA over native DNA as a template. Thus began the search for an activity with a more favourable intracellular location and template preference. Such an activity was found by several investigators; Mantsavinos (1964) for regenerating rat liver; Mazia & Hinegardner (1963) for sea urchin embryos. The obsession with purification of an enzyme with such characteristics hampered for some time a true appraisal of the situation relating DNA polymerase activity to the DNA replicative state of the source under study.

2.10 ii Multiple forms of DNA polymerases in eukaryotes and the effect of isolation conditions on their subcellular location.

In general, the major DNA polymerising activities found in most eukaryotic sources studied is a large (> 100,000 daltons) denatured DNA-preferring species found predominantly in the cytoplasmic-soluble fraction and first characterised as a distinct species by Yoneda & Bollum (1965), and a small (< 100,000 daltons)

native DNA-preferring species found predominantly in nuclear fractions. A survey of eukaryotic DNA polymerases characterised to date is shown in Table 2. Most of the groups quoted in Table 2 have attempted to characterise all of the activities present in the cell. The appearance of both species in either the soluble or nuclear fractions has been reported by several groups (see Table 2). The smaller species is found in soluble preparations always as the minor activity and could be due to nuclear leakage, or to a real physiological effect. Baril et al. (1971) find it attached to a ribosomal fraction in rat liver. Brun et al. (1974) find a dimeric form (50,000 daltons) of a cytoplasmic monomer (25,000) in their nuclei. Chiu & Sung (1972a) show that its level in the soluble fraction of rat brain cerebral cortex decreases after birth paralleling the fall in DNA synthetic capacity of that tissue. Further studies have shown that it becomes particulate (Chiu & Sung, 1972b), the significance of which is unknown. The reverse situation, that of finding the large species in the nucleus may have deeper implications. The obvious explanation is that it is a result of cytoplasmic contamination, which is the belief of Chang & Bollum (1972a) who go to great lengths to purify their nuclei free of it. Other groups, (Weissbach et al., 1971 ; Ove et al.; 1973; Sedwick et al., 1972; Wallace et al., 1971) find significant levels of it in nuclear fractions, in fact Sedwick et al. (1972) suggest that the large nuclear species may be different from the cytoplasmic one in its ability to use a native DNA template with large gaps in it. It is interesting to note the correlation of the similarity of techniques used to 'purify' nuclei to look at their endogenous DNA synthetic capacity and the DNA polymerases they contain. Use of hypertonic sucrose isolation media reduces the correlation of

TABLE 2. LARGE AND SMALL DNA POLYMERASES IN EUKARYOTIC CELLS

SOURCE	ESTIMATED M.W.		NUCLEAR (N) OR CYTOPLASMIC (C)	
	LARGE	SMALL	LARGE	SMALL
Rat liver	$2x10^5$	-	C	C
Rat liver & hepatomas	$2x10^5$	$6x10^4$	C	C
Rat brain	$2x10^5$	$3-4x10^4$	C	N&C[a]
Rat liver & hepatomas	$2.5x10^5$	$2.5x10^4$	C&N[b]	N&C[c]
Rabbit tissues	$1x10^5$	$3-4x10^4$	C&N	N&C
Several tissues	$1x10^5$	$4.5x10^4$	C	N&C[a]
HeLa cells	$1.5x10^5$	$5x10^4$	C&N	N
Rat liver	$4x10^5$	$6.5x10^4$	C	N&C
Rat tissues	$2x10^5$	$1x10^5$	C&N	N&C[a]
PHA stimulated lymphocytes	$1.5x10^5$	$3x10^4$	-	-
KB cells	$1.2x10^5$	$4x10^4$	C&N	N
Chick embryo	$1.4x10^5$	$2x10^4$	C	N
Calf thymus	$2x10^5$	$1.5x10^5$	-	-
Rat liver & hepatomas	$1.5x10^5$	$3-4x10^4$	C&N	N&C[a]
Human bone marrow	$1.5x10^5$	$4.5x10^4$	C	N
Chick embryo	$1.5x10^5$	$5x10^4$ $2.7x10^{4e}$	C	N&C[e]

KEY: a - cytoplasmic traces
b - in regenerating liver
c - ribosome bound
d - nuclear species prefers *E. coli* exonuclease III treated activated DNA
e - nuclear species is a dimer of cytoplasmic

DNA TEMPLATE PREFERENCE		REFERENCE
LARGE	SMALL	
Denatured	Native	Bellair (1968)
Denatured	Native	Iwamura et al. (1968)
Denatured	Native	Chiu & Sung (1971, 1972a,b)
Denatured	Native	Baril et al. (1970, 1971, 1973)
Activated	Activated	Chang & Bollum (1971b)
Activated	Activated	Chang & Bollum (1972a)
Activated	Activated	Weissbach et al. (1971)
Activated	Activated	Haines et al. (1971)
Denatured	Native	Wallace et al. (1971)
Activated	Activated	Smith & Gallo (1972)
Activated[d]	Activated	Sedwick et al. (1972)
Denatured	Native	Wicha & Stockdale (1972)
Denatured	Denatured	Komparler et al. (1973)
Denatured C Native N	Native	Ove et al. (1973)
-	-	Coleman & Hutton (1973)
Denatured	Native	Brun et al. (1974)

in vitro to in vivo synthesis of the nuclei (see page 14) and also depletes them of their large DNA polymerase species.

The large and small species are similar in some respects. They are both replicative i.e. they require all four dNTPs and DNA for maximum activity. Several groups report that the small species is capable of significant incorporation (40 - 60%) with less than four triphosphates. Sedwick et al. (1972) examined this phenomenon and revealed that it is probably due to the incorporation of only a few nucleotides at each point of incorporation. The reason for this limitation is not clear. It could be due to the inability to displace the other DNA strand at a nick or inability to replicate long single-stranded regions, a general finding for the small enzyme. The magnesium and pH optima of the two species vary but this may be due to the template used in the assay rather than a difference of physiological significance (Chang & Bollum, 1971b). Their response to salt also varies from source to source, the large species being generally more sensitive to high levels of salt in the assay. The polynucleotide best used as template may give an insight into the DNA structure used by the enzymes in vivo. The ability of the small enzyme to copy RNA templates has been reported by several groups (Stavrianopolous et al, 1972a,b; Chang & Bollum, 1972a; Haines et al., 1972 and Brun et al., 1974). This effect may be due to a less stringent demand on template sugar recognition by this enzyme compared to the larger species. The significance of this 'reverse transcriptase' type activity may not be valid, especially as distinct RNA dependent-DNA polymerases have been isolated from normal, uninfected cells. Furthermore the enzymes do not have any DNA template dependence (Ward et al., 1972; Bobrow et al., 1972; Fridlender et al., 1972). Chang &

Bollum (1972b) have shown in model studies that the large enzyme is capable of using oligoribonucleotide as well as oligodeoxyribonucleotide as primers for incorporation whereas the smaller enzyme is more selective, using only deoxy-primers. This points to a replicative role for the large species, being able to elongate RNA primed Okazaki pieces (see page 5).

Apart from size the two species have other differences. The larger species is inhibited more strongly by sulphydryl inhibitors than is the small species (Baril et al., 1971, 1973; Haines et al., 1971; Weissbach et al., 1971; Smith & Gallo, 1972). Their chromatographical behaviour suggests that at physiological pH, the smaller species is more negatively charged than the larger one, and in fact several groups have found the larger enzyme to have a low affinity for DNA either in DNA cellulose columns, (Haines et al., 1971) or in sucrose gradients (Yoneda & Bollum, 1965) under a variety of salt conditions, which may explain the ease with which it may be washed out of nuclei during more stringent nuclear purification techniques (DNA polymerase III from E. coli has been shown to have a low binding affinity for DNA).

2.10 iii Association of DNA polymerase activity with membranous material

Evidence supporting association of DNA polymerase with the nuclear membrane is circumstantial in nature. Yoshida et al. (1971) isolated a nuclear membrane fraction from calf thymus containing DNA polymerase activity but on washing to remove residual DNA virtually all of the activity was lost. A similar preparation was isolated by Yoshikawa-Fukada & Ebert (1971) who showed that when the membrane-chromatin complex was allowed to carry out the polymerising reaction

with added precursors the polymerase was released from the complex. When a calculation is made of the percentage activity associated with the membrane (Kay et al., 1972) it seems to correlate with the percentage DNA isolated with the membrane.

Baril et al. (1970, 1971) using normal and regenerating rat liver showed that virtually all of their soluble DNA polymerase activity (after removal of nuclei, mitochondria and microsomes) was associated with smooth membrane, as suggested by its ability to be sedimented by prolonged centrifugation and to band in a discontinuous sucrose gradient at the density of smooth membranes. In contrast, Poulson et al. (1973) using similar fractionation techniques with rat intestinal mucosa, showed that most of the soluble activity was not membrane associated and the little that was, was shown to be of mitochondrial origin. Burriss-Garrett & Bollum (1973) show that under their conditions the soluble activity from rat liver was not associated with particles with M.W. greater than 500,000 daltons, and that ability to find it associated with such large particles may be an artefact of a concentrative method of preparation. Furthering Baril's work, Novak and Elford (1973) have shown that a similar smooth membrane fraction from rat tissues and hepatomas contains a small amount of DNA which is capable of stimulating the endogenous synthetic ability of the smooth membrane preparation but such DNA can only be isolated from sources active in DNA synthesis. More recently Westergaard & Johnson (1973) have isolated what they term a replication complex from ethidium bromide treated Tetrahymena pyriformis. It contains a DNA polymerase, a RNA polymerase, a deoxyribonuclease and a RNA-linked DNA fragment similar in size to the RNA-primed Okazaki pieces in E. coli. (see page 5).

2.10 iv Inter-relationship between the large and small DNA polymerases

The first suggestion of an inter-relationship between the two polymerases was put forward before they had been properly characterised as being distinct. Keir (1965) proposed a theory based on the evidence at the time that the denatured DNA-preferring activity found in cytoplasm was an 'altered' form of the native-preferring 'intact' form found increasingly in rapidly proliferating tissues. With the discovery of their M.W. difference attempts were made to reduce the large species to an activity resembling the smaller species. Bellair (1968) demonstrated the presence of two DNA polymerase activities when he chromatographed a rat liver soluble preparation on Sephadex G-200 run in 1M NaCl. The major activity eluted in the void volume (200,000 daltons) and preferred denatured DNA as template; the minor activity eluting later (100,000 daltons) and preferring native DNA. In preparations run without salt all of the activity eluted in the void volume. In contrast, later studies by Furlong & Gresham (1971), using Walker 256 rat hepatoma, showed that the M.W. of the cytoplasmic enzyme could not be reduced by 1M NaCl, nor DNase nor RNase suggesting that it is not association with a small oligo-nucleotide primer which gives it its preference for denatured DNA. Furthering this work Holmes & Johnston (1973), using a partially purified soluble enzyme from rat liver, also showed that its size was not reduced by salt concentrations up to 1M NaCl. They also showed no reduction by Brij 58, a non-ionic detergent capable of dispersing lipid, ruling out residual membrane attachment. Glycerol gradients revealed a smaller M.W. than agarose gel chromatography suggestive of assymmetry. (c.f. E. coli polymerase III star, page 11),

Holmes et al., (1973) improved their purification and showed that a band of M.W. 54,000 was prevalent on sodium dodecyl sulphate (SDS) gels of their purest fractions. The enzyme would not enter their gels under non-denaturing conditions.

Recently Hecht (1973a,b) using mouse testes, and Lazarus & Kitron (1973) using baby hamster kidney cells, have managed to achieve production of a small active species comparable to the nuclear one from several sources, by treating a large soluble species with salt (0.125M NH_4Cl by Hecht, 0.5M NaCl by Lazarus & Kitron). Further, Hecht (1973b) has perhaps revealed the in vivo conversion point by showing that nuclear membranes fractions contain both activities as well as an intermediate-sized activity. Chang & Bollum (1972c) have shown that the large and small polymerase species from a variety of mammalian sources are susceptible to antibody created against the large species purified from calf thymus gland. E. coli DNA polymerases I and II and calf thymus terminal transferase are not affected. This suggest a relationship not only between the large and small species but also interspecies similarities. The cross-reaction could be due to common sites which are important for enzyme activity in all the species rather than an extensive structural relationship.

2.10 v Variation in DNA polymerase activity with differences in in vivo DNA synthetic rates.

Before this section is embarked upon distinction must be drawn between the variation in DNA synthesis due to the existence of DNA synthetic and non-synthetic phases within the growth cycle of an average dividing cell, and the variation due to leaving or entering this cycle, which manifests itself in many ways including cell differentiation (developing tissues) and the induction to growth

of quiescent systems by chemical or physical means. Into the latter category comes regenerating liver, stimulation of lymphocytes by phytohaemagglutinin (PHA) and dilution of contact-inhibited cells in culture. Also in this category comes the phenomenon of tumour formation, which can be either natural or artificially induced. Investigations into the synthesis of DNA and its control in such tissues can be useful in determining what has gone wrong in a cancerous condition.

(a) Variation in DNA polymerase with cell differentiation

As cells differentiate they normally reduce their proliferative ability and therefore DNA synthesis and cell division decline in importance as the cells concentrate on their differentiated function. O'Neill & Strohman (1969, 1970) have shown that when chick embryo muscle cells fuse to become mature multi-nucleate cells, their DNA synthetic capacity is reduced. They also showed that they lose 90% of their soluble DNA polymerase activity. Wicha & Stockdale (1972) have demonstrated that 90% of that activity is the large denatured DNA-preferring species. Ove et al. (1970), studying the development of rat liver from 6 days before to 10 days after birth reveal a correlation between fall in DNA synthetic ability and the large soluble species. Chiu & Sung (1972a,b) used two different areas of rat brain which develop at different times relative to birth. Cerebral cortex is almost completely developed at birth whereas the cerebellum grows rapidly until the 6th day after birth and then declines. In the cerebral cortex the large soluble species which is high in the foetus falls to a low level after birth but in the cerebellum it peaks at 6 days then decreases to an almost undetectable level in the adult. During these changes the small species varies only slightly

in both tissues and in fact is shown to become particulate as the rat develops towards adulthood, the total cellular amount remaining constant (Chiu and Sung, 1972b).

(b) DNA polymerase changes in induced systems and tumours compared with the quiescent state

There are probably many secondary control differences between induced systems such as regenerating liver and tumour cells but for the purpose of this study they will be grouped under the same category as having been stimulated into a proliferative state from a previously quiescent one. Many investigators have established that when liver is made to regenerate by partial hepatectomy, its soluble DNA polymerase is higher (Bollum & Potter, 1958). Many groups have since shown this increase to be due to the large species (Iwamura et al., 1968; Ove et al., 1969; Baril et al., 1971, 1973; Chang & Bollum, 1972d; Novak & Elford, 1973). Workers looking at nuclear activities in regenerating liver report contrasting results. Where only one activity, the small species, has been found in nuclei no growth response is detected (Chang & Bollum, 1972d). However Chiu et al. (1973) have shown a growth-responsive increase in a small tightly bound species. Association of the large species with the nucleus during regeneration has been reported (Wallace et al. 1971; Baril et al., 1973), although Wallace et al. were able to wash their nuclei free of it agreeing with Chang & Bollum (1972a) that it may be increased cytoplasmic contamination. Lynch & Lieberman (1973) do not size their activities but suggest that an activity which remains behind after extraction of nuclei by the non-ionic detergent Brij 58 increases during regeneration, and in fact after solubilisation this residual activity is subject to similar inhibition by araCTP as is the endogenous DNA synthesising activity

of isolated regenerating nuclei. In contrast the Brij-extractable activity is not. Chang et al. (1973) working with cultured mouse L cells show that as the cells enter and leave the stationary phase of growth, it is the large soluble species and not the small nuclear one which fluctuates with DNA synthetic differences. Similar correlations of large polymerase enzyme with growth differences are seen in tumours (Iwamura et al., 1968; Baril et al., 1971, Ove et al., 1969, 1973; Novak and Elford, 1973); however Ove et al., (1973) in hepatomas and Chiu et al., (1973) in DAB induced tumours also find a small tightly bound species which is higher in tumours. Chiu et al. (1973) also find a large species appearing in the nuclei of their DAB induced tumours. This enzyme appears to prefer a polyribonucleotide template. An interesting finding by Springgate and Loeb (1973) is that of a DNA polymerase from leukemic cells which makes more mistakes during incorporation using a homopolymer template than that from normal leucocytes. The implications of this in tumour progression are profound.

(c) Cell cycle variations of DNA polymerase activity and intracellular location

In order to study the variation in any parameter throughout the cell cycle the population of cells under study must be synchronised so that they are all at the same stage at the same time. Cell synchrony is only possible where most uncontrollable external influences have been removed so that once they have been aligned they will not become too asynchronous before they can be studied. Hence the invaluable contribution of cell culture techniques to this sort of study. Cell culture enables a uniform population of cells to be manipulated under the control of the investigator.

Disadvantages are the unknown effect of prolonged sub-culturing on the stability of the culture and the degree of correlation which can be made with the tissue of origin. Methods of inducing synchrony employed are based on using drugs to inhibit a particular step i.e. DNA synthesis or cell division, to enable all the cells to accumulate at the inhibition point. Varying degrees of synchrony can be obtained with these techniques but the danger is that the cells develop unbalanced growth and the results gained from such experiments must be interpreted with caution. A certain degree of synchrony can be obtained by releasing cells from a quiescent state but a study of the first cell cycle is complicated by overall growth increases. Preferred are those techniques which minimally disturb the system such as selection, by physical means, of cells at a particular stage such as mitosis (easily removed by shaking from a monolayer) or early interphase (separation on sucrose gradient).

The first cell cycle studies on DNA polymerase were done using the DNA synthesis inhibitor fluorodeoxyuridine (FdU). Littlefield & McGovern (1963) and Gold & Helleiner (1964), using thymidine reversed FdU inhibited mouse L cells, both showed that, as DNA synthesis was allowed to continue, soluble DNA polymerase activity fell and particulate (nuclear) activity rose. Lindsay et al. (1970) confirmed this using aminopterin inhibited mouse L929 cells, and also showed that the increasing particulate activity had a template preference similar to the soluble enzyme. They also showed that the nuclear activity declined after S-phase. A more recent study by Furlong et al. (1973) has supported these findings. They used hamster fibroblast cells in culture, which had been selectively removed at metaphase following a brief exposure to the mitotic inhibitor,

colcemid. A reproducible fraction (5-10%) of the DNA polymerase activity was found to remain firmly bound to the nuclear pellet after repeated cycles of freezing and thawing, which were shown to remove contaminating cytoplasmic marker enzymes. The specific activity of this firmly bound nuclear DNA polymerase was found to increase during S-phase in proportion to DNA synthesis. On solubilisation it was found to be large and to prefer a denatured DNA template. The initial soluble activity and that solubilised by freeze-thawing did not increase during S-phase. Madreiter et al. (1971) synchronised L cells using the mitotic selection method and also found an increase in particulate-associated activity during S-phase. Their soluble activity results were less conclusive. Schindler et al., (1972) showed little difference in total cell homogenate activity as the cell cycle progressed in mitotically selected murine mastocytoma cultures. On studies using nuclei capable of in vitro DNA synthesis (Friedman and Mueller, 1968), Friedman (1970) reported that these nuclei isolated from amethopterin-inhibited thymidine-released HeLa cells contain a denatured-preferring DNA polymerase activity which remained high in S-phase. The soluble activity remained constant until after S-phase when it rose. Later kinetic studies on the solubilised activity were consistent with there being two polymerase species (Friedman, 1969).

The foregoing reports have suggested that a denatured DNA-preferring soluble-like (possibly large) species is associated with nuclei during S-phase. No evidence is presented to suggest whether it is preferential synthesis of nuclear-associated enzyme or translocation of pre-existing cytoplasmic enzyme. Using the naturally synchronous divisions of early developing sea urchin embryos, Loeb and his co-workers have resolved this point, at least for their system. During

early development, when rapid DNA synthesis is going on, sea urchin embryos appear to redistribute their DNA polymerase activity from cytoplasm to nucleus (Fansler & Loeb, 1969). Later, Loeb and Fansler (1970) demonstrated that there is no preferential synthesis of DNA polymerase compared with total cellular protein of which there is very little turnover (Fry & Gross, 1970). As there is also no increase in total DNA polymerase over the period, it must represent a true translocation from cytoplasm to nucleus. In a later report Fansler & Loeb (1972) show a reversible association of DNA polymerase with the nucleus during the cell cycle, the enzyme associating with chromosomes as they unfold from mitosis, and remain bound until after S-phase when it dissociates.

2.11 Other activities implicated in eukaryotic DNA replication

The absence of a properly developed system of genetics for eukaryotes has ruled out an analysis similar to that in prokaryotes where particular factors are known to be involved in replication. Recently Jeggo et al. (1973) isolated a temperature-sensitive mutant of the smut fungus Ustilago maydis which contained a heat labile DNA polymerase. However most other enzymes described with activities similar to those known to be involved in prokaryote replication can only be implied to have a role in eukaryotes. Herrick & Alberts (1973) have isolated a nucleic acid helix-unwinding protein from calf thymus which can stimulate the large calf thymus DNA polymerase although not specifically. Cultured mammalian fibroblasts contain a protein which is present in greater amounts in growing compared with resting cells and has an affinity for single-stranded DNA (Tsai & Green, 1973). (c.f. Gene 32 protein of T4 phage, see page 13). The evidence for a mechanism of discontinuous chain growth for eukaryotes is not so clearly

defined as in prokaryotes and the role of RNA primers and gap-filling is still speculative. RNase H and DNA ligase activities have been demonstrated but elucidation of their in vivo role will have to await further biochemical analysis and a better characterised eukaryotic genetics - as will, of course unequivocal proof of which DNA polymerase species is the DNA replicase.

3. AIMS OF THE PRESENT WORK

The initial aim was to determine if there was a relationship between the two types of DNA polymerising activities shown to be present in nuclear and supernatant fractions prepared from cultured mouse L929 cells (Lindsay and Adams, 1968; Adams & Lindsay, 1969; Lindsay et al., 1970). As the supernatant activity preferred denatured DNA as primer whereas the nuclear one preferred native, a comparison was drawn with the hypothesis postulated by Keir (1965), that the supernatant activity was an 'altered' form of the nuclear one rendered inactive on a native DNA template in non-proliferative states. An activity capable of converting the soluble to the nuclear one was sought in crude cell fractions. Specific enzymic and physical techniques were employed to investigate the biochemical nature of the supernatant activity particularly because of its unusually high M.W. and the suggestion by contemporary reports that it may be attached to membrane (Baril et al., 1970, 1971).

Another approach was to study how the two activities changed their levels and intracellular location in response to DNA synthetic changes, with a view to gaining a better understanding of their role in DNA replication. To this end, an isolated nuclear system was developed which incorporated 5' dNTPs into DNA in a manner which correlated with the DNA replicative ability of the cells of origin.

The properties of this nuclear synthesis and of the DNA polymerases extractable from these nuclei were compared. To demonstrate which DNA polymerase species was involved, the effect of specifically extracting a particular enzyme on the endogenous synthetic ability of the nuclei was investigated. When extraction was complete i.e. all assayable nuclear DNA polymerase activity had been removed, attempts were made to reactivate the nuclei by adding back extracts containing different DNA polymerase species. The validity of the reactivated endogenous synthesis was checked by comparison of sucrose gradient profiles of the products in each case.

II MATERIALS

1. CHEMICALS

N-ethylmaleimide, sodium p-aminosalicylate and ammonium sulphate (specially low in heavy metals for enzyme work) were products of British Drug Houses Ltd., Poole, Dorset, England. Most of the other inorganic chemicals used in the present work were also obtained from this source and were AnalaR grade wherever possible.

Aminopterin, sodium 7-deoxycholate (DOC), Brij 58 (Polyoxyethylene 20 cetyl ether) and Tris (hydroxymethyl) aminomethane (Trizma base) were obtained from the Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Hyamine hydroxide (1M in methanol) and 2, 5 diphenyloxazole (PPO) were purchased from Koch-Light Laboratories, Colnbrook, Bucks., England.

Triton X-100 (polyoxyethylene octyl phenol) was a product of Rohm & Haas (U.K.) Ltd., Croydon, England.

p-Bis (o-methylstyryl) benzene, (bis MSB) was obtained from Eastman-Kodak Company, Kirkby, Lancs., England.

2. NUCLEIC ACID PRECURSORS

2.1 Unlabelled

The deoxyribonucleoside 5'-triphosphates (dATP, dGTP, dCTP and dTTP) and the deoxyribonucleoside 5'-diphosphates (dADP, dGDP, dCDP and dTDP) all as sodium salts were purchased from P-L Biochemicals Incorporated, Milwaukee, Wisconsin 53205, U.S.A.

The ribonucleoside 5'-triphosphates (rATP, rGTP, rCTP, and rUTP) all as sodium salts were obtained from the same source as above.

1-β-D-arabinofuranosylcytosine 5'-triphosphate (araCTP) was a product of the Sigma Chemical Co., St. Louis, Missouri, U.S.A.

2.2 Radioactive

[Me-^{3}H] dTTP, [Me-^{3}H] dTDP and [2-^{14}C] thymidine were all purchased from the Radiochemical Centre, Amersham, England.

3. BIOLOGICAL MATERIALS

3.1 Nucleic Acids

Salmon testis DNA was obtained from Worthington Bio-chemical Corporation, Freehold, New Jersey, U.S.A.

Yeast whole cell RNA was obtained from British Drug Houses Ltd., Poole, Dorset, England.

3.2 Proteins and Enzymes

Bovine serum albumin (BSA) and haemoglobin (Horse heart) were obtained from the Sigma Chemical Co., St. Louis, Missouri, U.S.A., as were the enzymes E. coli alkaline phosphatase, bovine pancreatic ribonuclease-A and bee venom phospholipase-A.

E. coli DNA polymerase was purchased from the Boehringer Corporation (London) Ltd., England.

Urease from Jack Beans was obtained from the Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.

3.3 Cell culture

L929 mouse fibroblast cells (Sanford et al., 1948) were obtained from the American Type Culture Collection (A.T.C.C.). They were maintained in minimal essential Eagle's medium (with double the original vitamin content - see Paul, 1970) supplemented with 10% (v/v) calf serum (i.e. EC10 medium) in the presence of streptomycin and penicillin (100 units of each per ml of medium). Materials for cell culture were supplied by Flow Laboratories Ltd., Irvine, Scotland and Bio-Cult Laboratories Ltd., Paisley, Scotland. Cultures were routinely checked for contamination by bacteria and pleuropneumonia-like organisms (PPLO).

4. CHROMATOGRAPHIC MATERIAL

Whatman CF2 Cellulose powder, Whatman No.1 chromatography paper, No.1 and 3MM filter circles (2.5 cm in diameter) were purchased from H. Reeve Angel and Co. Ltd., London, England.

Sephadex G-200 and Sepharose 4B were products of Pharmacia Fine Chemicals, Uppsala, Sweden as was Blue Dextran 2000, a high molecular weight dye for column calibration.

5. MISCELLANEOUS

Hiflo Super Cel (Celite) was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England.

Cellulose nitrate ultracentrifuge tubes were purchased from Beckman Ltd., Palo Alto, California, U.S.A.

Minicon B-15 concentrators (15,000 M.W. cut-off, maximum volume, 5 ml) were supplied by Amicon, High Wycombe, Bucks., England.

III METHODS

1. SOLUTIONS

1.1 Buffers

Unless otherwise stated Tris-buffer was used throughout the present work. Solutions were buffered by adding the appropriate volume of a stock solution of 1M Trizma base in water which had been previously adjusted to pH 7.5 using HCl (1M Tris HCl, pH 7.5). After dilution the pH was checked and re-adjusted to 7.5 if necessary.

Buffer A consisted of 20mM Tris HCl, pH 7.5 containing 5mM 2-mercaptoethanol.

Buffered-sucrose used for preparation of cell fractions consisted of Buffer A containing 0.25M sucrose (isotonic buffer).

Standard column buffer, used for gel filtration studies was comprised of Buffer A with KCl at a concentration of 0.15M.

1.2 Scintillation Fluid

Toluene/PPO scintillator was prepared by dissolving PPO at 0.5% (w/v) in AnalaR toluene.

Triton/Toluene scintillator was prepared by dissolving PPO at 0.5% (w/v) and bis MSB at 0.05% (w/v) in a solution containing 35% (v/v) Triton X-100 and 65% (v/v) AnalaR toluene.

2. ESTIMATION OF PROTEIN, DNA AND RNA

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

DNA was estimated by the method of Burton (1956) using salmon testis DNA as standard.

RNA was measured by the method of Schneider (1957) using yeast whole cell RNA as standard.

3. PREPARATION OF BIOLOGICAL MATERIAL

3.1 Logarithmically growing L929 cells

L929 cells in the logarithmic stage of growth were obtained by setting up Winchester bottles, each containing 20-40x10^6 cells, and growing the cells as monolayer cultures with constant rotation, for 3-4 days at 37°C, with one change of medium.

3.2 Stationary L929 cells

Stationary L929 cells were obtained by setting up Winchester bottles, as above, and allowing the cells to reach confluence. Generally cells grown for 7-10 days with two or three changes of medium were used for the present work.

3.3 L929 cells in the DNA-synthetic or S-phase

S-phase L929 cells were obtained by one of the two following methods:-

(i) Winchester bottles, each containing 80-100x10^6 cells, were set up using stationary L929 cells. After 8 h of growth, aminopterin, adenosine and glycine were added to final concentrations of 2x10^{-7}M, 2x10^{-4}M and 10^{-4}M respectively. After a further 16 h growth, thymidine was added at a final concentration of 5x10^{-6}M. 2 h later the cells were harvested when it was shown, in a separate experiment, autoradiographically, that 90% of the cells were incorporating exogenously added ^{3}H-thymidine into their DNA (Lindsay et al., 1970).

(ii) Cells were set up initially as in (i) and harvested 17-19 h later when it was shown by the criterion in (i) that 80% of the cells were making DNA (Lindsay et al., 1970).

3.4 Preparation of nuclear and soluble fractions from L929 cells

Cells, at the required growth stage, were rinsed with ice-cold balanced salt solution (BSS - see Paul, 1970), scraped off in

BSS and centrifuged at 600g for 5 min at 4°C. All subsequent operations were carried out at 0-4°C. The cells were washed twice by resuspension in 10-20 volumes of Buffered-sucrose (see Methods, section 1.1) followed by centrifugation as before. The cells were then resuspended in 5 volumes of Buffered-sucrose and disrupted by homogenisation, using a Potter-Elvehjem homogeniser with a close-fitting Teflon pestle and glass tube (Made by Sireica, Jamaica, New York, U.S.A.). After every 3 strokes, the cells were checked for breakage by phase-contrast microscopy and re-homogenised if necessary. The homogenate was then centrifuged at 800g for 10 min. For preparation of high-speed supernatant (S2) the low-speed supernatant (S1) from the previous step was centrifuged at 105,000g for 1 h in a Spinco 40 rotor. The nuclear pellet (N1), obtained by centrifuging the homogenate, was washed a further twice, by resuspension in 10-20 volumes of Buffered-sucrose and centrifugation at 800g for 10 min, giving rise to N2 and N3 nuclear preparations. Fig.1 outlines the procedure from the homogenate stage.

3.5 Alternative method of nuclear isolation from L929 cells

The steps for the preparation of N2 fraction in the previous section were followed with the difference that Buffered-sucrose was replaced with a solution containing 10mM Tris HCl pH 7.8, 1mM EDTA, 4mM $MgCl_2$ and 6mM 2-mercaptoethanol (Hershey et al., 1973a).

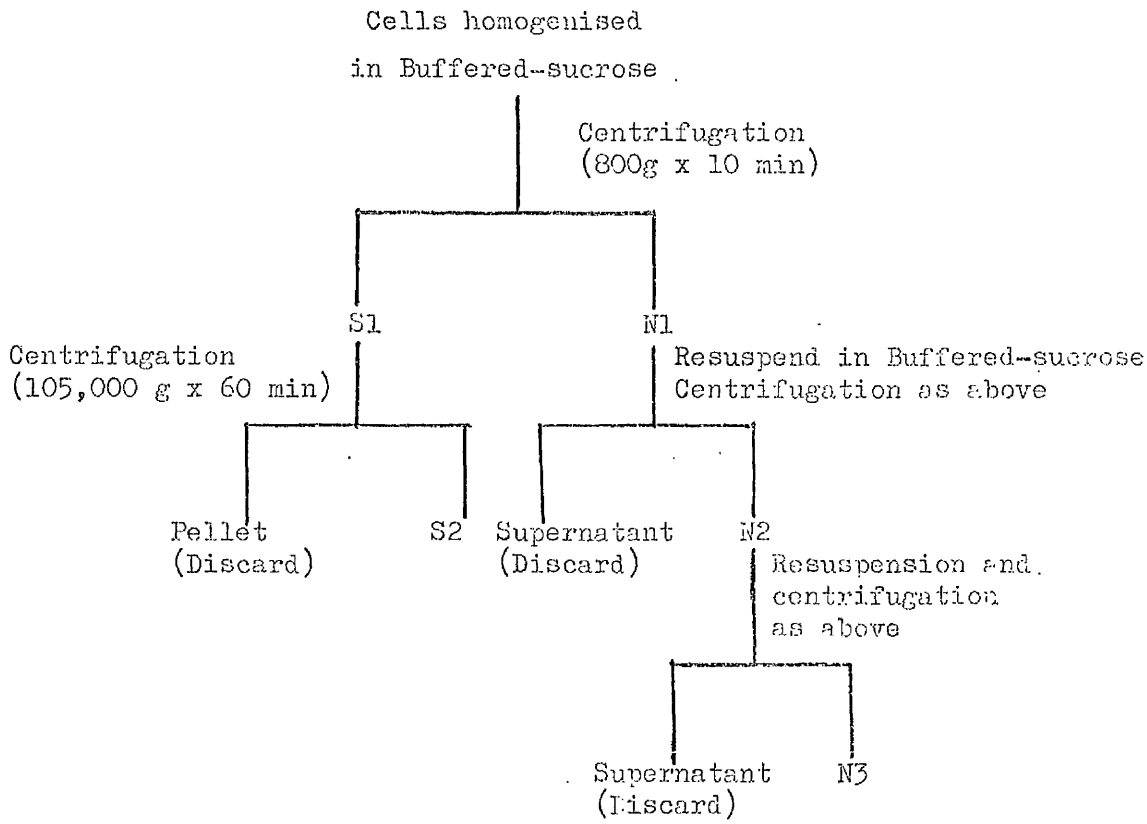
L929 cells proved difficult to break by homogenisation in the above solution and the resultant nuclei were contaminated with cytoplasmic granular material on examination by phase-contrast microscopy.

3.6 Concentration of cell fractions

Concentration of samples for column chromatography etc. was

Fig. 1

Preparation of nuclear and supernatant fractions of L929 cells



N1, N2 and N3 are successively washed nuclear fractions.

S1 and S2 are low and high speed supernatant fractions respectively.

achieved by one of the following two methods:-

(i) Ammonium sulphate (5.3 g per 10 ml) was added gently, with stirring to the sample to be concentrated. After leaving for 15 min after addition of all the $(NH_4)_2SO_4$, the sample was centrifuged at 17,000 g for 20 min. The resultant precipitate was dissolved in standard column buffer to approximately 1/5 of the original volume, dialysed for two periods of 1 h against 100 volumes of standard column buffer and centrifuged at 17,000 g for 20 min.

(ii) The sample was dialysed against standard column buffer as in (i) and added to a compartment of a Minicon B-15 concentrator (volume 5 ml). It was left in the refrigerator at 4°C until the volume was reduced to approximately 1/5 of the original sample.

3.7 Methods of nuclear extraction

Several methods were used to extract nuclei depending on whether the extract, the extracted nuclei, or both were required for further studies. All of the following procedures were carried out at 0-4°C.

i Extraction Method I

To the nuclear pellet was added, dropwise with constant stirring, an equal volume of a solution containing the extracting agent at twice the extraction concentration. The resulting suspension was allowed to extract for 10 min, then centrifuged either at 11,500 g for 20 min, when the maximum volume of extract was required, or at 800 g for 10 min. To obtain extracted nuclei free of extractable material, they were washed by resuspension in 10 volumes of Buffer A containing the extracting agent and centrifuged at 800 g for 10 min. To remove extracting agent the washing was repeated with Buffer A. The resultant

extracted nuclear pellet was then suspended in Buffer A as required.

ii Extraction Method II

Extraction Method II was devised as a milder alternative to I. It involves dialysis of a suspension of nuclei, for two periods of 1 h against 100 volumes of Buffer A containing extracting agent. The method is advantageous as it allows extraction to take place gradually and with minimal mechanical damage to nuclei. It was used principally to study the feasibility of nuclear reconstitution after extraction. After the extraction-dialysis, continued dialysis against Buffer A would allow reconstitution without the harmful effects of mechanical separation and resuspension.

The extract and exhaustively extracted nuclei can be obtained after the extraction-dialysis by removal from the dialysis bag, and treatment as described under Extraction Method I.

iii Extraction Method III

This procedure was devised for rapid and efficient production of exhaustively extracted nuclei free of extracting agent. Nuclei in a suspension of extracting agent, obtained by either of the two previous methods, were layered on top of a discontinuous sucrose gradient containing 10 volumes of Buffer A plus extracting agent and 10% sucrose, and 2 volumes of Buffer A plus 15% sucrose. The nuclei were then centrifuged at 800 *g* for 10 min. The extract, which remained on top, was carefully removed with a Pasteur pipette. The remaining liquid was removed by aspiration being careful not to contaminate the extracted nuclear pellet with solution containing extracting agent. The exhaustively extracted nuclei were then resuspended in Buffer A as required.

3.8 ^{14}C pre-labelling of S-phase L929 cell DNA

Cells were set up as described in Methods, section 3.3 (ii). After 16 h of growth, 1.5μCi of [2-^{14}C] thymidine, of specific activity 62mCi/mmole, was added to the medium (100 ml). After 1 h, the radioactive medium was replaced with pre-warmed non-radioactive medium, and the cells harvested 1 h later.

3.9 Preparation of native and denatured DNA solutions

Most samples tested for DNA polymerase activity were assayed with both native and denatured DNA. DNA (salmon testis) was dissolved at 1-2 mg per ml in 0.05M KCl by gently stirring at 0-4°C. Denaturation was achieved by heating at 100°C for 10 min, followed by rapid cooling in an ice-water mixture.

4. ENZYME ASSAYS

4.1 Assay for DNA polymerase activity, using deoxyribonucleoside triphosphates

The basic assay system was that of Shepherd & Keir (1966). The sample was incubated for 1 h at 37°C in a total volume of 0.25 ml containing 20mM Tris HCl pH 7.5, 6mM $MgCl_2$, 60mM KCl, 0.4mM EDTA, 12mM 2-mercaptoethanol, 400μg/ml salmon testis DNA (either native or denatured) and 0.2mM dATP, dGTP, dCTP and [Me-^{3}H] dTTP at a specific activity of 20μCi/μmole.

The reaction was terminated by the addition of 0.05 ml of 2M NaOH and reincubated for at least 1 h at 37°C. 0.1 ml portions were spotted onto Whatman 3MM filter circles (2.5 cm diameter) and washed six times in 5% (w/v) trichloroacetic acid containing 50mM $Na_4P_2O_7$ (10 ml per filter) and then dried with ethanol and ether. The DNA was then dissolved by heating with 0.5 ml of 1M Hyamine hydroxide for 20 min at 60°C in a counting vial. 5 ml of toluene/PPO scintillator was added to the vial and the radioactivity incorporated estimated by

counting the vial in a Phillips liquid scintillation counter, pre-programmed for estimating ^{3}H in homogeneous solution. DNA polymerase activity is expressed as d.p.m. [^{3}H] dTMP incorporated per hour.

4.2 Assay for DNA polymerase activity using deoxyribonucleoside diphosphates

The assays were conducted in exactly the same way as with deoxyribonucleoside triphosphates, except that diphosphates were substituted for the corresponding triphosphates and [Me-^{3}H] dTDP replaced [Me-^{3}H] dTTP. 1.5mM ATP was also present.

4.3 Assay for nucleoside diphosphokinase activity

To assay the conversion of dTDP into dTTP during DNA polymerase assays with deoxyribonucleoside diphosphates as precursors in the presence of ATP, 100µl portions of the polymerase incubation mixtures were pipetted into 0.4 ml of ice-cold 5% trichloroacetic acid. The samples were centrifuged at 800g for 10 min and 50µl samples of the supernatant (containing acid-soluble material) were spotted onto sheets of Whatman No 1 paper (46x57 cms), 10 cm from the top. The chromatogram was developed for 20 h by descending chromatography with isobutyric acid, ammonia (sp. gr. 0.88) 0.1M EDTA and water (100:4.2:1.6:55.8 by volume) as solvent (Krebs & Hemms, 1953).

After chromatography, the paper was dried and examined under ultra-violet light. Spots corresponding to dTTP and dTDP, as judged by simultaneously chromatographed markers, were cut out and counted in Toluene/PPO scintillator, as described in Methods, section 4.1. The percentage conversion of dTDP to dTTP was determined.

4.4 Assay for DNA synthesis by isolated nuclei

Assay system A

Nuclei were assayed at 37°C for varying times by suspension in a final volume of 0.2 ml containing 20mM Tris HCl pH 7.5, 10mM $MgCl_2$, 10mM KCl, 0.4mM EDTA, 10mM 2-mercaptoethanol, 2mM ATP, 0.2mM dATP, dGTP and dCTP and 0.05mM [Me-^{3}H] dTTP at a specific activity of 200 µCi/µmole. The reaction was terminated by addition of 0.05 ml of 5M NaOH and after incubation for 1 h at 37°C, 0.25 ml of 2% SDS solution containing 4mM EDTA and 6% p-aminosalicylate was added. The mixture was heated at 70°C for 30 min to disaggregate the nuclei. 2 ml of ice-cold 5% trichloroacetic acid containing Hiflo Super Cel (at 20g per litre) was added followed by 3 ml of 5% TCA containing 50mM $Na_4P_2O_7$ (TCA/PPi). The samples were kept in an ice-water mixture for 10 min and then centrifuged at 800 g for 10 min. The pellet was washed once more with 5% TCA/PPi and transferred with thorough washing to a Whatman No 1 filter circle (2.5 cm in diameter) in a Millipore microanalysis filter holder (No. XX10 025 03). The filter had been prelined by addition of 2 ml of 5% TCA containing Hiflo Super Cel (20g/l). The pad of Hiflo Super Cel, containing the precipitated sample was then washed three times with 15 ml of 5% TCA/PPi, once with 15 ml of absolute alcohol and twice with 3 ml of ether. The pad was then scraped from the filter into a counting vial and the radioactivity incorporated estimated as in Methods, section 4.1.

Assay system B

The assay is basically that described by Hershey et al. (1973a). Nuclei were assayed at 37°C for varying times by suspension in a final volume of 0.2 ml containing 40mM Tris HCl pH 7.5, 100mM NaCl, 10mM $MgCl_2$, 0.67mM EDTA, 4mM 2-mercaptoethanol, 5mM ATP, 0.1mM dATP, dGTP, dCTP and [Me-^{3}H] dTTP at a specific activity of 50µCi/µmole. The reaction was terminated by addition of 1 ml of 50mM $Na_4P_2O_7$ followed by 5 ml of

ice-cold 0.5M perchloric acid containing 0.005M $Na_4P_2O_7$. If the amount of nuclei used was low, 100µg of salmon testis DNA was added as carrier. After being left for 10 min in ice, the samples were centrifuged at 800 g for 10 min. The resultant pellet was dissolved in 0.5 ml of 1M NaOH (heating at 37°C if necessary) and the acid-precipitation step repeated. This procedure was repeated three more times. The final pellet was suspended in 1 ml of 0.5M PCA, heated at 70°C for 30 min, allowed to cool and centrifuged for 10 min at 800 g. The supernatant was transferred to a counting vial containing 10 ml of Triton/Toluene Scintillator and counted in a Phillips liquid Scintillation counter pre-calibrated for measuring ^3H in Triton/Toluene/Aqueous emulsions.

For both systems, the DNA content of the nuclei was measured and the activity expressed as pmoles TMP incorporated/mg DNA.

4.5 Assay for cytochrome oxidase activity

Cytochrome oxidase activity was measured exactly as described by Cooperstein & Lazarow (1951). The activity was expressed as ΔE_{550} per hour at 37°C.

4.6 Assay for E.coli DNA polymerase

E. coli DNA polymerase, used as a gel filtration column marker, was assayed exactly as for L929 DNA polymerase activity described in Methods, section 4.1 with denatured DNA as primer.

4.7 Assay for E. coli alkaline phosphatase

Alkaline phosphatase from E. coli was assayed basically as described by Garen & Levinthal (1960).

5. FRACTIONATION PROCEDURES

5.1 Gel filtration on Sephadex G-200

Sephadex G-200 was pre-swollen and equilibrated according to

the supplier's instructions. The gel was then packed into columns (final dimensions in the range of 50-57 cm X 0.9 cm). After each column preparation, the void volume and the elution volumes of several markers were determined using Blue Dextran (BD), E. coli DNA polymerase (EDP), E. coli alkaline phosphatase (AP) and haemoglobin (Hb). EDP and AP were assayed as described in Methods, sections 4.6 and 4.7 respectively. BD and Hb were determined by measuring the extinction at 600 nm and 440 nm respectively. The flow rate was 6.0 - 6.2 ml per hour and 0.9 - 1.1 ml fractions were collected. All procedures were carried out at 0 - 4°C.

5.2 Gel filtration on Sepharose 4B

The swelling and equilibration were carried out according to the suppliers instructions and columns packed as described for Sephadex G-200 (Methods, section 5.1). Urease was used as a marker and was measured by extinction at 280nm. Flow rates and fraction collection were as described for Sephadex G-200.

5.3 DNA-cellulose chromatography

Native DNA-cellulose was prepared using salmon testis DNA by the method of ~~Alberts et al.~~ Litman, (1968). The final product was shown to have 6.43µg DNA bound per mg of cellulose. Samples in Buffer A were applied to a packed column of DNA-cellulose (3x1.5 cm) at 4°C and allowed to stand for 10 min to permit binding. The unbound eluate was then collected and the column washed with 10 volumes of Buffer A. Continuous gradient elution with increasing KCl concentration was then performed and 2 ml fractions collected. The flow rate was 6.0 - 6.2 ml per hour.

5.4 Alkaline sucrose gradient centrifugation

Samples were layered onto linear 5-20% sucrose gradients containing 0.1M NaOH and 1.0M KCl and centrifuged at 63,500 g for 16 h at 4°C in

a SW25 rotor. Gradients were harvested by putting a tube through the sucrose to the bottom of the tube and withdrawing material at a rate of 2.5 ml/min into 1.0 - 1.2 ml fractions.

To determine the total radioactivity, samples were dried down, solubilised in hyamine and counted in Toluene/PPO scintillation fluid as described in Methods, section 4.1.

Acid insoluble radioactivity was measured by precipitation and washing with 5% trichloroacetic acid using Hiflo Super Cel as described in Methods, section 4.4.

IV RESULTS

1. DNA POLYMERASE ACTIVITY OF L929 CELLS

The presence of DNA polymerase activity in cultured mouse fibroblast L cells has been reported previously by several investigators (Littlefield et al., 1963; Gold & Helleiner, 1964; Lindsay & Adams, 1968). Using similar cell fractionation procedures as employed in the present work, Lindsay & Adams (1968) showed that nuclei isolated from logarithmically growing L929 cells exhibited a 2-5 fold preference for native DNA as template, whereas a soluble fraction was 3-4 times more active in the presence of heat-denatured DNA. A comparison of some of the properties of the two activities has been documented (Lindsay, J.G., Ph.D. Thesis, University of Glasgow, 1969). The activities have similar pH optima and respond in the same way to changes in magnesium and potassium ion concentration in the assay. Apart from DNA template preference and cellular distribution, the two activities differ in their response to omission of one or more of the four deoxyribonucleoside 5' triphosphates in the assay. Nuclear DNA polymerase is able to incorporate at 80% of the control rate with omission of one triphosphate and at 40% in the presence of only dTTP. Soluble activity, however, behaves in a more replicative manner being reduced to less than 15% of control rates in the presence of only dTTP.

2. DNA POLYMERASE SPECIES PRESENT IN SOLUBLE AND NUCLEAR FRACTIONS FROM L929 CELLS

Preliminary purification studies in this and other laboratories suggested that there may be more than one DNA polymerase species present in eukaryotic cells. The inter-relationship and intracellular distribution of these species has been the subject of much controversy in recent years (See Table 2 of Introduction).

2.1 Soluble DNA polymerase activity

A high-speed supernatant fraction from logarithmically growing cells prefers heat-denatured DNA confirming previous findings. When applied to a column of Sephadex G-200, and eluted with standard column buffer (0.15M KCl, 20mM Tris HCl, pH 7.5, 5mM mercaptoethanol), the bulk of the activity elutes in the void volume, suggesting that its molecular weight is in excess of 200,000 daltons (Fig.2). Very little, if any, lower M.W. activity is found. The activity still retains its preference for denatured DNA although this is reduced. This preferential loss of activity with denatured DNA is probably due to dilution on the column, as verified by the results shown in Fig.3, where the activity of a soluble preparation is measured as a function of protein concentration in the assay. It is seen that as the protein concentration decreases, so too does the ratio of the ability to use denatured DNA compared with native. The reason for this is unknown but it appears to be reversible i.e. concentration of supernatant samples for column application results in DNA polymerase activity with an increased denatured to native ratio. It may represent a concentration dependent co-operative binding effect of either the DNA polymerase enzyme itself or of an unknown factor responsible for its ability to use a denatured DNA template.

2.2 Nuclear DNA polymerase activity

A nuclear preparation from logarithmically growing L929 cells exhibits DNA polymerase activity with a slight native DNA template preference. The assayable DNA polymerase activity of such nuclei is shown to be completely extractable by treatment with 0.4M KCl (Table 3). This treatment was shown by microscopic examination to leave the nuclei still whole, though somewhat damaged, with some of their contents extruding. The DNA polymerase activity is shown to

Fig. 2

Fractionation of DNA polymerase activity present in a soluble fraction of L929 cells by gel filtration on Sephadex G-200

A high speed supernatant fraction (S2) prepared as described in Methods, section 3.4 from logarithmically growing cells is concentrated approx. 5-fold by ammonium sulphate precipitation (Methods, section 3.6). A 1 ml sample, containing 8.3 mg of protein is then applied to a Sephadex G-200 column and eluted with standard column buffer (Methods, section 1.1), as described in Methods, section 5.1. The fractions obtained are then assayed for DNA polymerase activity as in Methods, section 4.1.

—⊙— DNA polymerase activity with native DNA

—○— activity with denatured DNA

—◑— denotes coincident points

—◇— concentration of protein

The fraction in which the following markers peak is marked thus (▽).

BD	-	Blue Dextran	200,000
EDP	-	*E. coli* DNA polymerase	109,000
AP	-	*E. coli* alkaline phosphatase	80,000
Hb	-	Haemoglobin	65,000

Fig. 2

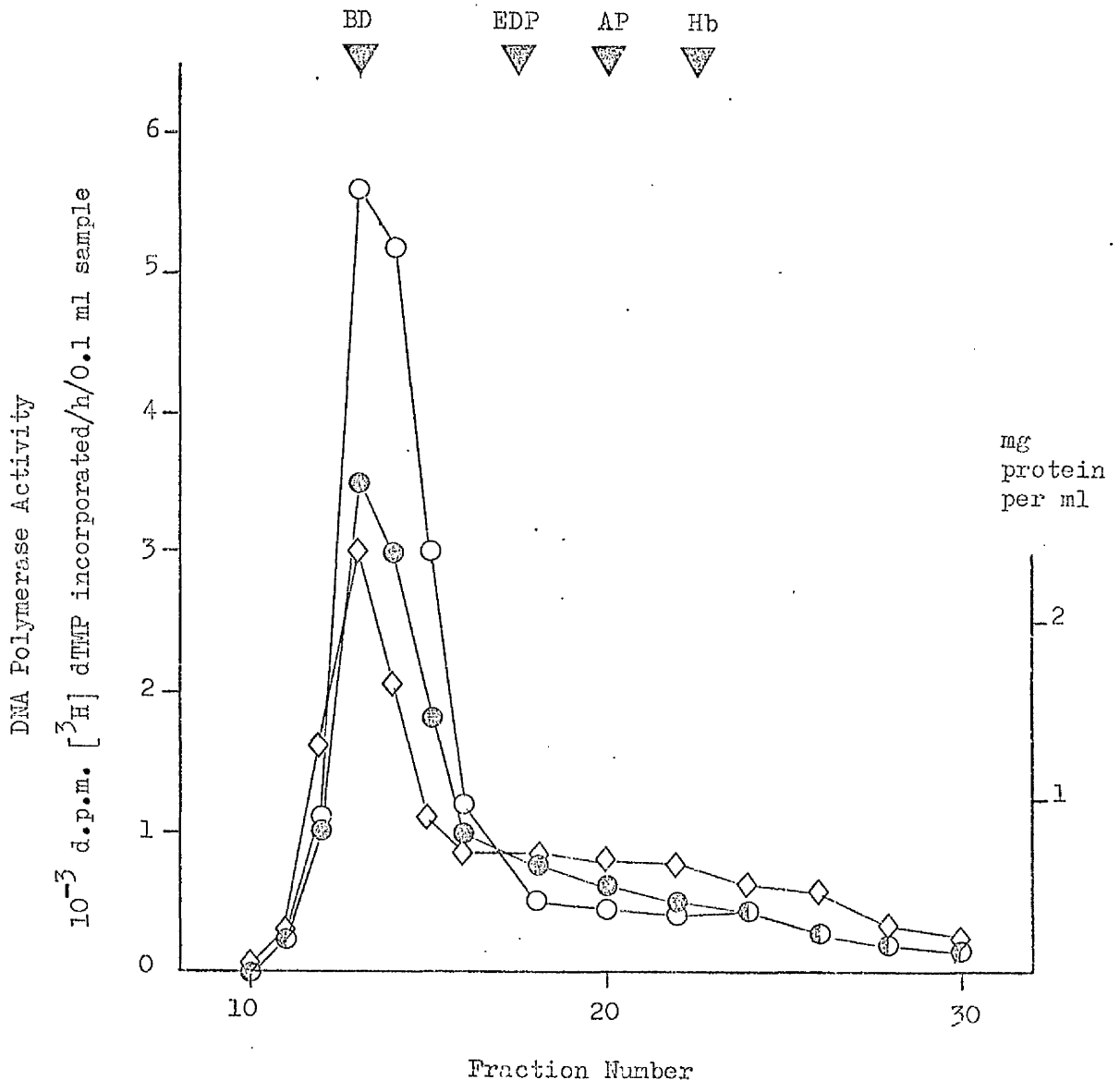
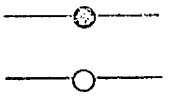

BD
EDP
AP
Hb
DNA Polymerase Activity
10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample
mg protein per ml
0
1
2
3
4
5
6
1
2
10
20
30
Fraction Number

Fig. 3

Effect of protein concentration on the DNA polymerase activity present in soluble fractions of L929 cells

The standard DNA polymerase assay is employed and various amounts of a concentrated S2 fraction (7.5 mg protein/ml), prepared as previously described, are added as indicated opposite.



activity with native DNA

activity with denatured DNA

Fig. 3

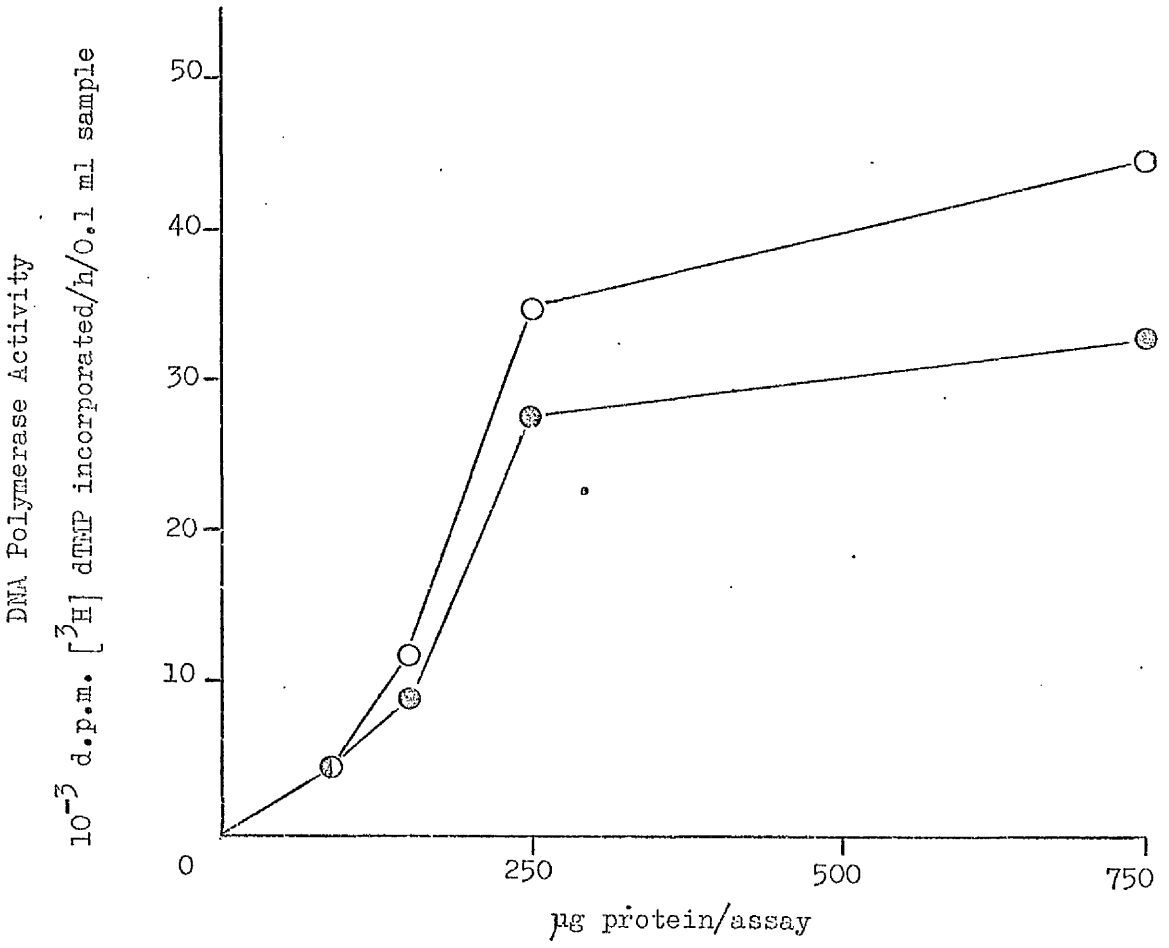

DNA Polymerase Activity
10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample
50
40
30
20
10
0
250
500
750
µg protein/assay

Table 3

Extraction of DNA polymerase activity from nuclei prepared from logarithmically growing cells

Equal aliquots of nuclei prepared from logarithmically growing cells (N3 - prepared as describe in Methods, section 3.4, are extracted with 0.4M KCl, salmon testis DNA (400µg/ml) and water (Extraction Method I, Methods, section 3.7). The total DNA polymerase activity of the resultant extracts and exhaustively extracted nuclear pellets is then determined after appropriate additions to normalise the KCl and DNA concentration in the extracts.

Extracting agent	Total DNA polymerase activity 10^{-3} d.p.m. [^{3}H] dTMP incorporated/h			
	Nuclear pellet		Extract	
	native DNA	denatured DNA	native DNA	denatured DNA
Water	77.3	77.0	zero	zero
0.4M KCl	zero	zero	208.0	192.4
DNA (400µg/ml)	72.6	73.2	93.5	66.5

be activated 2-3 fold on extraction. This effect may be attributed to the inability of exogenously added DNA to successfully act as template for all the available activity within the nuclei. The ability of native DNA (at the concentration present in DNA polymerase assays) to extract nuclear DNA polymerase is also shown in Table 3. As suspected the DNA only removes about half of the activity extractable by 0.4M KCl but the residual activity is only slightly reduced.

A 0.4M KCl extract of nuclei from logarithmically growing cells, is shown on Sephadex G-200, to exhibit multiple DNA polymerase peaks (Fig.4). By comparison with markers, the approximate molecular weights in daltons are as follows: peak I ($>200,000$); peak II (110-200,000); peak III (65,000-80,000); and a shoulder (IV) ($<65,000$). Peak I has a preference for denatured DNA and is similar in this respect and in molecular size to the soluble species. There is some evidence (See Discussion, Section 3) that peak I from nuclei and the soluble species are the same enzyme but unequivocal proof will have to await purification which has proved difficult because of the enzyme's instability and the small amounts of material available. Peaks II, III and IV all show a marked preference for native DNA and varied in amount in different preparations. Peak III was always the major peak, the other two often appearing as shoulders. Distribution and size considerations point to a monomer, dimer, tetramer relationship but this has yet to be firmly established.

3. ABILITY OF THE NUCLEAR DNA POLYMERASES TO USE DEOXYNUCLEOSIDE 5' DIPHOSPHATES AS PRECURSORS FOR DNA SYNTHESIS

The basic assumption that 5' dNTPs, in the freely soluble form, as used in *in vitro* DNA polymerase assays, are the immediate *in vivo* activated form of the 5' dNMP moieties incorporated during

Fig. 4

Fractionation of DNA polymerase activity present in a 0.4M KCl extract of nuclei from L929 cells by gel filtration on Sephadex G-200

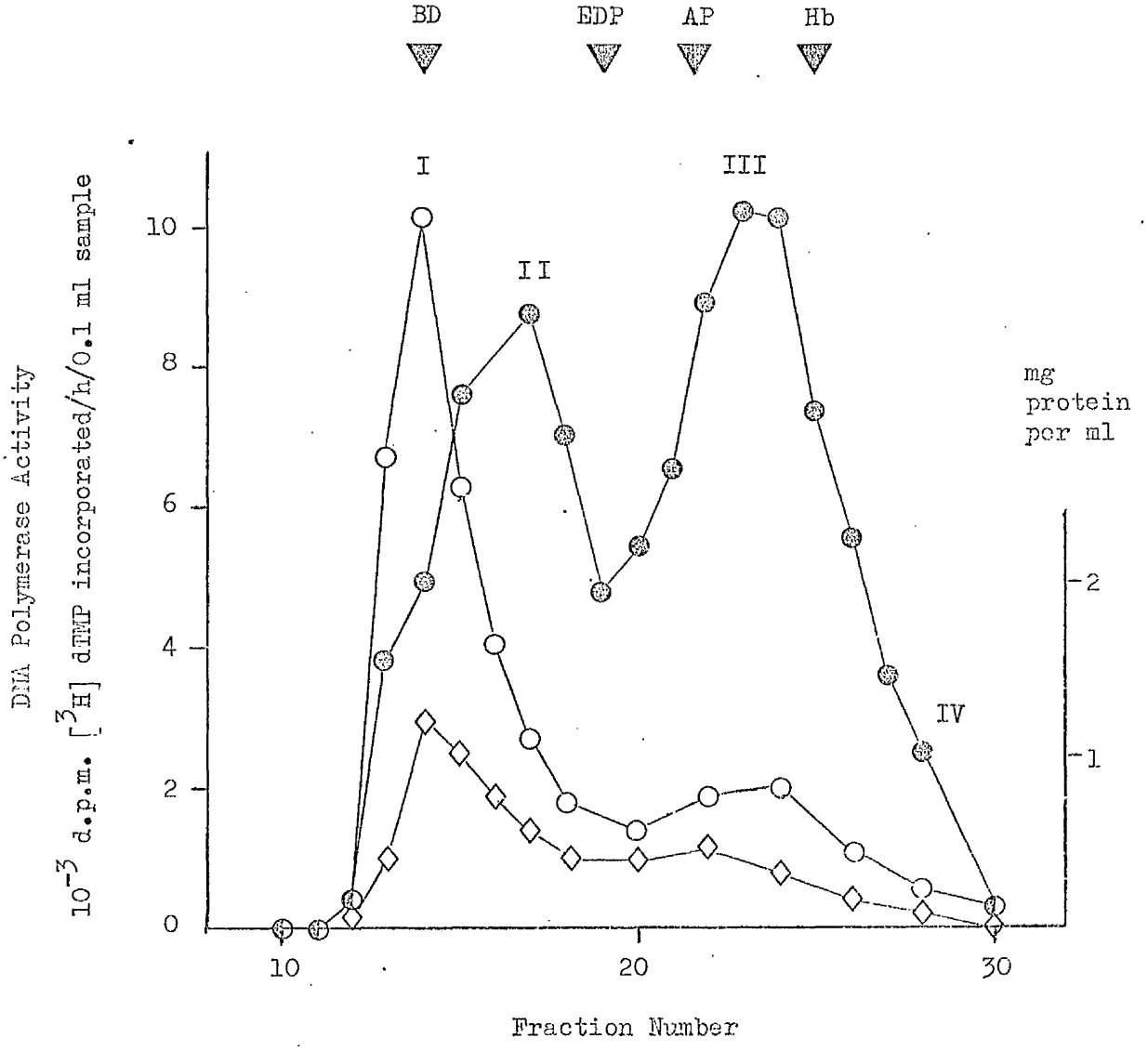
A 0.4M KCl extract of nuclei (N3) from logarithmically growing cells is prepared as described in Table 3, except that the maximum amount of extract is obtained by centrifugation at 11,500 g for 20 min. The extract is then concentrated approx. 5-fold as previously described, and a 1 ml sample, containing 9.1 mg of protein, is fractionated on a Sephadex G-200 column as described for Fig. 2.

——●——	DNA polymerase activity with native DNA
——○——	activity with denatured DNA
——◑——	denotes coincident points
——◇——	concentration of protein

Markers are as described in the legend to Fig. 2

I, II, III and IV refer to peaks of DNA polymerase activity as discussed in the text.

Fig. 4


BD
EDP
AP
Hb
I
II
III
IV
DNA Polymerase Activity
10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample
mg protein per ml
0
2
4
6
8
10
1
2
10
20
30
Fraction Number

DNA replication, has been questioned by Werner (1971) for E. coli (See Introduction, Section 1.3). Work with a partially pure DNA polymerase fraction from the nuclei of L929 cells also questioned this basic assumption (Henderson, M.A.L., Ph.D. Thesis, University of Glasgow, 1972). These studies showed that dNDPs, in the presence of a phosphate donor, ATP or dNTPs, were incorporated in a manner inconsistent with them passing through the free deoxytriphosphate pool. However, no conclusion as to the actual precursor involved could be made because of the presence of nucleoside diphosphokinase (NDPK) activity in the preparations. dNDPs in the absence of a phosphate donor were poor precursors.

The ability of the multiple nuclear DNA polymerase species, separable by gel filtration, to incorporate dNDPs in the presence of ATP is shown in Fig.5. Comparison with the standard triphosphate incorporation shows that use of dNDPs plus ATP is restricted to the later fractions of the column peaking at ~70,000 based on internal markers. Incorporation is only about a third of the standard dNTP assay. When NDPK activity is assayed across the column it is seen to co-elute with the ability of DNA polymerase to use dNDPs (Fig.6), whereas in the first few fractions which lack NDPK, but which exhibit extensive peak I DNA polymerase activity, there is negligible incorporation with dNDPs. It is possible that the smaller nuclear native DNA-preferring DNA polymerase species have an associated NDPK activity (See Miller & Wells (1971) for prokaryotic NDPK-associated DNA polymerases) or that the NDPK activity fortuitously co-elutes with it, imparting an artefactual activity. It is evident, however, that there is no NDPK activity associated with peak I nuclear DNA polymerase and that dNDPs cannot act as precursors for incorporation by this enzyme.

Fig. 5

Ability of the DNA polymerase species present in a 0.4M KCl nuclear extract to use deoxynucleoside 5' diphosphates as precursors in the presence of ATP

A 0.4M nuclear extract is fractionated on Sephadex G-200 as described in Fig. 4. The resulting fractions are assayed by the standard method and with the 5'dNTPs being replaced by 5'dNDPs and 1.5mM ATP. (Methods, section 4.2).

(a) DNA polymerase activity with native DNA

——●—— 5'dNTPs as precursors

——▲—— 5'dNDPs as precursors

(b) DNA polymerase activity with denatured DNA

——○—— 5'dNTPs as precursors

——△—— 5'dNDPs as precursors

Markers are as described in the legend to Fig. 2.

Fig. 5

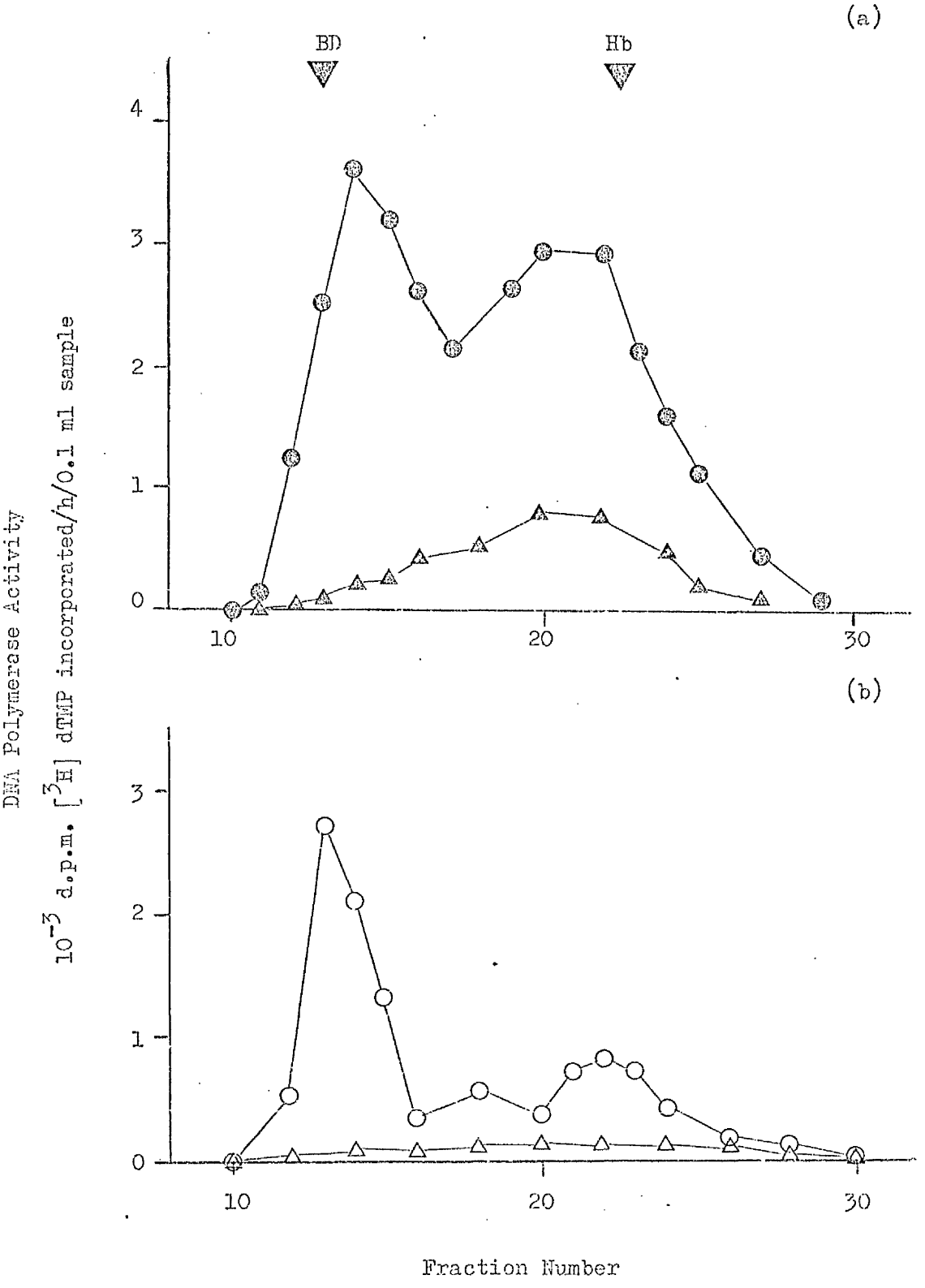

(a)
BD
Hb
4
3
2
1
0
10
20
30
(b)
3
2
1
0
10
20
30
DNA Polymerase Activity
10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample
Fraction Number

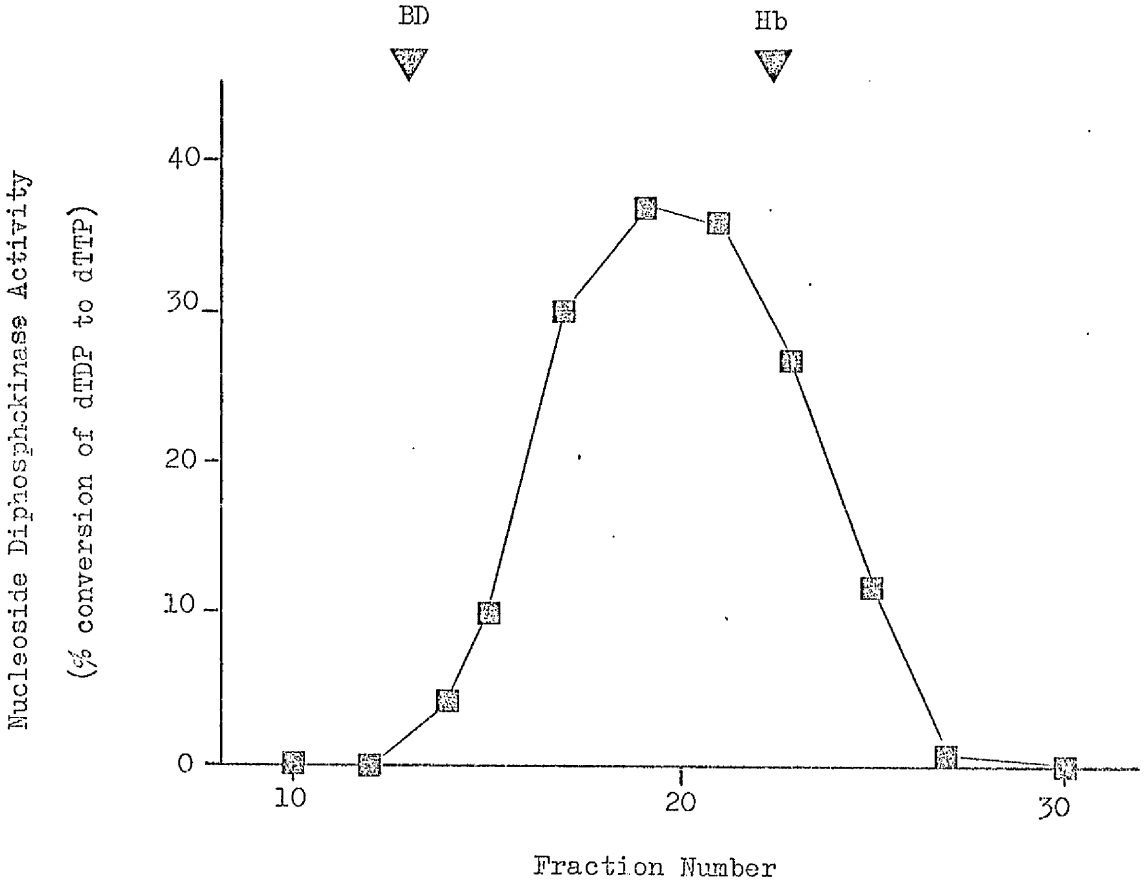
Fig. 6

Fractionation of nucleoside diphosphokinase activity present in a 0.4M KCl nuclear extract by gel filtration on Sephadex G-200

A 0.4M KCl nuclear extract is fractionated on Sephadex G-200 as previously described and the fractions assayed for nucleoside diphosphokinase activity (■) as described in Methods, section 4.3.

Markers are as described in the legend to Fig. 2.

Fig. 6


BD
Hb
40
30
20
10
0
10
20
30
Fraction Number
Nucleoside Diphosphokinase Activity
(% conversion of dTDP to dTTP)

To rule out the possibility of all four dNTPs acting in a manner more specific than merely as phosphate donors, the effect of the four dNTPs replacing ATP on dNDP incorporation for the first part of the Sephadex G-200 column (the region containing peak I DNA polymerase activity but no NDPK activity) is shown in Fig.7. Lack of stimulation verifies that dNDP incorporation is not demonstrable in DNA polymerase fractions lacking NDPK activity.

4. EFFECT OF RIBONUCLEOSIDE 5' TRIPHOSPHATES ON THE DNA POLYMERASE ACTIVITY OF SOLUBLE AND NUCLEAR FRACTIONS

One of the principal enigmas of *in vitro* DNA polymerase reactions is the inability of all DNA polymerases, so far isolated, to incorporate dNTPs in the absence of pre-existing 3'OH primed ends. The mystery of how these 3'OH ends arise *in vivo* has resulted in many hypotheses being put forward (See Introduction, Section 1.3). The most favourable explanation seems to be that DNA polymerase is primed *in vivo* by short pieces of RNA. Whether these are synthesised by already characterised RNA polymerases (or DNA polymerases under different conditions), or by an as yet unknown activity is not known. The effect of adding rNTPs to the standard assays carried out by crude soluble and nuclear fractions from L929 cells is shown in Table 4. As can be seen, no stimulation is demonstrated with native DNA as template which cannot be accounted for by increased dNTPs. With denatured DNA, as template, a 25% stimulation is seen which is peculiar to rNTPs. As three of the four rNTPs are effective alone this does not support a role for them as RNA precursors, but rather as effective phosphate donors, the differing abilities being due to differing nucleoside diphosphokinase specificities. Therefore it appears that no RNA synthesising activity capable of stimulating DNA polymerase by providing 3'OH primed ends is detectable in these

Fig. 7

Effect of replacing ATP by all four deoxynucleoside 5' triphosphates on the incorporation of diphosphates by the high molecular weight DNA polymerase species present in a 0.4M KCl nuclear extract

The procedure is identical to that for Fig. 5, with the exception that fractions are also assayed with all four unlabelled 5'dNTPs (0.2mM each) replacing ATP.

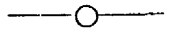
(a) DNA polymerase activity with native DNA

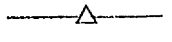

5'dNTPs


5'dNDPs + ATP


5'dNDPs + unlabelled 5'dNTPs

(b) DNA polymerase activity with denatured DNA


5'dNTPs


5'dNDPs + ATP

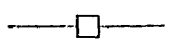

5'dNDPs + unlabelled 5'dNTPs

Fig. 7

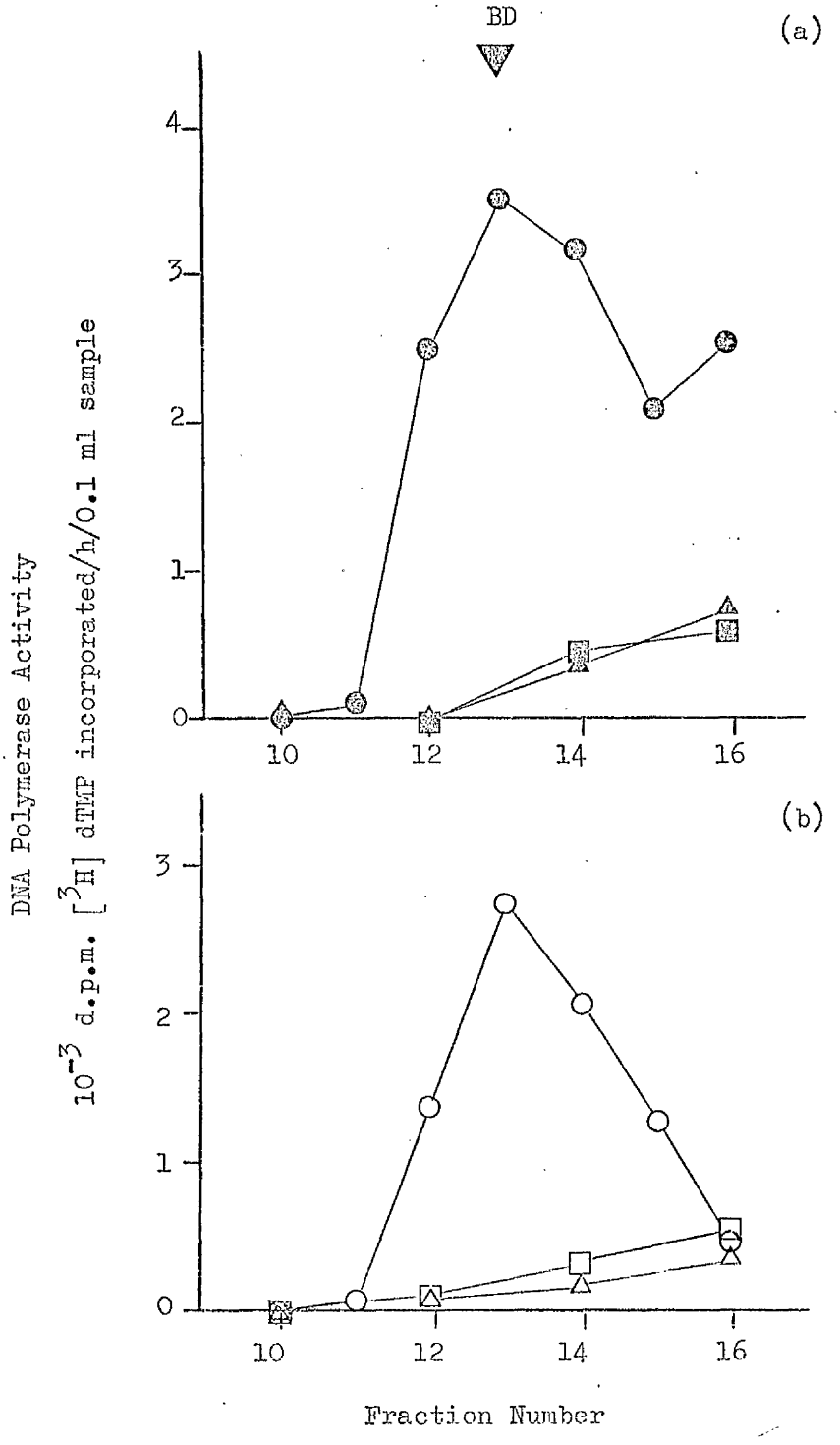

BD
(a)
(b)
DNA Polymerase Activity
10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample
0
1
2
3
4
10
12
14
16
Fraction Number

Table 4

Effect of ribonucleoside 5' triphosphates on the DNA polymerase activity of soluble and nuclear fractions

A high-speed supernatant fraction (S2) and a 0.4M KCl nuclear extract, obtained as previously described, are assayed for DNA polymerase by the standard method with the appropriate additions as shown below.

DNA polymerase activity

10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample

	High speed supernatant fraction		0.4M KCl nuclear extract	
	native DNA	denatured DNA	native DNA	denatured DNA
Control	7.5	13.5	4.8	6.3
+ dNTPs (0.2mM)	9.4	23.3	5.5	7.3
+ rNTPs (0.2mM)	9.5	30.6	5.1	10.5
+ rGTP (0.2mM)	9.5	21.0	4.5	7.9
+ rATP (0.2mM)	10.0	28.8	4.8	9.6
+ rCTP (0.2mM)	11.8	32.8	4.4	6.9
+ rUTP (0.2mM)	9.9	27.7	4.7	6.6

fractions under the conditions used.

5. RELATIONSHIP BETWEEN THE LARGE AND SMALL DNA POLYMERASE SPECIES OF L929 CELLS

When a nuclear-located, native DNA-preferring, DNA polymerase activity was discovered the soluble, or cytoplasmic, denatured DNA-preferring species was considered to be an 'altered' form of the 'intact' replicating conformation (Keir, 1965). This led to suggestions of a structural relationship between the two types of activity. On finding that the soluble enzyme was very much larger than the nuclear native DNA-preferring enzyme, it was considered possible that the smaller species may be bound up within the larger one and in some way rendered less able to use native DNA as template. This led to various speculations as to what was responsible for the size of the larger species and for its loss of ability to use native DNA. As the number of actual enzyme molecules present as each species is not known, it is realised that it is only speculation that the enzyme loses the ability to use native DNA and not that it gains the ability to use denatured DNA. However, the ability to produce smaller active fragments, similar in size to the smaller nuclear species, by treatment of the soluble enzyme would be good evidence in favour of a structural relationship between the two types of activity.

Two approaches were adopted to this end. Firstly, assuming that the soluble species represented a precursor of the nuclear one, an activity capable of facilitating an in vivo conversion was sought in cell fractions. Secondly, other more indirect physical, chemical and enzymic methods were employed to probe the biochemical nature of the soluble species. The criterion for conversion was the elution of DNA polymerase activity, on Sephadex G-200, in the region of the

smaller nuclear species i.e. ~70,000, after subjection of a high-speed supernatant preparation to various treatments. Before application to the column, treated samples were checked for recovery of activity and a change of template preference. If the column profile of the treated sample showed other than expected recoveries or appearance of smaller active fragments, the untreated control sample was tested on the column.

5.1 Conversion activity in cell fractions

Fig. 8a shows the Sephadex G-200 profile of a high-speed supernatant fraction which has been self-incubated for 1 h at 37°C. There is no significant appearance of DNA polymerase activity in the fractions eluting after the void volume peak of activity suggesting that there is no activity capable of converting the soluble enzyme into smaller fragments in the soluble fraction itself. Preferential loss of denatured DNA-primed activity is found. This confirms previous findings (Henderson, M.A.L., Ph.D. Thesis, University of Glasgow, 1972), that the DNA polymerase activity from nuclei of L929 cells is more thermolabile when assayed with denatured DNA than with native DNA.

The Sephadex G-200 profile of a high-speed supernatant preparation which has been incubated for 1 h at 37°C in the presence of a nuclear fraction from logarithmically growing cells is shown in Fig. 8b. The nuclei were pre-extracted with 0.4M KCl to remove all nuclear DNA polymerase species which might give rise to artefactual conversion activity. As Fig 8b shows, no conversion was detectable ruling out the presence of such an activity in nuclear preparations treated this way. It is realised that the activity sought may have been extracted from nuclei by 0.4M KCl, but difficulty in reproducing the pattern and amount of the smaller nuclear DNA polymerases on Sephadex G-200 would detect only a large increase in

Fig. 8

Sephadex G-200 profiles of DNA polymerase activity present in soluble fractions after incubation with certain cell fractions

Concentrated high speed supernatant fractions (S2) from logarithmically growing cells, prepared as previously described, are treated as shown below and a 1 ml sample fractionated on Sephadex G-200 as for Fig. 2. The resultant fractions are assayed for DNA polymerase activity.

(a) Self-incubated for 1 h at 37°C.

(b) Incubated for 1 h at 37°C with nuclei, obtained from the same cells as the supernatant fraction, which have been exhaustively extracted with 0.4M KCl using Extraction Method I, Methods, section 3.7. After incubation the nuclei were removed by centrifugation at 800 g for 10 min.

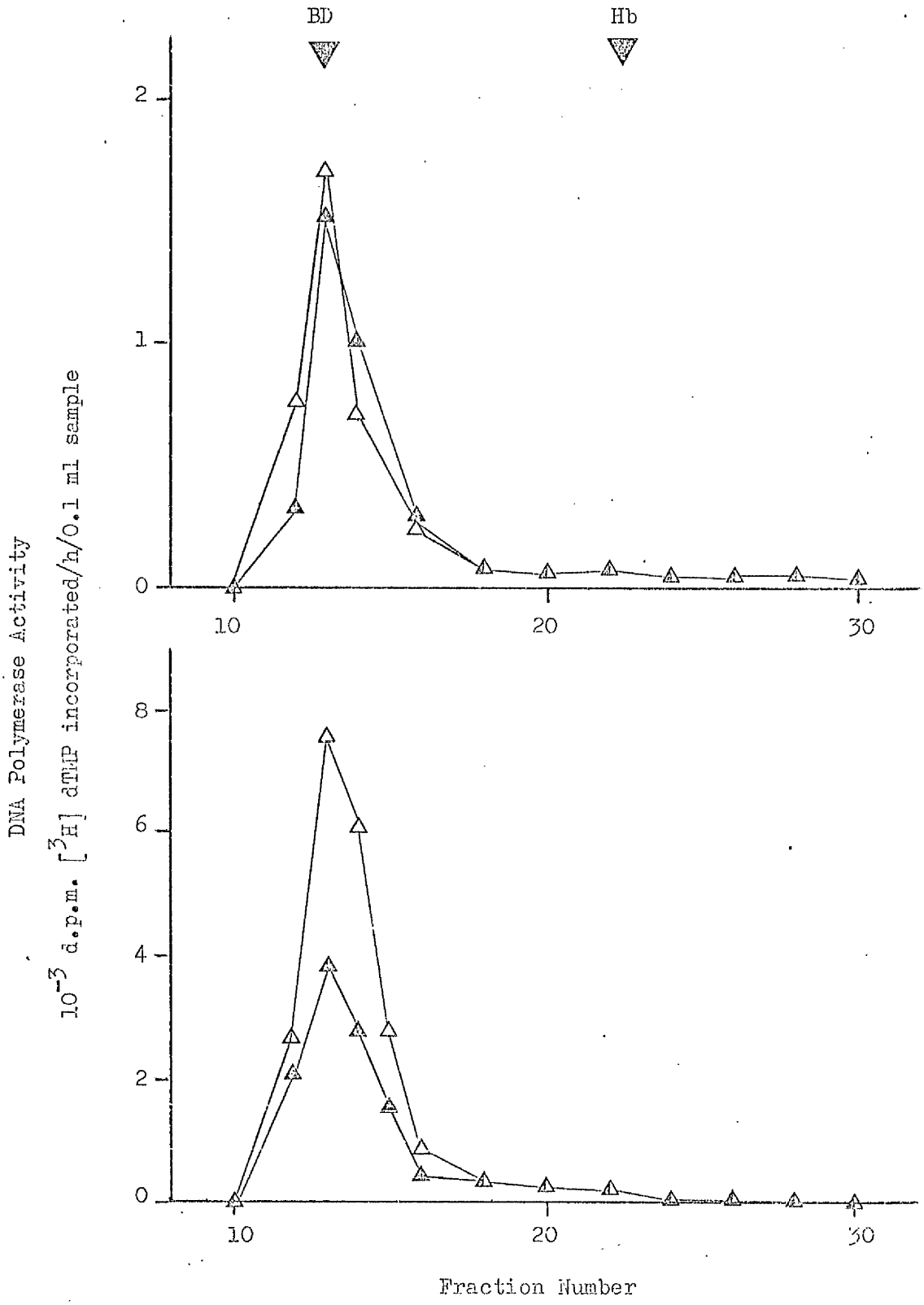
—▲— DNA polymerase activity with native DNA

—△— activity with denatured DNA

Note: (a) and (b) are different high speed supernatant preparations

Markers as in Fig. 2.

Fig. 8


BD
Hb
2
1
0
10
20
30
8
6
4
2
0
10
20
30
DNA Polymerase Activity
10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample
Fraction Number

such species. The situation is more desirable where no small species are detectable initially as with a high-speed supernatant fraction.

The approach does not rule out binding of the newly-generated small species within the nuclei which are sedimented from the sample prior to column application. Activity recoveries however suggest that no great loss in soluble activity is occurring.

5.2 Biochemical nature of the soluble species and conversion by indirect means

Ultracentrifugation and agarose gel chromatography studies in this and other laboratories have suggested that the soluble species in eukaryotic cells is very large and may in fact be associated with cellular material such as membrane (Haines et al., 1971; Holmes & Johnston, 1973; Baril et al., 1970, 1971). Burriss-Garret & Bollum (1973) disagree with this and claim that the finding of the soluble DNA polymerase activity associated with large particles is created by the concentrative method of isolation used by the other workers. The apparent association gives the activity a high M.W. which may be in the range of $0.5 - 1.0 \times 10^6$ or even higher.

Fig. 9 shows a Sephadex G-200 profile of the DNA polymerase activity in a high-speed supernatant fraction from L929 cells which has been pre-treated with 0.5M KCl and eluted from the column using standard column buffer containing 0.5M KCl. As demonstrated in Fig. 9, when fractionated by gel filtration in the presence of 0.5M KCl, soluble activity loses its activity completely but no smaller species are generated. However, if the salt concentration is reduced without separation, then more than 70% of the activity is recoverable (Inset, Fig. 9). Therefore 0.5M KCl appears to dissociate some necessary factor from the soluble activity. These results agree with the findings of Haines et al., (1971) who show that the soluble activity

Fig. 9

Fractionation of soluble DNA polymerase activity on Sephadex G-200 in the presence of 0.5M KCl

A 1 ml aliquot of a high speed supernatant fraction (S2), prepared as before, is dialysed against 100 vols of standard column buffer with KCl at a concentration of 0.5M, and then fractionated on Sephadex G-200 as before, in the presence of 0.5M KCl.

As control, a 1 ml sample of the same S2 fraction is treated exactly as described in Fig. 2.

The resulting fractions from both columns are then assayed for DNA polymerase activity (with the appropriate additions to ensure that the final concentration of KCl in all assays is the same).

—△— activity with native DNA

—△— activity with denatured DNA

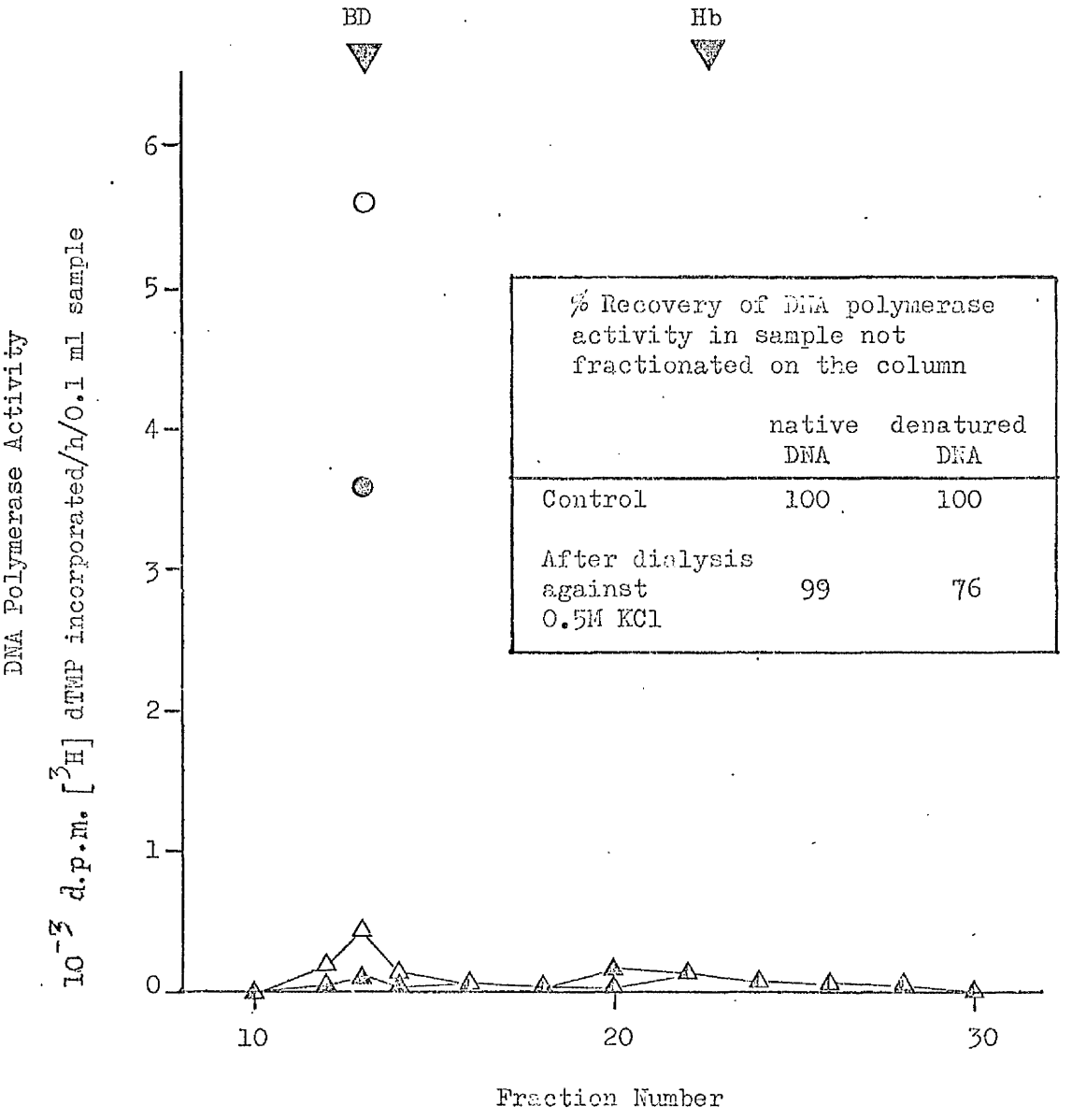
of fractions from column run in 0.5M KCl.

The control profile is similar to that in Fig. 2 and so only the DNA polymerase activity of the peak tube with native (●) and denatured (○) DNA is shown.

The inset opposite shows the % recovery of DNA polymerase activity after treatment with 0.5M KCl but without fractionation.

Markers are as in Fig. 2.

Fig. 9



% Recovery of DNA polymerase activity in sample not fractionated on the column		
	native DNA	denatured DNA
Control	100	100
After dialysis against 0.5M KCl	99	76

from rat liver is severely inhibited by fractionation on agarose gels in 2M NaCl. However several investigators using the procedure of Bellair (1968) find substantial amounts of activity after gel filtration in the presence of 1M NaCl (Furlong & Gresham, 1971; Ove et al., 1969).

Figs 10a and 10b show that treatment with pancreatic RNase or phospholipase for 1 h at 37°C is unable to release small active fragments from the soluble enzyme.

Sodium deoxycholate (DOC), a detergent known to disperse lipoprotein complexes, has an inhibitory effect on the soluble enzyme, more so with a denatured DNA template (Fig. 11). Inclusion of DOC, at slightly inhibitory concentration (0.03%) in the column buffer does not result in production of nuclear-like species (Fig.12). RNase and DOC both give rise to a shoulder eluting 1 or 2 fractions after the void volume peak of soluble activity. Although the significance of this minor activity remains to be assessed, its existence does not affect the conclusion that no nuclear-like activity is generated.

Attempts at better separation of the nuclear species by Sephadex 4B gel filtration in order to investigate this intermediate region proved unsuccessful. However by reference to a urease marker (480,000 daltons) it was shown that peak I nuclear DNA polymerase has at least a M.W. of 500,000 daltons (Fig.13).

To demonstrate whether the soluble activity was associated with low density lipoprotein (resistant to phospholipase) such as membraneous material, sucrose gradient density flotation studies were done. As is shown in Table 5, no DNA polymerase is associated with material of the density of mitochondria or less (e.g. smooth membrane).

The foregoing results suggest that the soluble species probably

Fig. 10

Sephadex G-200 profiles of soluble DNA polymerase activity after enzymic treatment

Aliquots of a concentrated S2 fraction, prepared as before, are incubated for 1 h at 37°C with

(a) 50 µg/ml pancreatic ribonuclease

(b) 50 µg/ml phospholipase

1 ml samples are then fractionated on Sephadex G-200 as described for Fig. 2, and the fractions assayed for DNA polymerase activity

—△— with native DNA

—△— with denatured DNA

(▩) and (□) show the RNA concentration in the void volume fraction, before and after ribonuclease treatment respectively.

Markers are as in Fig. 2.

Fig. 10

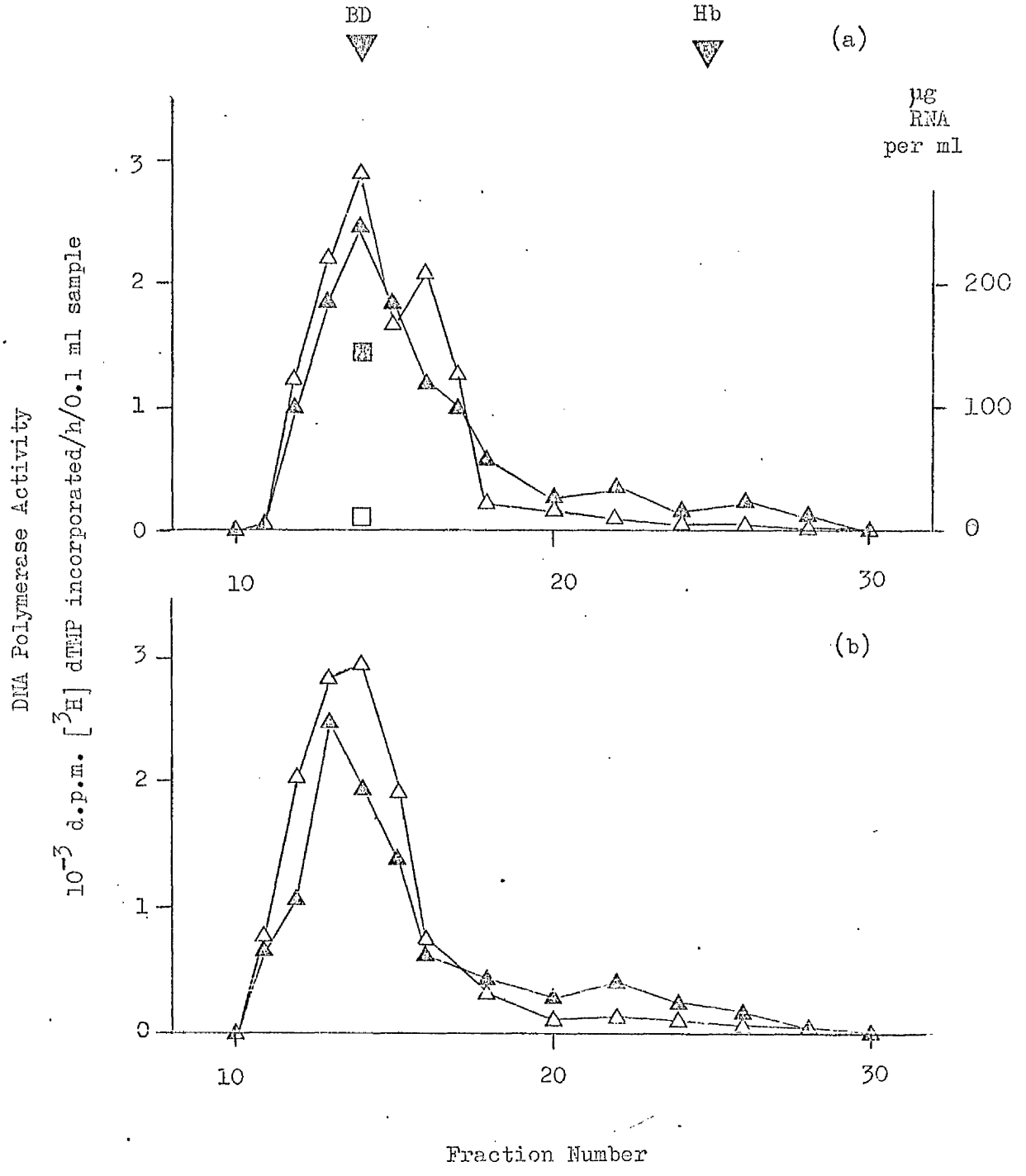

BD
Hb
(a)
µg RNA per ml
200
100
0
3
2
1
0
10
20
30
(b)
DNA Polymerase Activity
10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample
Fraction Number

Fig. 11

Effect of sodium deoxycholate on the soluble DNA polymerase activity

The DNA polymerase activity of an S2 fraction, prepared as before, is assayed with sodium deoxycholate (DOC) present at the concentrations shown opposite.

—▲— DNA polymerase activity with native DNA

—△— activity with denatured DNA

Fig. 11

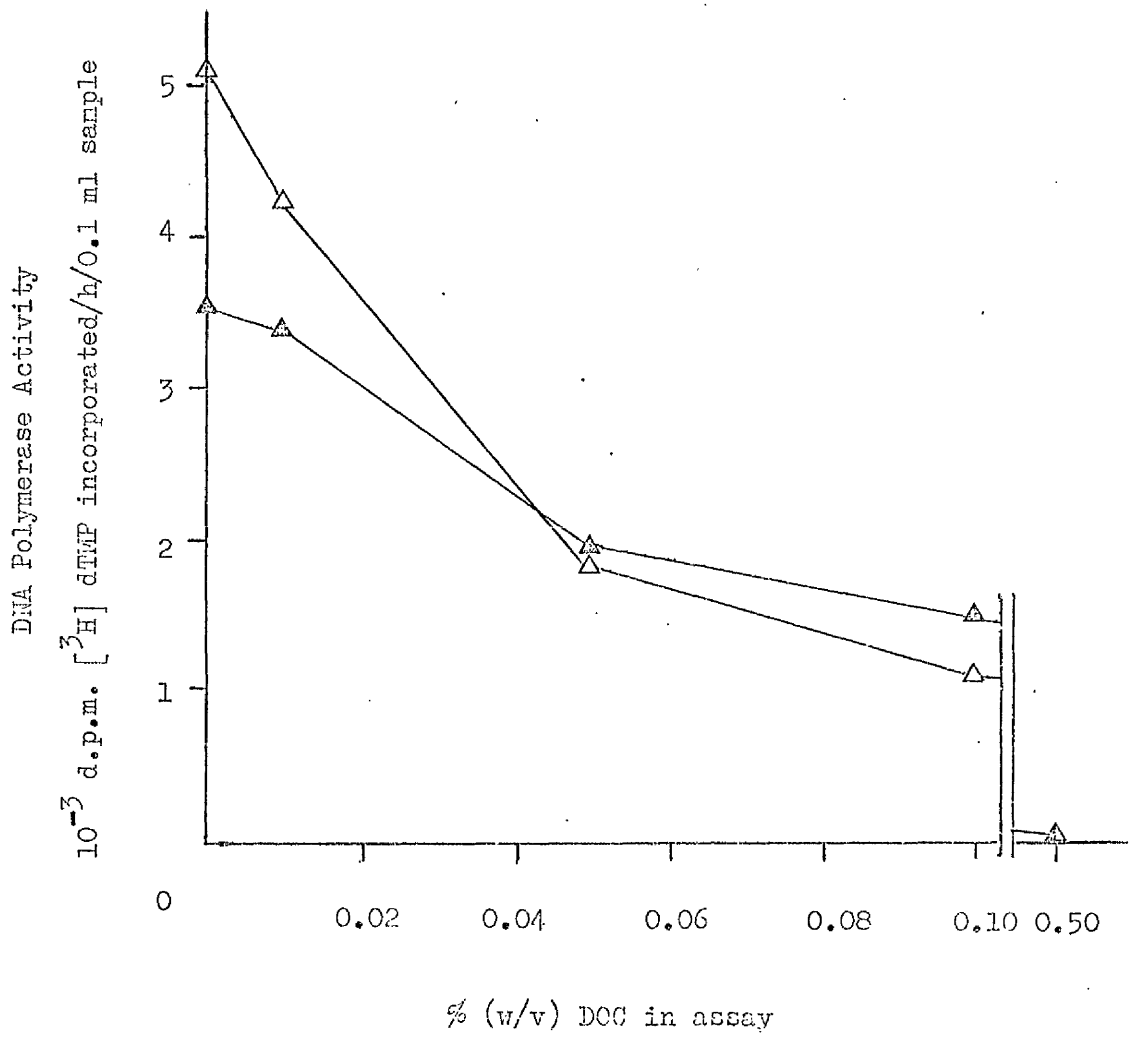

DNA Polymerase Activity
10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample
5
4
3
2
1
0
0.02
0.04
0.06
0.08
0.10
0.50
% (w/v) DOC in assay

Fig. 12

Fractionation of soluble DNA polymerase activity on Sephadex G-200 in the presence of sodium deoxycholate

A concentrated S2 fraction, prepared as before, is made 0.03% (w/v) with respect to sodium deoxycholate (DOC) by the addition of an appropriate amount of 10% (w/v) DOC solution. A 1 ml sample is then fractionated on Sephadex G-200 as before with 0.03% (w/v) DOC present in the standard column buffer. The resulting fractions are assayed for DNA polymerase activity.

—△— with native DNA

—△— with denatured DNA

—△— denotes coincident points

Markers are as before.

Fig. 12

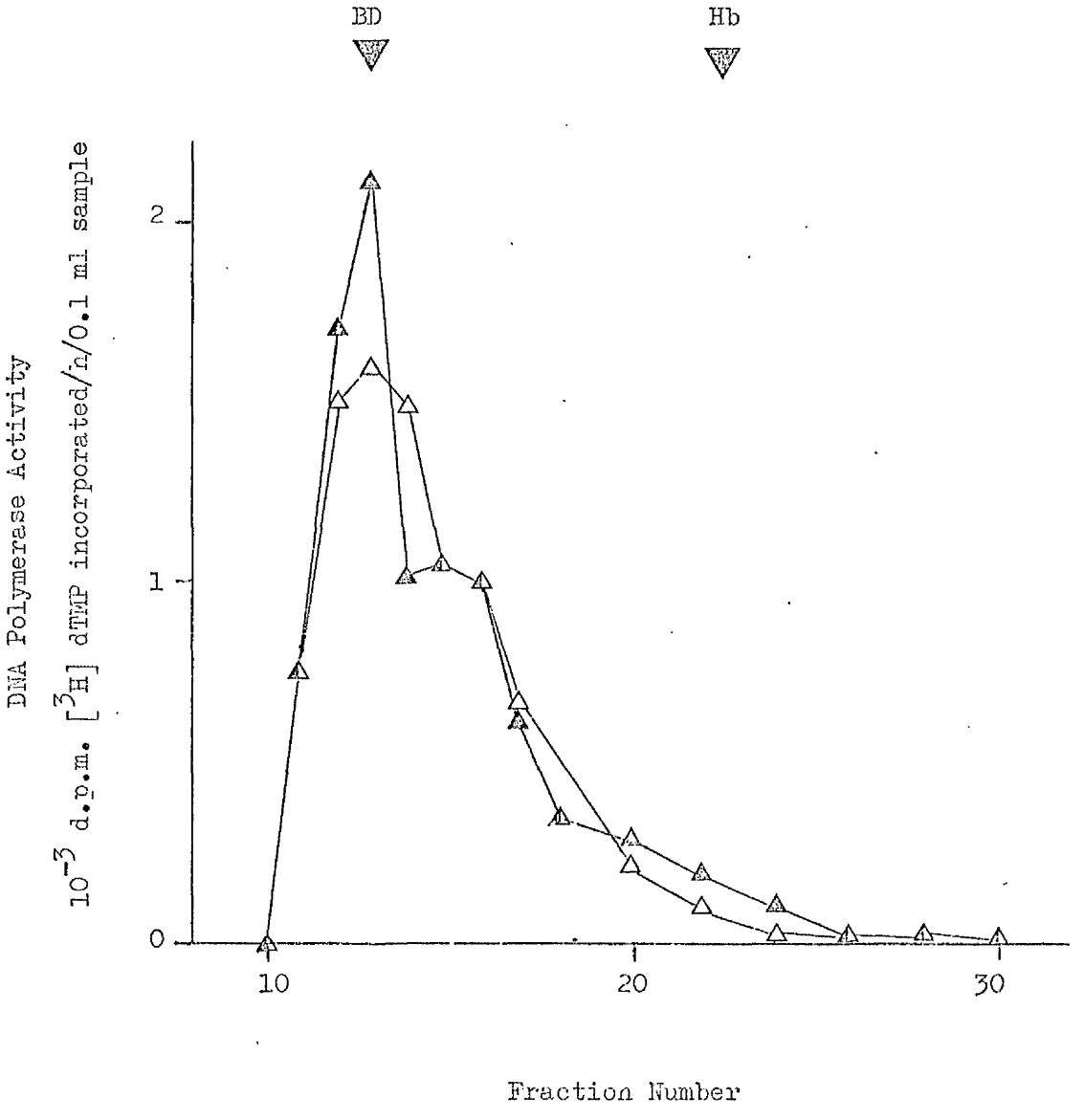

BD
Hb
DNA Polymerase Activity
10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample
2
1
0
10
20
30
Fraction Number

Fig. 13

Fractionation of DNA polymerase activity present in a 0.4M KCl extract of nuclei by gel filtration on Sepharose 4B

A 0.4M KCl nuclear extract is obtained as previously described and fractionated on Sepharose 4B as described in Methods, section 5.2. The fractions obtained are assayed for DNA polymerase activity.

—⊙— with native DNA

—○— with denatured DNA

—◐— denotes coincident points

—◇— concentration of protein

The fraction in which a urease marker (M.W. 480,000 daltons) elutes is marked thus (▽).

Fig. 13

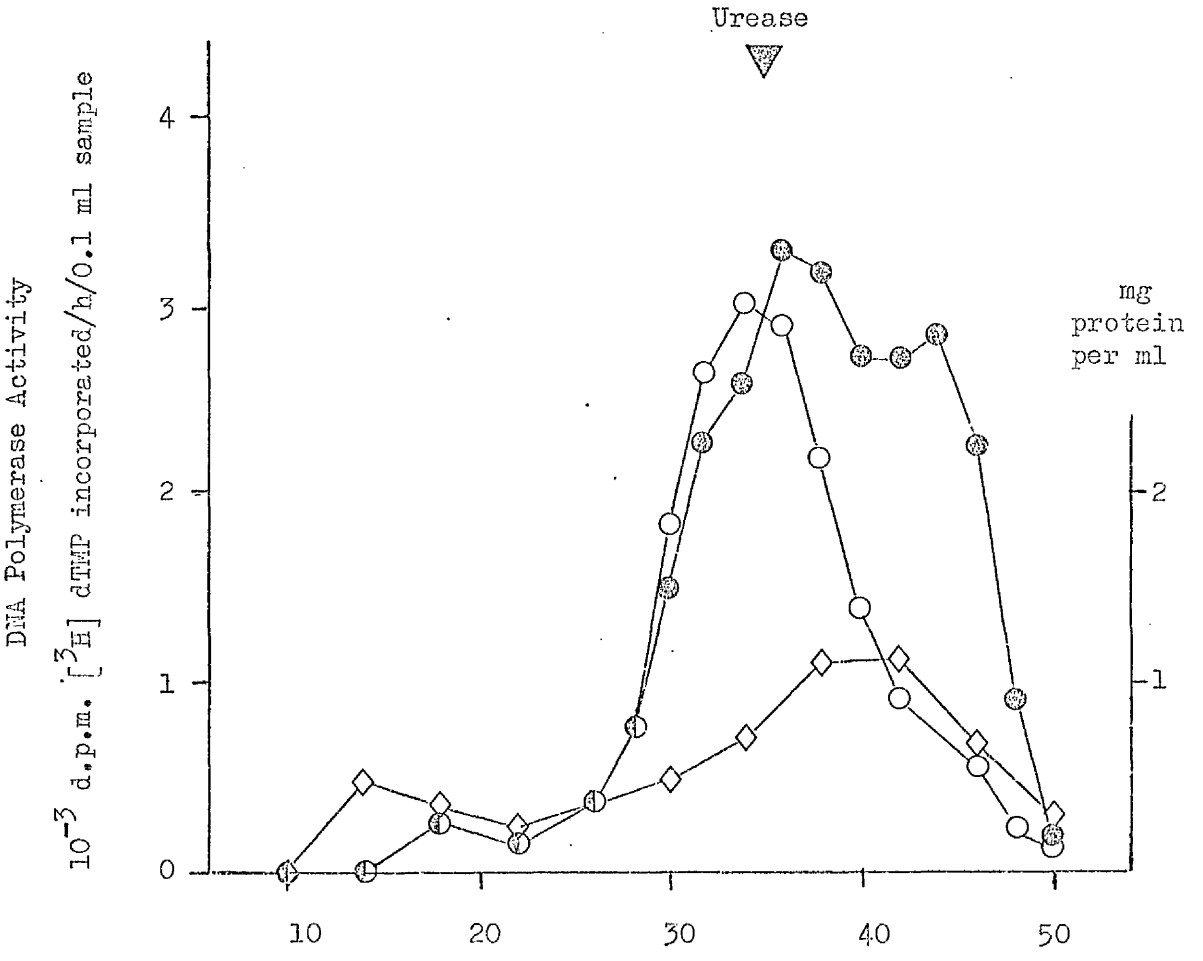

Urease
DNA Polymerase Activity
10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample
mg protein per ml
0
1
2
3
4
10
20
30
40
50

Table 5

Fractionation of DNA polymerase activity present in a soluble fraction by discontinuous sucrose gradient density flotation

A sample of concentrated S2, obtained as before, is made 60% (w/v) with respect to sucrose and overlaid carefully and consecutively with 45, 35, 25 and 10% (w/v) sucrose. The total sample is then centrifuged for 1 h at 100,000 *g* using a Beckman SW 40 rotor. A 1 ml sample is carefully removed, using a pasteur pipette, from each sucrose interface and assayed for DNA polymerase activity.

Sucrose interface	Density	Total DNA polymerase activity (10^{-3} d.p.m. [^{3}H] dTMP incorporated per hour)	
		native DNA	denatured DNA
10-25%	$>1.045<1.106$	zero	zero
25-35%	$>1.106<1.145$	zero	zero
35-45%	$>1.145<1.218$	zero	zero
60%	$\geqslant 1.218$	68	297
Original sample	-	111	767

A parallel sample was run using a low-speed supernatant fraction (S1) obtained as described in Fig. 1. By assaying the corresponding sucrose interface fractions for cytochrome oxidase activity (Methods, section 4.5) mitochondria were shown to band at the 35-45% (w/v) sucrose interface (i.e. a density $>1.145<1.218$)

represents a protein aggregate (salt-dissociable) with perhaps traces of lipid (DOC results), although DOC could also dissociate hydrophobic protein interactions. The results diminish the possibility of low M.W. nuclear DNA polymerase existing as a sub-unit of the soluble species unless any conversion occurring, due to loss in soluble activity, results in a low M.W. species of dramatically reduced activity (i.e. with 0.5M KCl).

6. EFFECT OF DNA SYNTHETIC CHANGES ON THE DNA POLYMERASE SPECIES

Lindsay et al. (1970) have reported changes in the DNA polymerase activity exhibited by soluble and nuclear preparations of L929 cells, as the cells entered the DNA synthetic (S) phase from a previously quiescent phase where very little DNA synthesis was occurring. Their results showed that the DNA polymerase activity in the soluble fraction fell during S-phase whereas the nuclei gained activity during S-phase, more so with a denatured DNA template, confirming previous findings (Littlefield et al., 1963; Gold & Helleiner, 1964).

Gel filtration showed that only the large denatured DNA-preferring species was present in the soluble fraction throughout the growth changes described above.

Comparison of the Sephadex G-200 profiles of 0.4M KCl extracts of nuclei from stationary and S-phase cells demonstrates that the increased DNA polymerase activity of nuclei from S-phase cells, in the presence of denatured DNA, is due to increased peak I activity (Figs. 14a,b). In contrast the levels of the smaller native DNA-preferring peaks remain relatively unaffected by the change in DNA synthetic ability of the cell. These results, together with the previous findings, suggest that soluble DNA polymerase becomes associated with the nucleus when cells are synthesising DNA.

Fig. 14

Fractionation on Sephadex G-200 of DNA polymerase activity present in 0.4M KCl nuclear extracts of stationary and S-phase cells

Nuclei (N3) were prepared as before from equal numbers of stationary and S-phase cells, grown as described in Methods, section 3.2 and 3.3 respectively, and the 0.4M KCl extracts obtained therefrom (Extraction Method I, Methods, section 3.7) fractionated on Sephadex G-200 as previously described. The resulting fractions are assayed for DNA polymerase activity.

(a) S-phase cells

(b) Stationary cells

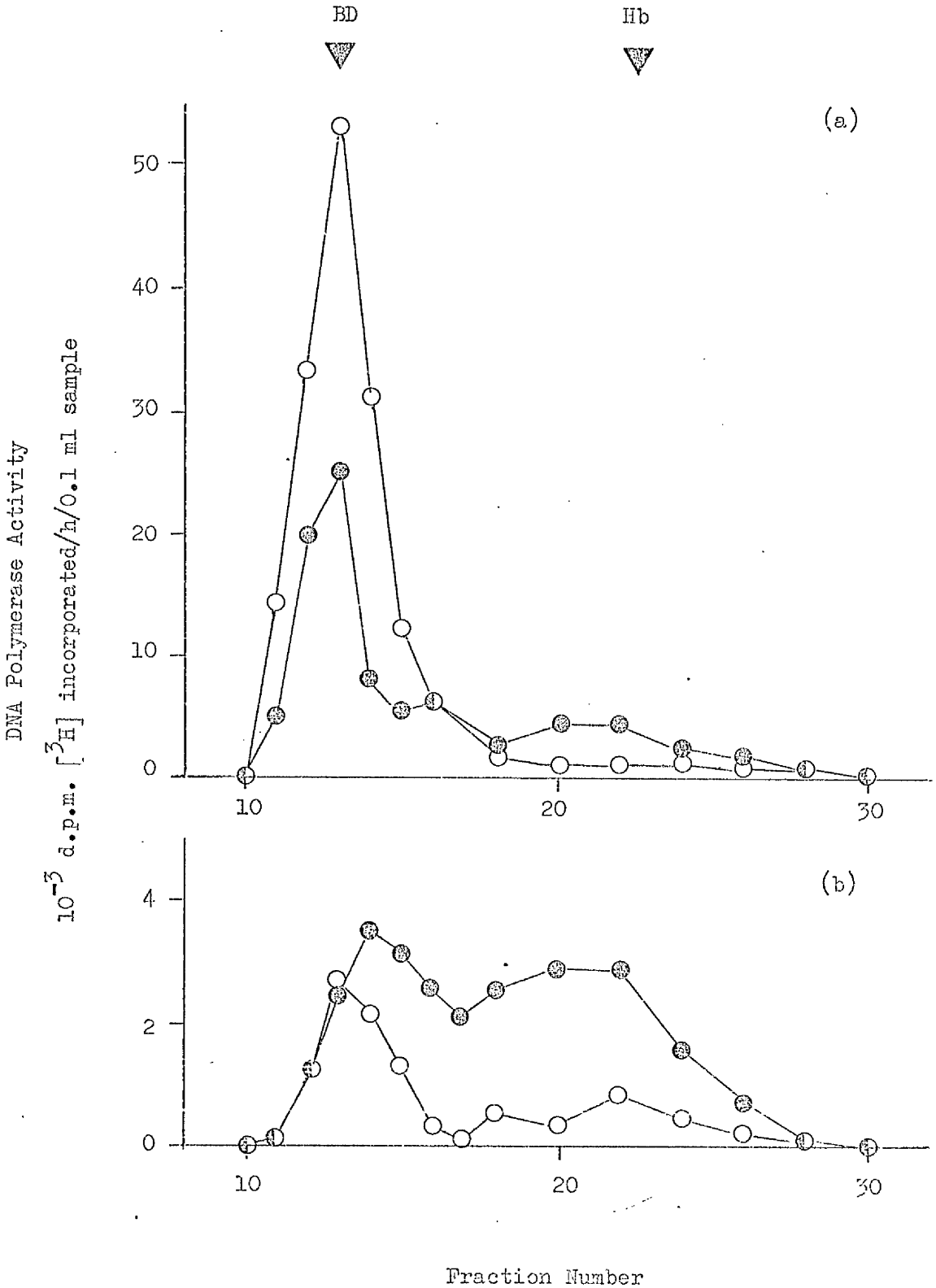
——●—— DNA polymerase activity with native DNA

——○—— with denatured DNA

——◑—— denotes coincident points

Markers are as before.

Fig. 14


BD
Hb
(a)
(b)
50
40
30
20
10
0
4
2
0
10
20
30
DNA Polymerase Activity
10^{-3} d.p.m. [^{3}H] incorporated/h/0.1 ml sample
Fraction Number

As a check to ensure that the increased amount of peak I activity in nuclei from S-phase cells was not simply due to contamination from the higher level of soluble DNA polymerase activity present in cytoplasm from S-phase cells, compared to that in stationary phase cells (Lindsay et al., 1970), a control cross-mixing experiment was carried out. Table 6 shows the effect on eventual nuclear DNA polymerase activity of exchanging the low-speed supernatant fractions, after the first nuclear sedimentation, during a parallel preparation of nuclei from stationary and logarithmically growing cells. As can be seen, nuclei from stationary cells do not exhibit significantly increased DNA polymerase activity when their isolation involved mixing with a low-speed supernatant from logarithmically growing cells containing substantial DNA polymerase activity. Similarly nuclei from logarithmically growing cells do not lose activity when mixed with supernatant from stationary cells during isolation. Therefore the increased peak I activity typical of nuclei from S-phase cells does not seem to be freely diffusible from cytoplasm to nucleus or vice versa under the conditions used. It is realised that contamination could occur before cell breakage, but the results of Lindsay et al. (1970) would disagree with this. They show that 24 h after release from stationary phase when the ability of nuclei to use denatured DNA has declined, the soluble DNA polymerase activity is even higher than during S-phase.

7. ABILITY OF SOLUBLE DNA POLYMERASE ACTIVITY TO BIND TO DNA

The possible association of soluble DNA polymerase with the nucleus has prompted experiments to discover the nature of the binding and why it increases during the period of DNA synthesis. One possibility is that the DNA, normally unavailable because of masking by histones and other nuclear proteins, becomes unmasked at

Table 6

Binding of soluble DNA polymerase activity to nuclei during preparation

Cell suspensions from both logarithmically-growing and stationary cultures, grown as before, are homogenised as usual (Methods, section 3.4). Duplicate aliquots are centrifuged as in Fig. 1, and the S1 fractions of one of the duplicates of the differing homogenates interchanged. After resuspension and 10 min equilibration, the nuclei are prepared as before. The resulting N3 and S2 fractions are assayed for DNA polymerase activity.

Fraction	DNA polymerase activity (10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample)	
	native DNA	denatured DNA
Stationary S2	< 20	< 20
Log. S2	580	1520
Stationary N3	640	< 20
Stationary N3 prepared via log. S1	680	120
Log. N3	1070	730
Log. N3 prepared via stationary S1	1220	930

specific regions enabling DNA polymerase to bind and to carry out its replicative function. This would predict a strong binding between 'naked' DNA and the enzyme.

Table 7 shows that soluble DNA polymerase activity is unable to bind to native salmon-testis DNA bound to a cellulose matrix even at very low ionic strength.

The results of further experiments, on the ability to sediment the soluble DNA polymerase as a DNA-enzyme complex, by centrifugation in low salt, are shown in Table 8. There is no increase in the percentage of soluble DNA polymerase activity sedimented in the presence of native or denatured DNA compared with a control containing no DNA. The control samples, however, showed that 60-70% of the activity was sedimented under the conditions used, suggesting that the soluble activity is of considerable size.

These results agree to some extent with the findings of Haines et al., (1971) for a partially pure rat liver soluble DNA polymerase preparation. They find a variable ability of the enzyme to bind to DNA-cellulose columns, either failing to bind at all or eluting at low ionic strength. Yoneda & Bollum (1965) also failed to detect binding of calf thymus soluble enzyme to native or denatured DNA, in low salt, on sucrose gradients.

8. EFFECT OF VARIOUS TREATMENTS ON THE SEDIMENTATION OF SOLUBLE DNA POLYMERASE ACTIVITY

As shown in the previous section, 60-70% of the DNA polymerase activity in a high-speed supernatant preparation is sedimented by centrifugation at 105,000 g for 7.5 h.

Fig. 15 shows the effect of KCl concentration on the percentage DNA polymerase activity sedimented. In the absence of KCl, 95% of the activity is sedimented whereas at 0.25M KCl, 70%

Table 7

Binding of soluble DNA polymerase to DNA cellulose

DNA cellulose chromatography (using native salmon testis DNA) is carried out as described in Methods, section 5.3. The sample is applied at zero KCl.

Fraction	Total DNA polymerase activity (10^{-3} d.p.m. [^{3}H] dTMP incorporated per hour)	
	native DNA	denatured DNA
Applied sample (S2)	105.2	463.4
Pooled unbound eluate	196.2	506.3
Fractions eluted with gradient from 0-0.7M KCl	zero	zero

Table 8

Sedimentation of soluble DNA polymerase activity attached to DNA

Native and denatured salmon testis DNA are added to equal aliquots of an S2 fraction (800 µg/ml final concentration) and the sample centrifuged at 105,000 *g* for 7.5 h to pellet the DNA. The percentage DNA polymerase activity pelleted is determined.

Sample	% DNA pelleted	% DNA polymerase activity pelleted	
		native DNA	denatured DNA
Control S2	-	59.6	70.3
+ native DNA	100	60.1	71.5
+ denatured DNA	93	58.5	57.0

Fig. 15

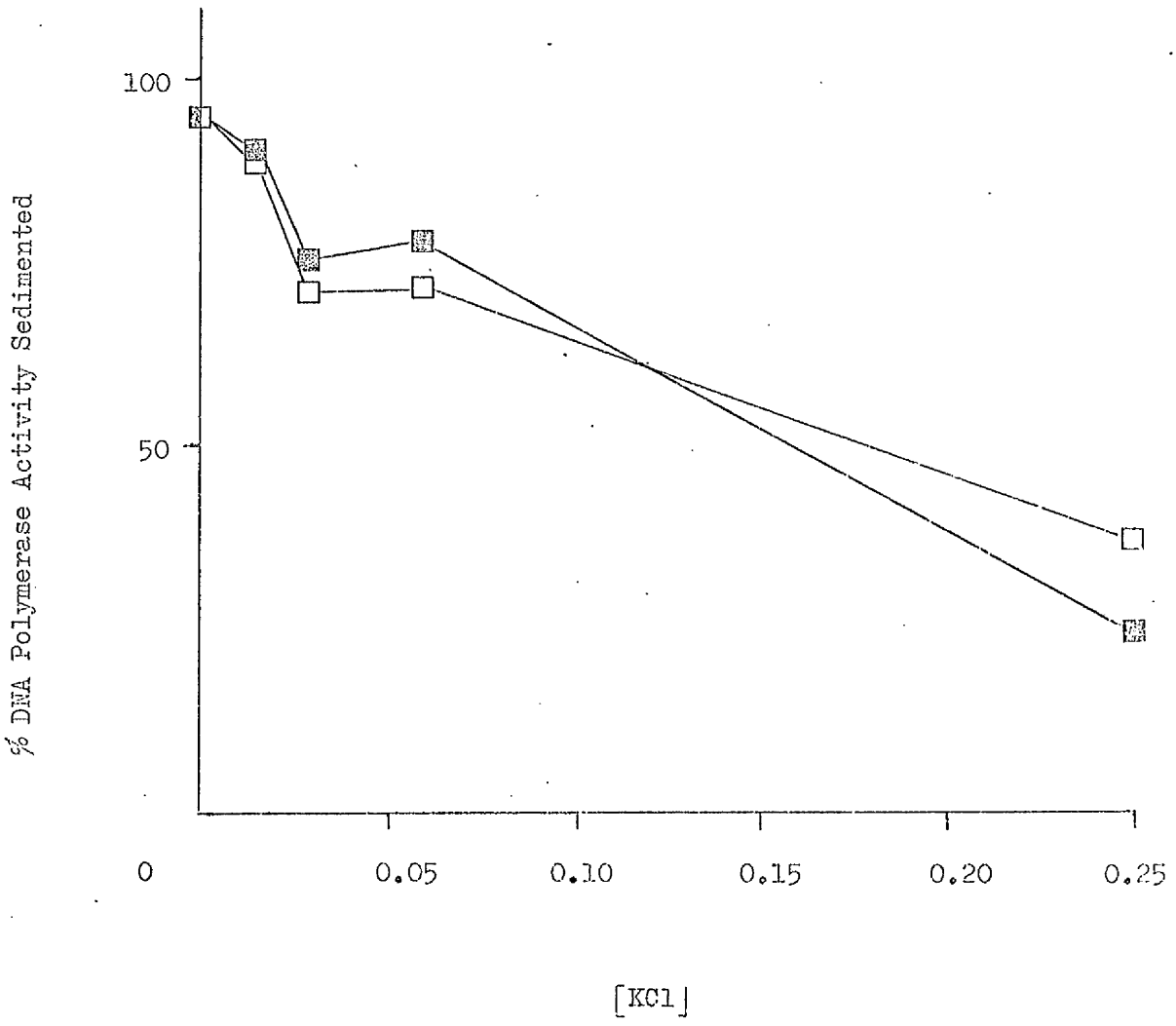
Effect of KCl concentration on the sedimentation of soluble DNA polymerase activity

Duplicate aliquots of an S2 fraction are adjusted to the KCl concentrations shown opposite. One of the duplicates is centrifuged at 105,000 g for 12 h and the DNA polymerase activities of the resulting supernatant fractions together with that of the un-centrifuged control determined. The percentage DNA polymerase activity sedimented at each KCl concentration is calculated .

——▩—— with native DNA

——□—— with denatured DNA

Fig. 15


% DNA Polymerase Activity Sedimented
100
50
0
0.05
0.10
0.15
0.20
0.25
[KCl]

of the activity remains in the supernatant after centrifugation, suggesting that it may form part of a salt-dissociable aggregation. This approach to the effect of salt on the size of the soluble enzyme differs from that in Results, section 5.2 where treatment with 0.5M KCl caused almost complete loss of enzyme activity. The method used in this section does not involve separation of dissociable factors.

Baril et al. (1970, 1971) proposed that the soluble DNA polymerase activity from regenerating rat liver is associated with a smooth-membrane fraction as demonstrated by its ability to be sedimented by prolonged centrifugation (78,000gfor 15 h), and to band at the density of smooth membranes on discontinuous sucrose gradients. In a later study they showed that it could be released from this membrane association by treatment with a solution containing the detergent Triton X-100. However in our hands, Triton X-100 does not affect the percentage DNA polymerase activity sedimented in a high-speed supernatant fraction from L929 cells (Table 9). Surprisingly, when the effect of Triton X-100 on the activity of DNA polymerase in high-speed supernatant preparations is measured (Fig.16), it was found that concentrations up to 10% (v/v), in the assay, inhibit only the activity with denatured DNA. Moreover, the degree of inhibition is only to ~50% which is reached at 1% (v/v).

A possible explanation of this effect could be the formation of micelles which are no longer inhibitory to the reaction. Thus the concentration of free Triton X-100 molecules may never rise above 1% (v/v). The critical micellar concentration for Triton X-100 was reported to be in the range 0.05-0.1% (v/v) (Helenius & Simons, 1972).

Table 9

Effect of Triton X-100 on the sedimentation of soluble DNA polymerase activity

An aliquot of S2, prepared as before, is made 0.15% (v/v) with respect to Triton X-100 and together with an untreated aliquot are centrifuged for 4 h at 105,000 g.

	% DNA polymerase activity sedimented	
	native DNA	denatured DNA
Untreated S2	46 ± 14	77 ± 13
S2 + 0.15% (v/v) Triton X-100	65 ± 14	90 ± 6

(Standard deviations are based on the result of two separate experiments each done in duplicate)

Fig. 16

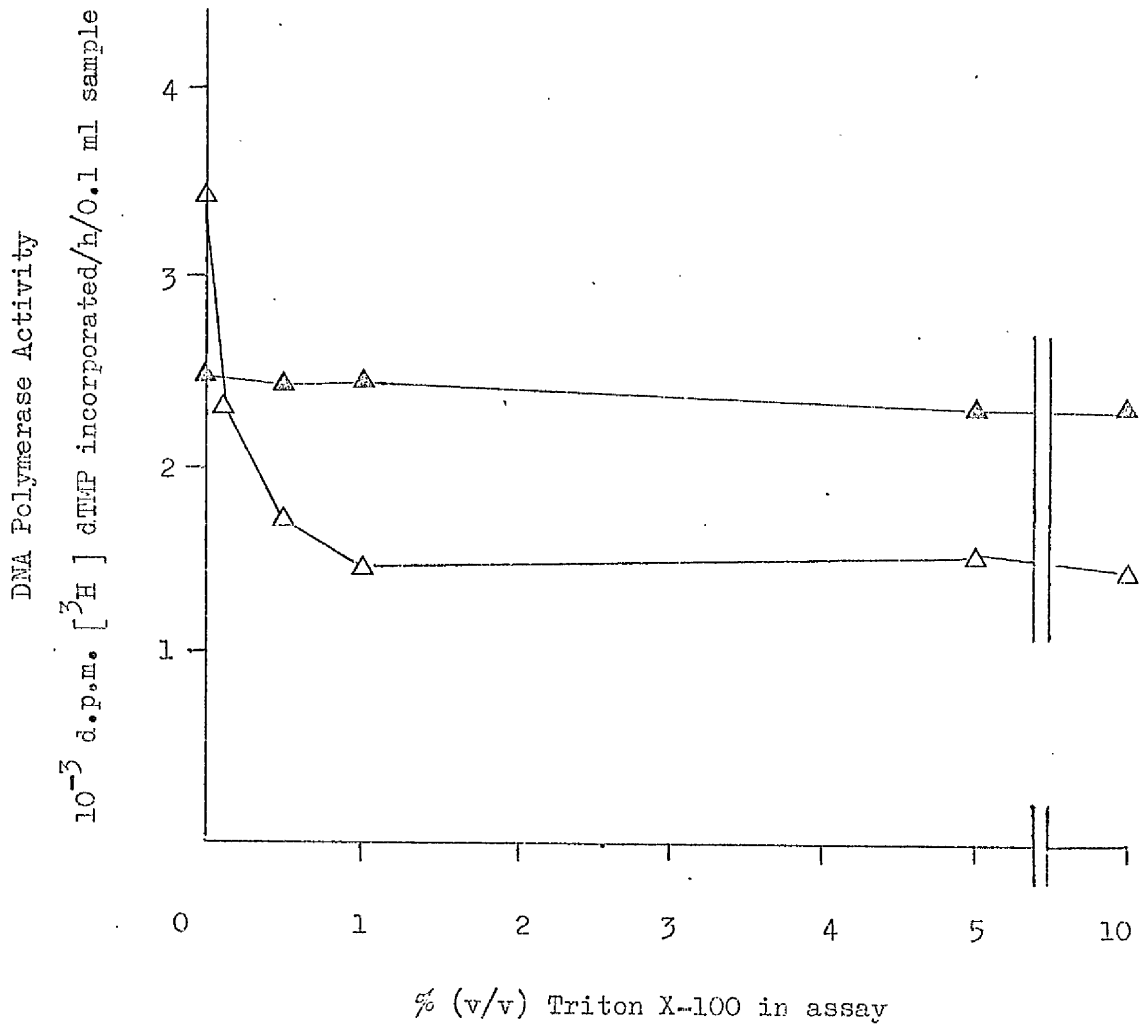
Effect of Triton X-100 on the soluble DNA polymerase activity

Increasing amounts of Triton X-100 are added to a standard assay of DNA polymerase activity present in a S2 fraction.

——△—— DNA polymerase activity with native DNA

——△—— with denatured DNA

Fig. 16


DNA Polymerase Activity
10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample
4
3
2
1
0
1
2
3
4
5
10
% (v/v) Triton X-100 in assay

The preferential inhibition by Triton X-100 of DNA polymerase activity with denatured DNA could reflect inhibition of a factor necessary for synthesis on a single-stranded DNA template or it could be affecting the assay indirectly merely by inactivating the denatured DNA template. Holmes & Johnston (1973) found that Triton X-100 rapidly inactivated a partially pure DNA polymerase enzyme from a rat liver supernatant fraction using an activated DNA template.

9. EFFECT OF KCl EXTRACTION ON THE ABILITY OF NUCLEI TO BIND DNA POLYMERASE ACTIVITY

The failure of DNA polymerase from a high-speed supernatant fraction to bind to DNA may suggest that another factor is necessary for the observed association with nuclei during S-phase. The synthesis of this factor may trigger the onset of DNA synthesis. The following set of experiments was designed to test if nuclei, after extraction with 0.4M KCl (shown previously to remove all assayable DNA polymerase activity) are capable of rebinding the DNA polymerase activity and, if so, if they are also capable of binding soluble activity. This latter capacity would reveal whether the predicted binding factor remains in the nucleus after extraction.

9.1 Ability of nuclei from S-phase cells to rebind DNA polymerase activity after KCl extraction

Fig 17a shows the effect of increasing KCl concentration on the amount of DNA polymerase activity extractable from nuclei of S-phase cells. Extraction is apparently complete by 0.4M KCl, as for nuclei from logarithmically-growing cells (See Table 3).

On reducing the salt concentration to zero by dialysis, the nuclei are capable of rebinding 40-60% of the DNA polymerase activity extractable by KCl concentrations in the range of 0.1-0.4M

Fig. 17

Extraction by KCl of DNA polymerase activity from nuclei of S-phase cells and the ability of the nuclei to rebind the extracted activity

Duplicate suspensions of nuclei (N3), prepared from S-phase cells as before, are extracted by dialysis (Extraction Method II, Methods, section 3.7) with the varying concentrations of KCl shown opposite. After dialysis, they are treated as follows:-

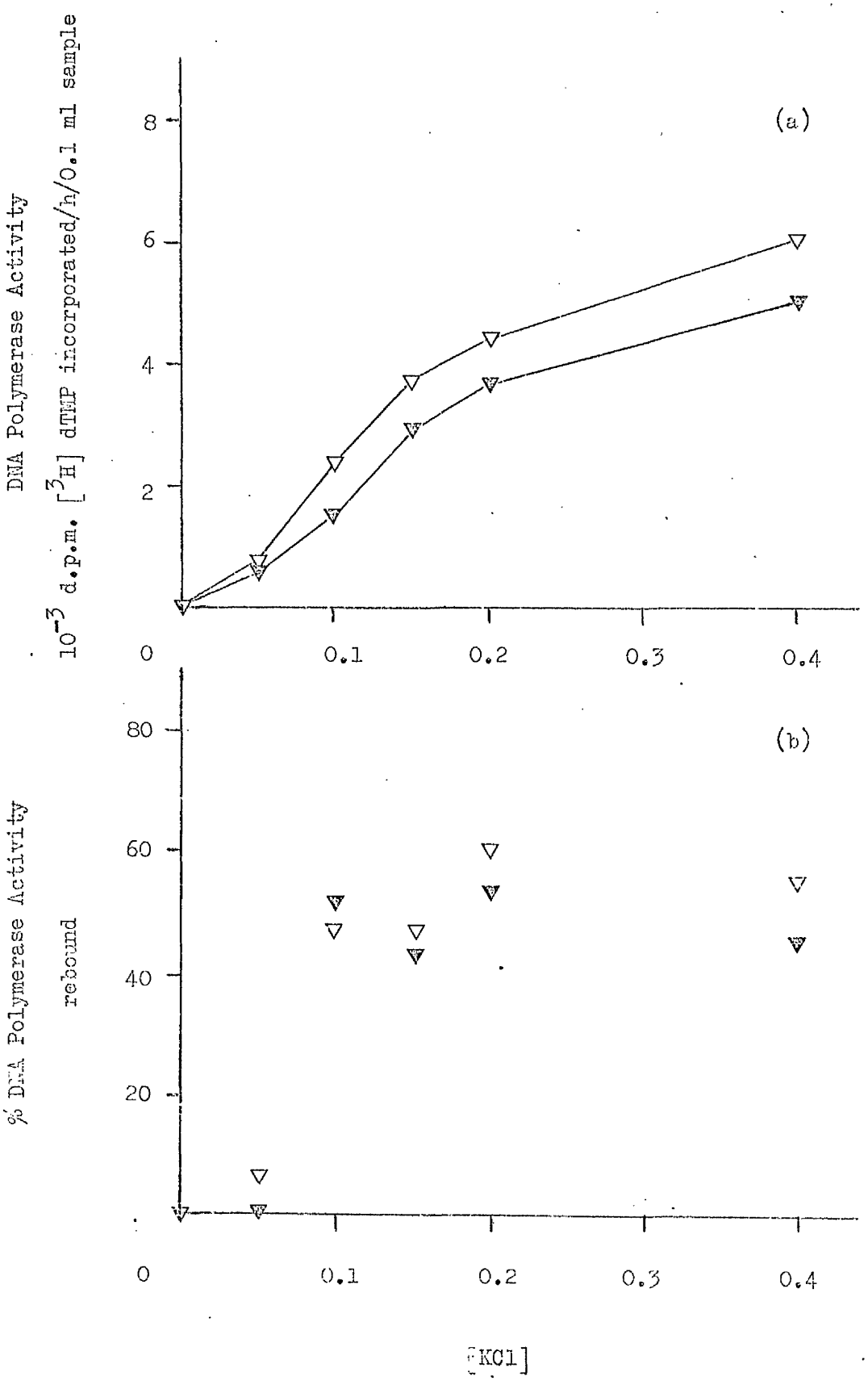
(a) One of the duplicates is centrifuged at 800 *g* for 10 min and the DNA polymerase activity in the extract determined.

(b) The other duplicate is further dialysed against 100 volumes of Buffer A alone (to reduce the salt) for 2x1h, then centrifuged as in (a) and the supernatant fraction assayed for DNA polymerase activity. The percentage DNA polymerase activity rebound by the nuclei is then calculated.

—▽— DNA polymerase activity with native DNA

—▽— with denatured DNA

Fig. 17


DNA Polymerase Activity
10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample
(a)
8
6
4
2
0
0.1
0.2
0.3
0.4
% DNA Polymerase Activity
rebound
(b)
80
60
40
20
0
0.1
0.2
0.3
0.4
[KCl]

(Fig. 17b). Above 0.4M KCl the nucleohistone complexes begin to disaggregate and the expanding DNA fibres destroy the nuclear integrity, as is seen by phase-contrast microscopic examination.

The ability of the nuclei to rebind DNA polymerase activity with a similar DNA template preference to that extracted, suggests that peak I nuclear activity is capable of rebinding, as well as the smaller nuclear species, which are known to have a greater binding affinity for DNA per se (Henderson, M.A.L., Ph.D. Thesis, University of Glasgow, 1972; Haines et al., 1971).

9.2 Ability of 0.4M KCl extracted nuclei from S-phase cells to bind soluble DNA polymerase activity

Fig. 18 reveals that nuclei from S-phase cells which have been extracted with 0.4M KCl, fail to bind significant DNA polymerase activity from a high-speed supernatant fraction. This pinpoints a fundamental difference between the enzyme in nuclear extract and supernatant preparations. It could signify the presence in extracts of a factor which is involved in the binding of high M.W. DNA polymerase to DNA.

9.3 The relative ability of 0.4M KCl extracted nuclei from stationary and S-phase cells to bind DNA polymerase activity from a 0.4M KCl nuclear extract of S-phase cells

Fig. 19 shows that 0.4M KCl extracted nuclei from stationary and S-phase cells are equally capable of rebinding DNA polymerase activity from a nuclear extract of S-phase cells, providing further evidence that the binding requirement resides in the nuclear extract from S-phase cells. Control experiments with nuclei from stationary cells which have not been extracted show that unless such nuclei have been previously extracted with KCl

Fig. 18

Ability of 0.4M KCl extracted nuclei from S-phase cells to bind soluble DNA polymerase activity

Nuclei (N3) from S-phase cells are exhaustively extracted with 0.4M KCl (Extraction Method II, Methods, section 3.7). Equal aliquots of an S2 fraction, which has been previously dialysed for 2x1h against 100 volumes of Buffer A + 0.4M KCl, are mixed with increasing amounts of the above nuclei, the highest amount of nuclei being derived from an equivalent number of cells as was the aliquot of S2. After 10 min equilibration, the suspensions are dialysed against Buffer A alone as above (to reduce the salt) and centrifuged at 800g for 10 min. The supernatant fractions are then assayed for DNA polymerase activity and the percentage bound in each case calculated.

—▽— with native DNA

—▽— with denatured DNA

Fig. 18

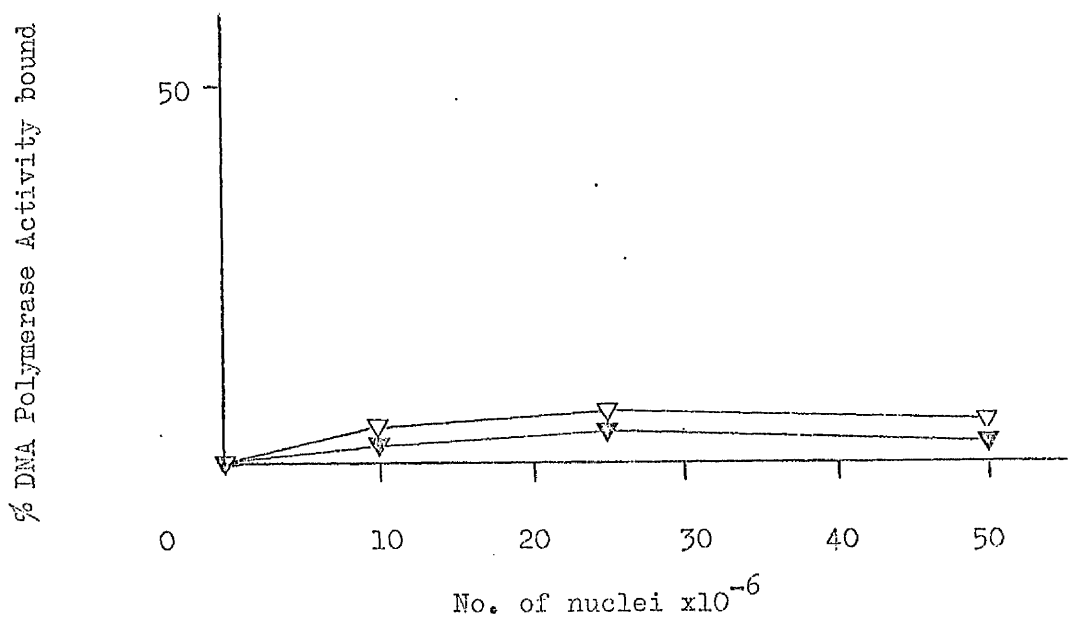
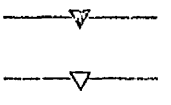

% DNA Polymerase Activity bound
50
0
10
20
30
40
50
No. of nuclei x10^{-6}

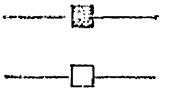
Fig. 19

Binding of DNA polymerase activity present in 0.4M KCl nuclear extracts from S-phase cells to nuclei from stationary and S-phase cells which have been pre-extracted with 0.4M KCl

Aliquots of a 0.4M KCl extract of nuclei derived from 20×10^6 S-phase cells are mixed with increasing amounts of 0.4M KCl exhaustively extracted nuclei (Extraction Method II, Methods, section 3.7), obtained from cells either in stationary or S-phase. The samples are then dialysed against Buffer A as before (to reduce the salt), centrifuged at 800 g for 10 min and the supernatant fractions assayed for DNA polymerase activity. The percentage bound in each case was calculated.



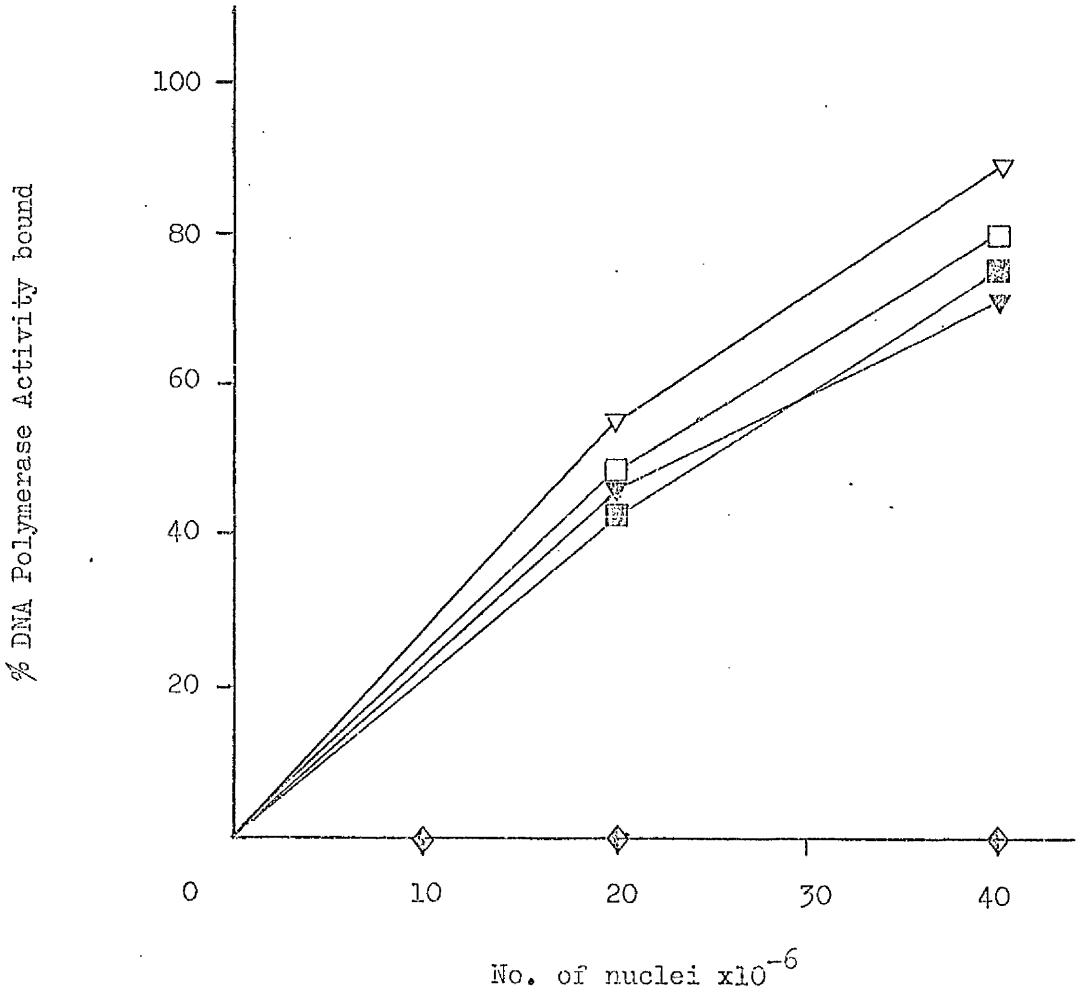
DNA polymerase activity bound by extracted S-phase nuclei — with native DNA; with denatured DNA



DNA polymerase activity bound by extracted stationary nuclei — with native DNA; with denatured DNA

DNA polymerase activity bound by unextracted stationary nuclei — with native DNA; with denatured DNA

Fig. 19


% DNA Polymerase Activity bound
100
80
60
40
20
0
10
20
30
40
No. of nuclei x10^{-6}

they will not bind DNA polymerase activity present in S-phase nuclear extract (Fig. 19). The significance of this finding is not clear. Lack of binding to unextracted nuclei from S-phase cells could merely be a result of saturation of binding sites, but nuclei from stationary cells contain very low levels of peak I nuclear activity, suggesting that some other factor is blocking binding. Removal of this factor (as achieved by 0.4M KCl extraction) could represent another pre-requisite before S-phase can be embarked upon.

10. DNA SYNTHESIS BY ISOLATED L929 NUCLEI

Nuclei isolated from S-phase cells have been shown to continue DNA synthesis for a short time in vitro in the absence of added DNA (Friedman & Mueller, 1968; Hershey et al., 1973a). Preliminary studies using nuclei from S-phase L929 cells prepared by the standard isolation technique revealed that nuclear preparations are able to synthesise DNA in the absence of added template DNA. The requirements for synthesis, (assay system A, Methods, section 4.4) are similar to those for DNA polymerase assays except that ATP is present, which was shown for other systems to be necessary for full expression of nuclear DNA synthetic ability.

Table 10 shows that homogenates exhibit 2-3 fold greater incorporation than once or twice washed nuclear pellets. The second wash does not appreciably reduce the incorporating ability of the nuclei, although the nuclei appear freer from cytoplasmic contamination as revealed by phase-contrast microscopic examination. Possible explanations for the higher incorporation by the homogenate could be the presence of soluble DNA polymerase activity utilising the endogenous DNA template, or other stimulatory factors reported to be present in the cytoplasm (Friedman & Mueller, 1968; Kidwell &

Table 10

DNA synthesis in the absence of exogenously added DNA by nuclear fractions at various stages of preparation

Nuclear fractions from S-phase cells are prepared as described in Fig. 1, and their endogenous DNA synthetic ability is measured using Assay System A (Methods, section 4.4).

Fraction	pmoles dTMP incorporated/30 min/mg DNA
Homogenate	69.0±20.0
N1 resuspended in buffered sucrose	24.4±5.0
N2 resuspended in buffered sucrose	25.2±3.5

Errors are a result of duplicate variation

Mueller, 1969; Hershey et al., 1973a). Nuclei from S-phase cells have a greater and more prolonged initial rate of DNA synthesis than do nuclei from stationary cells (Fig. 20). After a short time the rate of incorporation is reduced to a second rate, which is variable and does not appear to be dependent on the DNA replicative ability of the cells from which the nuclei are derived. After the initial growth dependent rate, synthesis could be due to incorporation at nicks in the DNA introduced during isolation and subsequent incubation (Kaufman et al., 1972; Hyodo & Ono, 1970a,b). Evidence for this derives from the fact that nuclei from stationary L929 cells can be made to increase their endogenous incorporating capacity by treatment with pancreatic DNase (R.L.P. Adams, personal communication).

10.1 Alternative assay conditions

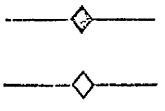
While this work was in progress, Hershey et al. (1973a), using an isolated nuclear system from HeLa cells, claimed to have achieved initial rates of endogenous incorporation approximating to the same order of magnitude as the *in vivo* rate of DNA synthesis in these cells. Such high levels had never been seriously approached by previous eukaryotic *in vitro* systems.

A comparison of the assay conditions of Hershey et al. (assay system B) with those already described (assay system A), is shown in Fig. 21. It is seen that nuclei exhibit much greater endogenous DNA synthesis using the conditions of Hershey et al. The main difference between the two systems is the higher ionic strength and ATP concentration of assay system B, both shown by Hershey et al. to be essential for the high rates of *in vitro* DNA synthesis. Fig. 22 reveals that the optimum salt (NaCl or KCl)

Fig. 20

Comparison of the endogenous DNA synthetic abilities of nuclei from stationary and S-phase cells

A suspension in buffered-sucrose of nuclei (N3) isolated, as previously described, from stationary and S-phase cells is assayed for the ability to synthesise DNA in the absence of exogenously added DNA using Assay System A.



nuclei from S-phase cells

nuclei from stationary cells

Fig. 20

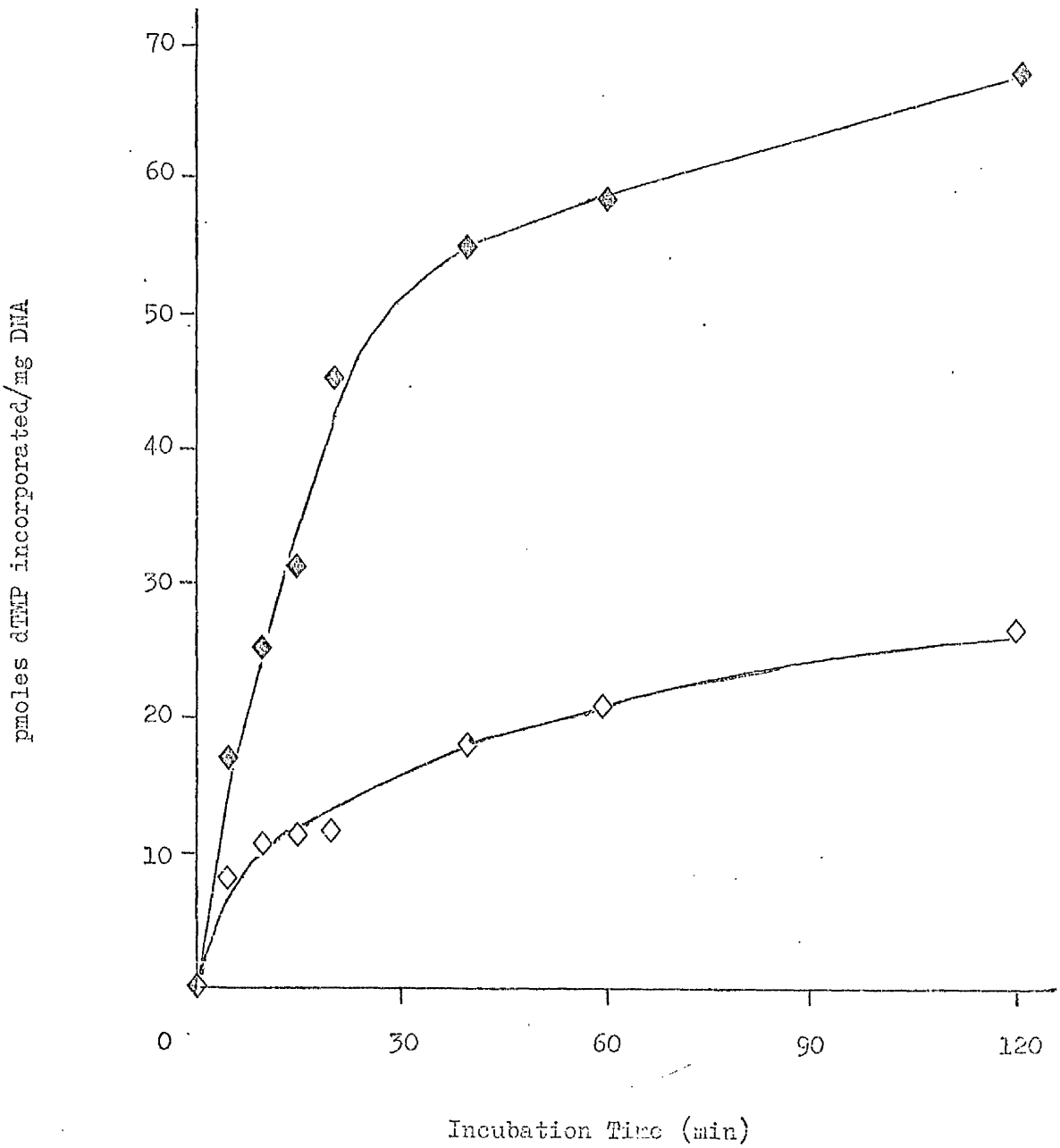

pmoles dTMP incorporated/mg DNA
70
60
50
40
30
20
10
0
30
60
90
120
Incubation Time (min)

Fig. 21

Comparison of assay systems on the endogenous DNA synthesising ability of nuclei from S-phase cells.

The endogenous DNA synthesising capacity of nuclei (N3) from S-phase cells is determined under the following two differing conditions of assay:-

—◇— Assay System A (Methods, section 4.4)

—◇— Assay System B (Methods, section 4.4)

Fig. 21

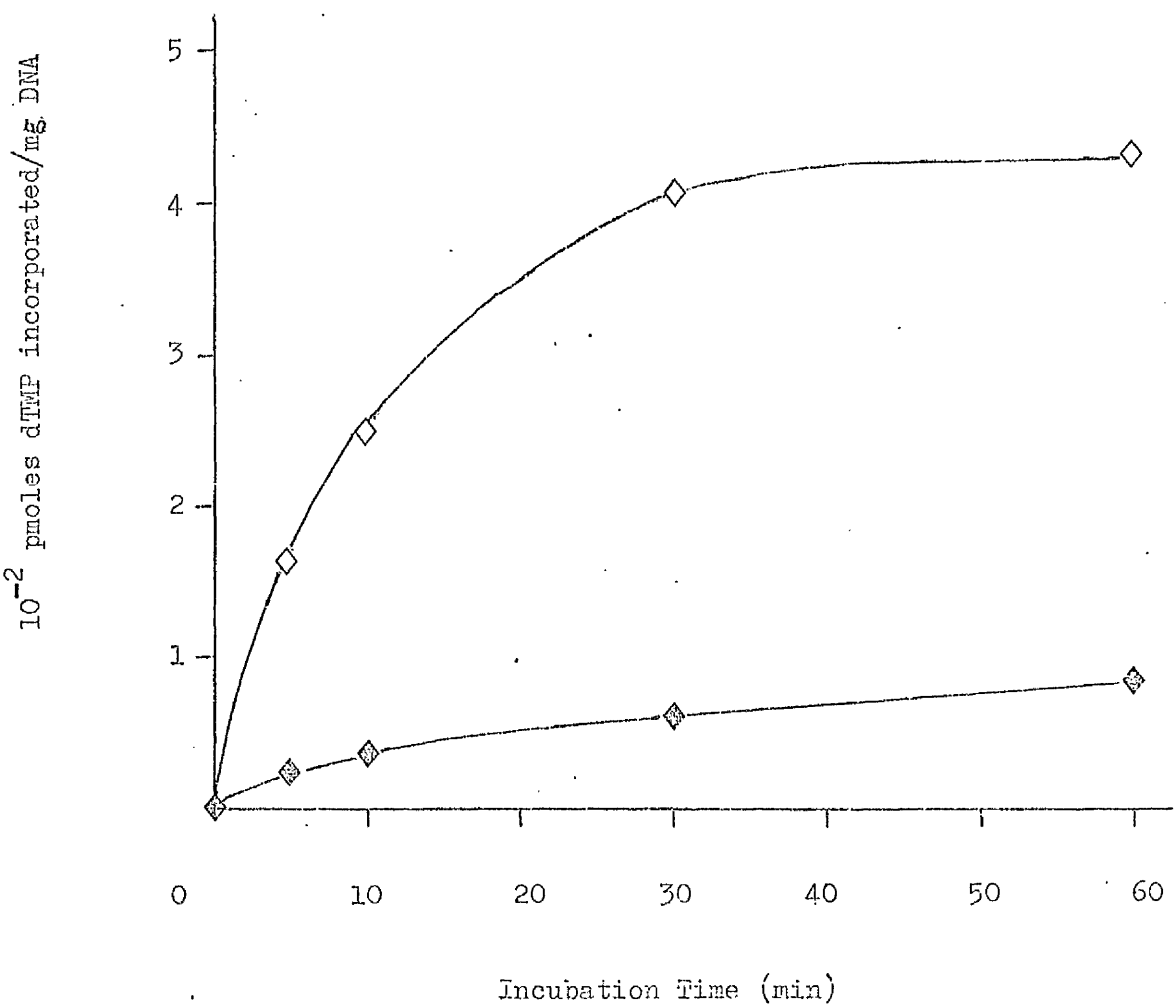
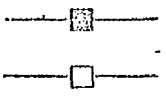

10^{-2} pmoles dTMP incorporated/mg DNA
5
4
3
2
1
0
10
20
30
40
50
60
Incubation Time (min)

Fig. 22

Effect of salt concentration on the endogenous DNA synthetic ability of nuclei from S-phase cells

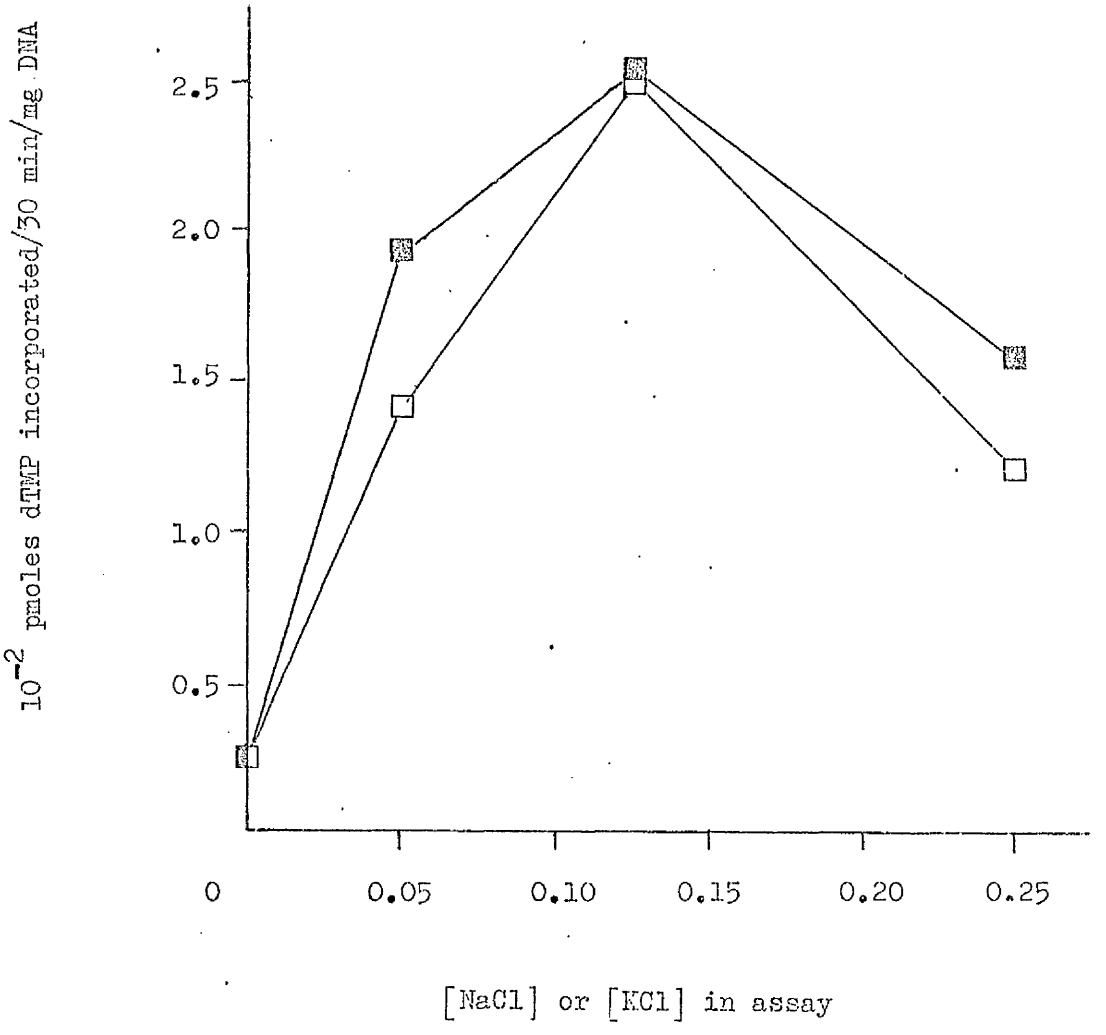
Nuclei (N3) from S-phase cells are assayed for endogenous DNA synthesising capacity using Assay System B in which the salt concentration (NaCl or KCl) is varied as shown opposite.



varying KCl

varying NaCl

Fig. 22


10^{-2} pmoles dTMP incorporated/30 min/mg DNA
2.5
2.0
1.5
1.0
0.5
0
0.05
0.10
0.15
0.20
0.25
[NaCl] or [KCl] in assay

concentration for L929 nuclei is around 100mM as used by Hershey et al. Incorporation by L929 nuclei prepared by the method of Hershey et al. (1973a) showed little difference compared to the standard isolation procedure (Fig. 23). Therefore it was decided to adopt assay system B, but to retain the existing procedure for nuclear isolation, which gave cleaner nuclei on microscopic examination. Furthermore adoption of a new nuclear preparation method would involve characterisation of the DNA polymerase activities present. Fig. 24 confirms that, using assay system B, the nuclei still reflect their in vivo capacity for DNA synthesis prior to isolation.

10.2 The product of DNA synthesis by isolated nuclei

Characterisation of the product synthesised during the endogenous nuclear incorporation may give a clue to the nature of the DNA synthesis taking place within nuclei. Alkaline sucrose gradient centrifugation reveals that the product of nuclear incorporation, after 5 min in vitro incubation, is in small pieces sedimenting near the top of the gradient, whereas the bulk DNA is larger, sedimenting further down (Fig. 25a). Fig. 25b, which shows the pattern obtained from unincubated nuclei, reveals that the incubation conditions do not reduce the size of the bulk DNA, as was the case for rat liver nuclei after in vitro incubation (Hyodo & Ono, 1970b). Fig. 25b also shows that the acid-soluble material (i.e. ^{3}H-TTP present during assay) peaks at the very top of the gradient, and is completely removed by the acid-washing procedure. The finding of the product as small pieces suggests that the nuclear DNA synthesis involves extension (or completion) of Okazaki-type fragments but not their ligation to bulk DNA. The lack of tritium label in the bulk DNA region of the gradient

Fig. 23

Effect of an alternative method of nuclear preparation on the endogenous DNA synthetic ability

Nuclei prepared by the established procedure (N3) (Methods, section 3.4) and by the procedure of Hershey et al. (1973a) as outlined in Methods, section 3.5 are assayed for endogenous DNA synthetic ability using Assay System B.

—◇— nuclei prepared by established method

—◇— nuclei prepared by method of Hershey et al.

Fig. 23

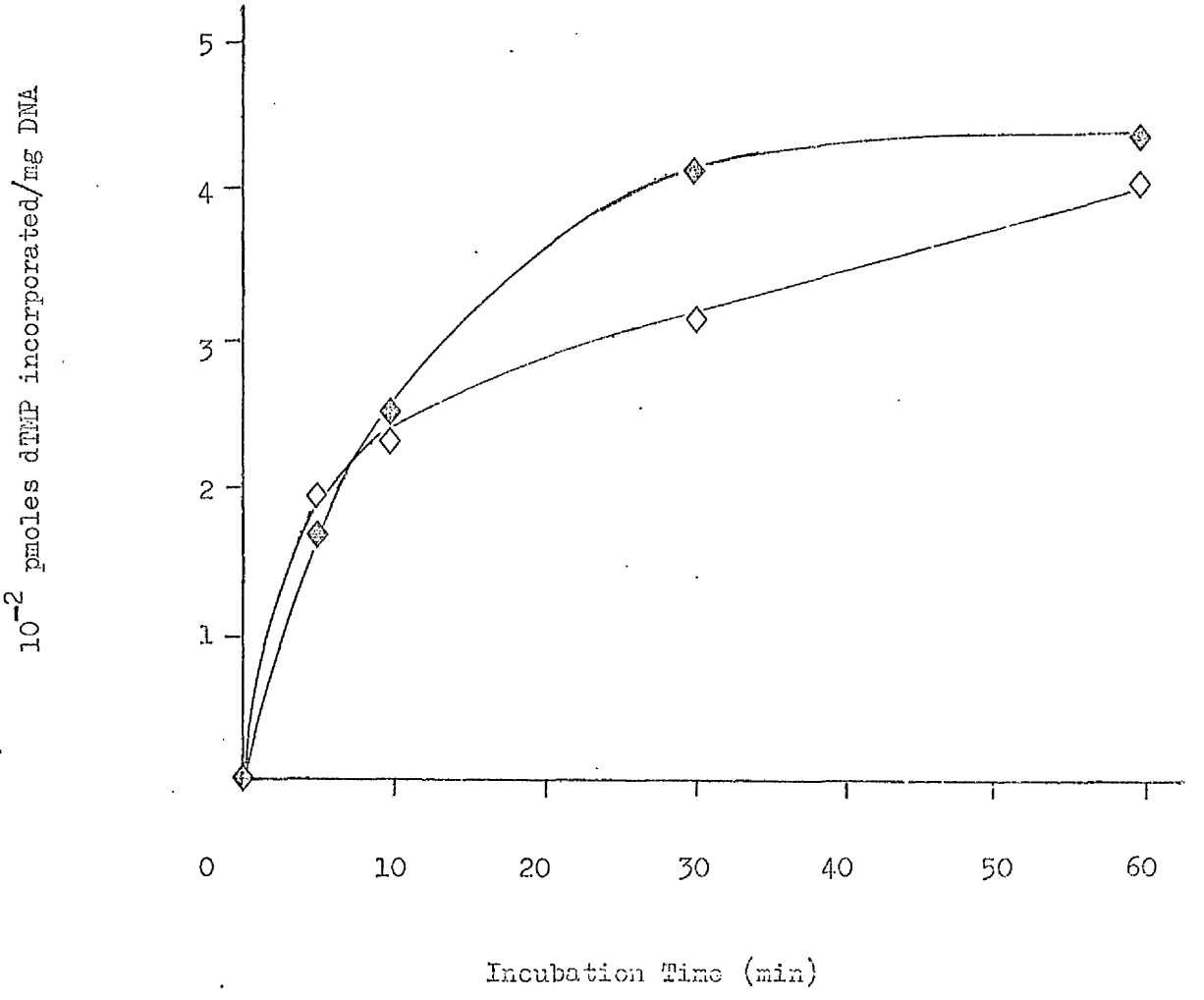
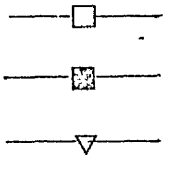

10^{-2} pmoles dTMP incorporated/mg DNA
5
4
3
2
1
0
10
20
30
40
50
60
Incubation Time (min)

Fig. 24

Endogenous DNA synthetic ability of nuclei isolated from cells at various times after release from stationary phase

Nuclei (H3) are prepared from cells harvested at the times shown after release from the stationary phase of growth (see Methods, section 3.3 ii), and assayed for endogenous DNA synthesising capacity after 5 and 30 min incubation using Assay System B.



after 5 min incubation

after 30 min incubation

% cells synthesising DNA *in vivo* (reproduced from Lindsay et al. (1970) - see Methods, section 3.3)

Fig. 24

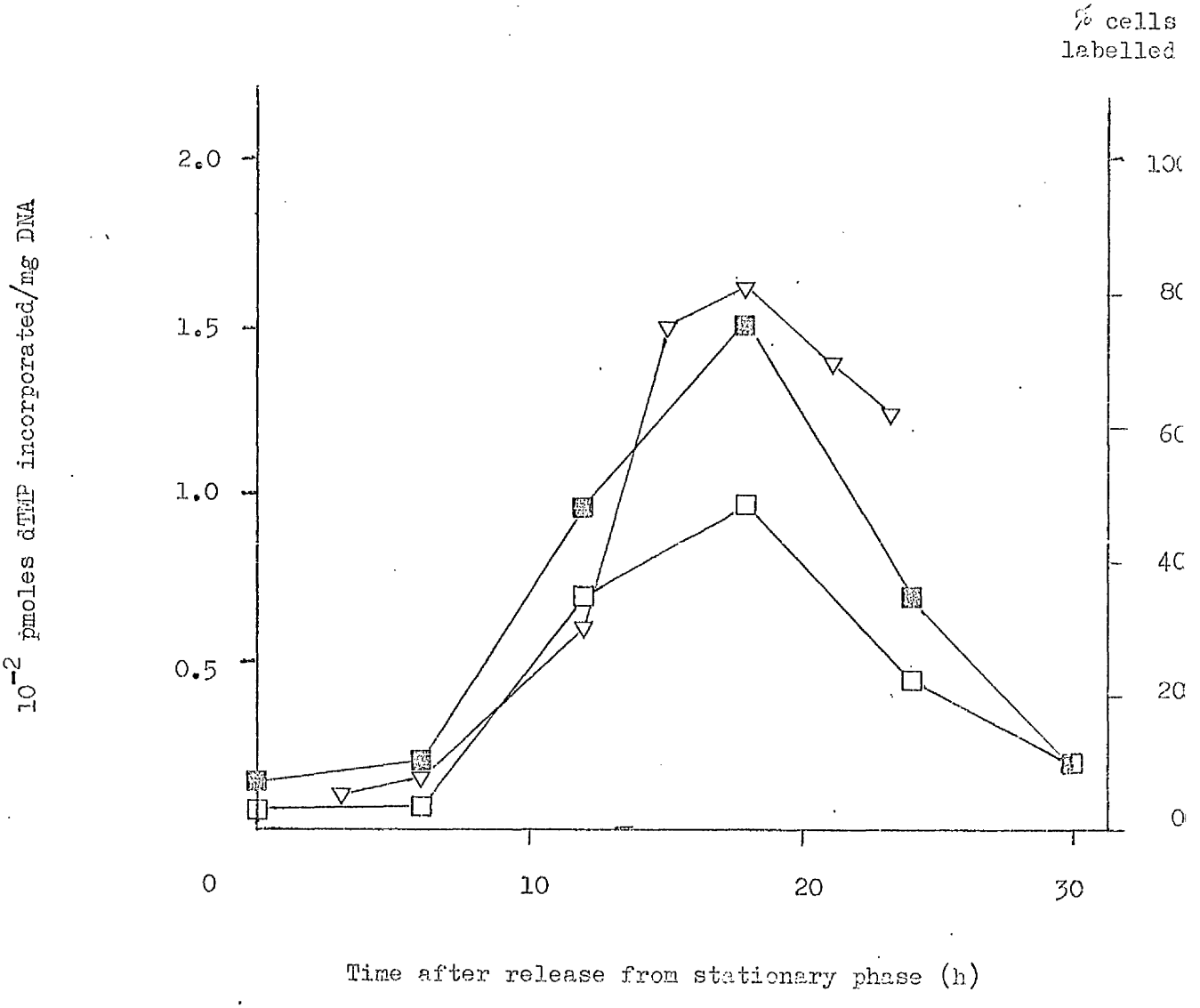

% cells labelled
10^{-2} pmoles dTMP incorporated/mg DNA
2.0
1.5
1.0
0.5
0
10
20
30
100
80
60
40
20
0
Time after release from stationary phase (h)

Fig. 25

Characterisation of the product of endogenous DNA synthesis by nuclei from S-phase cells on alkaline sucrose gradients

Nuclei (N3) from S-phase cells, pre-labelled with [^{14}C] as described in Methods, section 3.8, are incubated for 5 min in a final volume of 0.5 ml under the conditions of Assay System B, except that [^{3}H] dTTP is present at four times the specific activity. The reaction is terminated by addition of 1 volume of a solution containing 2% sodium dodecyl sulphate (SDS), 6% p-amino salicylate (PAS), 4mM EDTA and 10% n-butanol and heated at 70°C until the nuclei dissolve and the sample appears homogenous. The sample is then analysed on an alkaline sucrose gradient as described in Methods, section 5.4.

(a) Sample: Nuclei incubated for 5 min

——▣—— Acid-insoluble [^{3}H]-labelled material

——□—— Acid-insoluble [^{14}C]-labelled material

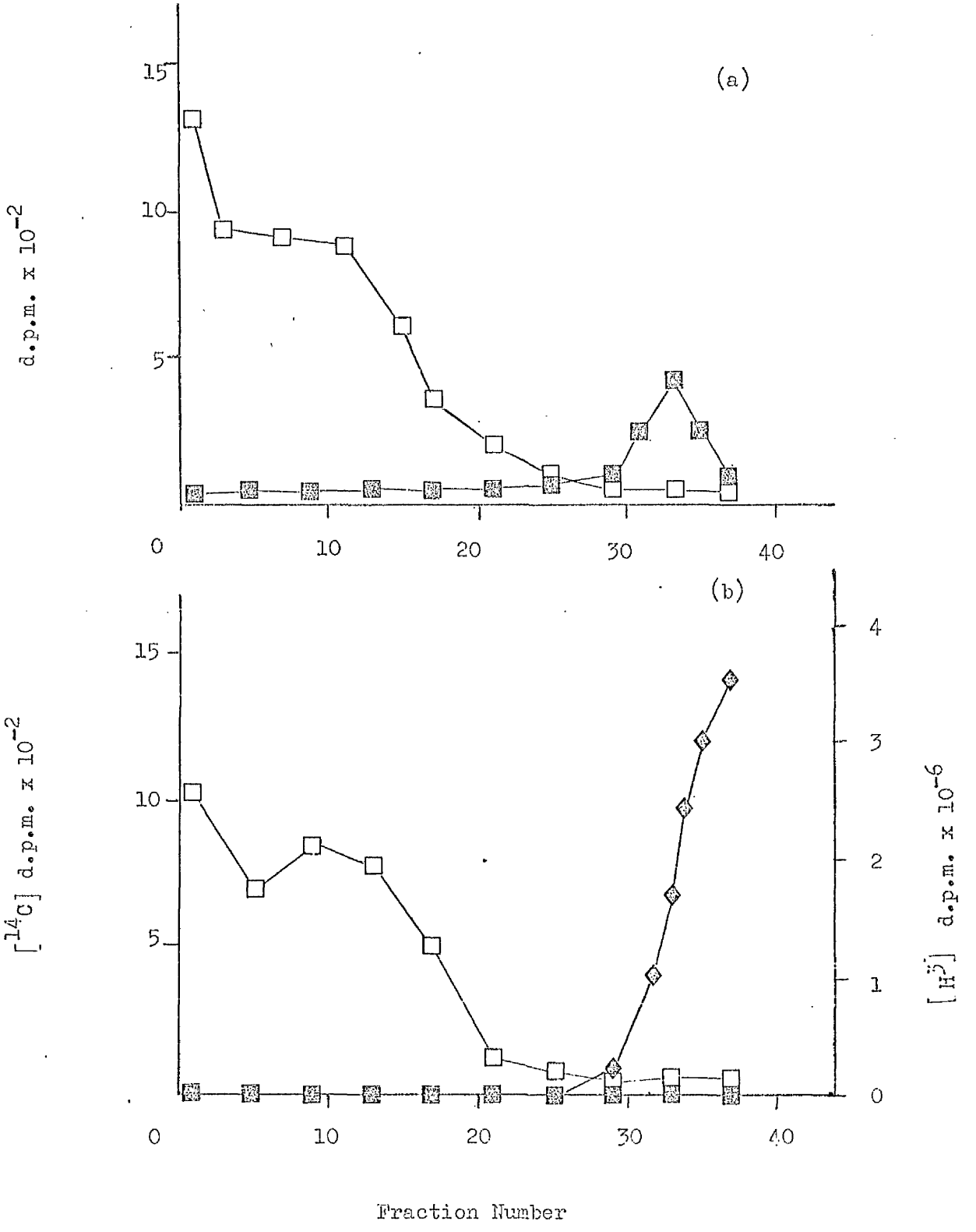
(b) Sample: Unincubated nuclei

——◇—— Total [^{3}H]-labelled material

——▣—— Acid-insoluble [^{3}H]-labelled material

——□—— Acid-insoluble [^{14}C]-labelled material

Fig. 25



(Fig. 25a) suggests that there is undetectable repair synthesis going on and that contribution to the product, in the first 5 min of incubation, by incorporation at non-specific nicks is negligible.

10.3 Factors limiting endogenous nuclear incorporation

One possible explanation for the rapid fall in the rate of incorporation by isolated nuclei (Fig. 20) is that Okazaki fragments, begun *in vivo* before nuclear isolation, are being finished and initiation of new fragments is not occurring. Another reason could be the necessary participation of a factor (normally synthesised afresh) which is thermolabile or thermodiffusible and is decaying (or diffusing) as the incubation progresses.

Various additions were made to the assay in an attempt to prevent the rapid fall in rate after 15 min of incubation (Table 11). Failure to prevent the fall in rate by addition of fresh assay mixture rules out exhaustion of an assay constituent. The addition of ribonucleoside triphosphates, implicated to be involved in the initiation of Okazaki fragments in prokaryotes (Sugino et al., 1972), does not delay the change in incorporation rate, suggesting that rNTPs are not sufficient for new fragment initiation to occur *in vitro*.

The addition of nuclei which had been incubated simultaneously, but under conditions which preclude any synthesis, i.e. absence of added triphosphate, produced no effect despite their apparent template potential (Table 11). It is possible that sufficient deoxyribonucleoside triphosphates were still present in these nuclei to allow completion of their Okazaki fragments, but a more likely explanation is the presence of a factor essential for continued nuclear incorporation, which is lost during incubation either by denaturation or diffusion.

A control experiment revealed that the thermolability was not due to loss of DNA polymerase, as activity with a denatured

Table 11

Effect of various additives on the fall-off in endogenous incorporation rate exhibited by nuclei from S-phase cells

Nuclei (N3) from S-phase cells are incubated under standard conditions (Assay System B). After 15 min incubation, the following additions are made and the eventual incorporation at 30 min measured:-

A - water (control)

B - rATP, rGTP, rCTP and rUTP (final assay concentration 0.1mM)

C - nuclei from S-phase cells which have been pre-incubated at 37°C for 15 min in the absence of assay mixture

D - fresh assay mixture

	% of control incorporation level at 30 min incubation
Nuclei incubated for 15 min	81±6
Nuclei incubated for 30 min with the following additions at 15 min:-	
+ 50ul A	100±10
+ 50ul B	85±10
+ 50ul C	115±20
+ 50ul D	120±10

Standard deviations are duplicate variations.

Footnote: Effect of incubation on the DNA polymerase activity of nuclei (C above) with denatured DNA

	(d.p.m. [^{3}H] dTTP incorporated/h/ 0.1 ml sample)
Before incubation	3560
After incubation	2920

DNA template was still present at 80% of its pre-incubation exogenously assayable level (Footnote, Table 11).

11. EFFECT OF VARIOUS INHIBITORS ON NUCLEAR DNA SYNTHESIS AND ON THE DNA POLYMERASE SPECIES PRESENT WITHIN THE NUCLEI

The effect of certain inhibitors on the rate of endogenous DNA synthesis by nuclei from S-phase cells and their effect on the separable DNA polymerase activities extractable from such nuclei, may give some insight into which enzyme is responsible for nuclear DNA synthesis.

Fig. 26 demonstrates that n-ethylmaleimide (NEM) and ara CTP are inhibitory to the endogenous reaction reducing incorporation to a third of control values. Triton X-100 is less inhibitory reducing incorporation to approximately 50%.

The effect of these same inhibitors on the DNA polymerases separable from a 0.4M KCl extract of S-phase nuclei is shown in Table 12. In this case, NEM completely inhibits peak I activity but only inhibits peak III by 25%. AraCTP also appears to be more inhibitory to peak I than to peak III. Triton X-100 has little effect on the activity of either of the activities when assayed with a native DNA template but reduces peak I activity to 40% when assayed with denatured DNA. The results suggest that peak I enzyme or a reaction similar to that carried out by peak I enzyme, is involved in endogenous incorporation by isolated S-phase nuclei. This is supported by the previous finding of a large amount of peak I activity associated with nuclei during the DNA synthetic phase (Results, section 6).

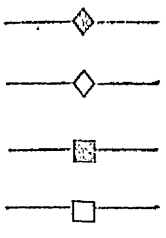
12. EFFECT OF VARIOUS EXTRACTING AGENTS ON NUCLEAR DNA SYNTHESIS

The ability to remove all of the DNA polymerase activity from nuclei and then to recover endogenous DNA synthesising ability by the addition of a particular nuclear species would be strong

Fig. 26

Effect of various inhibitors on the endogenous DNA synthetic ability of nuclei from S-phase cells

Nuclei (N3) from S-phase cells are assayed as before (Assay System B) with the following additions:-



none

0.2% (v/v) Triton X-100

1mM NEM

200μM araCTP

Fig. 26

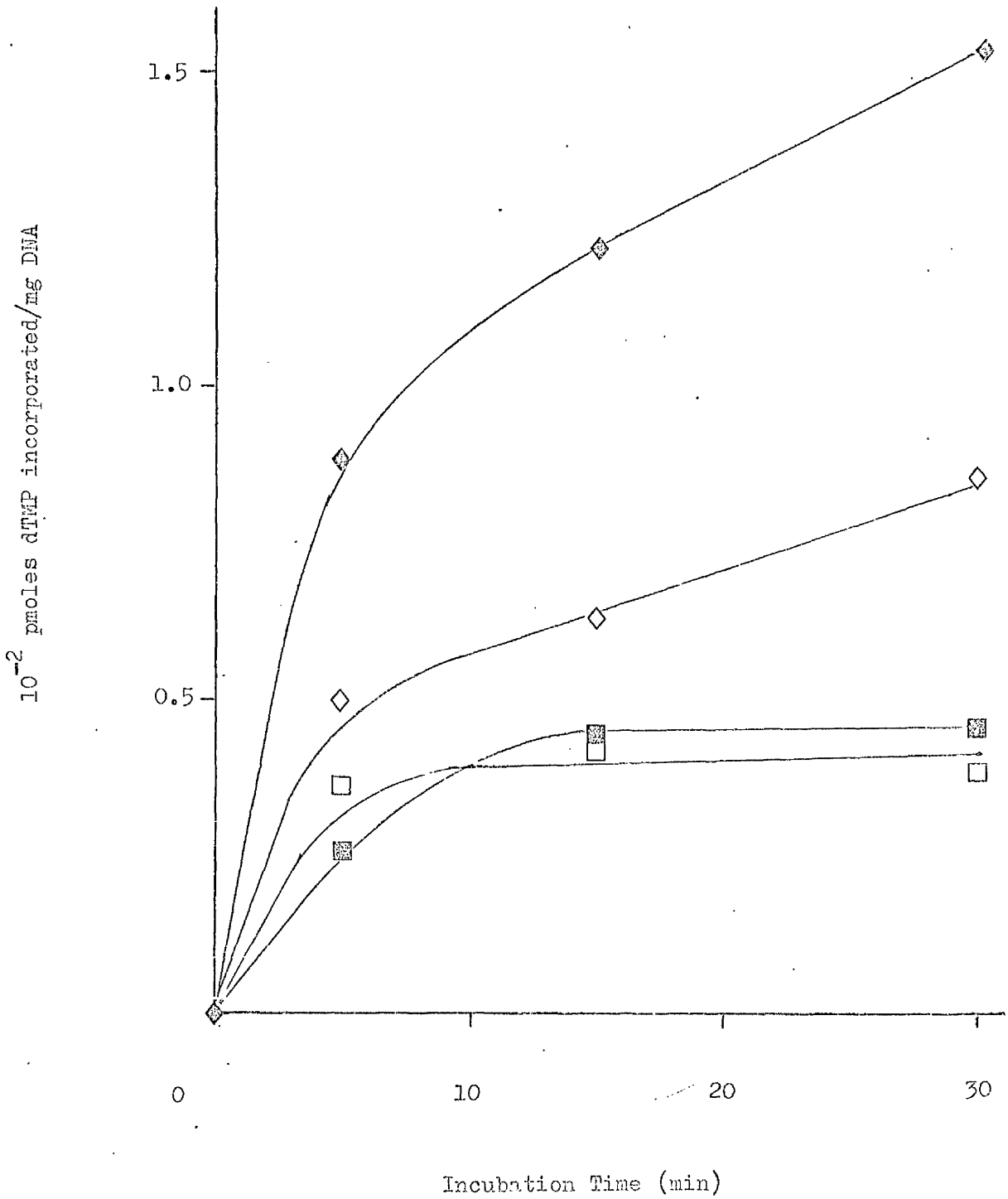

1.5
1.0
0.5
0
10
20
30
10^{-2} pmoles dTMP incorporated/mg DNA
Incubation Time (min)

Table 12

Effect of various inhibitors on the DNA polymerase species present in a 0.4M KCl extract of S-phase cells

A 0.4M KCl extract of nuclei (N3) from S-phase cells is fractionated by gel filtration on Sephadex G-200 as described for Fig. 4. The fractions representing the major peaks of DNA polymerase activity (peaks I and III - see Results, section 2.2) are assayed with and without the inhibitors shown below.

Inhibitor present	% inhibition of DNA polymerase activity		
	Peak I		Peak III
	native DNA	denatured DNA	native DNA
None	zero	zero	zero
10mM NEM	93	100	25
0.2% (v/v) Triton X-100	25	60	zero
200 µM araCTP	65	85	zero

evidence in favour of a role for that enzyme in nuclear incorporation and possibly DNA replication.

Another approach is to use milder extraction techniques, to specifically extract only one of the species present. A reduction of endogenous incorporation due to loss of one particular species may implicate that species in the replication process.

12.1 Effect of 0.4M KCl extraction on nuclear DNA synthesis

As expected, extraction of nuclei by 0.4M KCl, which was shown previously to remove all DNA polymerase activity, results in complete loss of nuclear DNA synthesising ability (Fig. 27).

Fig. 28 shows that when nuclei are extracted with 0.4M KCl, and the salt removed without separation of the extract from the nuclei, the resulting nuclei regain 50-60% of their original DNA synthesising activity. Alkaline sucrose gradient analysis demonstrates that the product of the nuclei, which have been reconstituted after subjection to 0.4M KCl extraction is in small pieces, suggesting that the type of DNA synthesis taking place in the reconstituted nuclei is similar to that being carried out by nuclei prior to extraction (Fig. 29).

Attempts to recover endogenous DNA synthesis, in nuclei which have been exhaustively extracted with 0.4M KCl, by mixing with various fractions containing DNA polymerase activity, proved disappointing. The treatments to which nuclei are subjected to exhaustively extract them i.e. centrifugation and resuspension in high salt, which is essential for proposed specific reactivation studies, results in almost complete loss of recoverability of endogenous DNA synthesis (Fig. 30). A possible explanation could be the destruction of a fragile DNA replication complex involving the integrity of the nuclear membrane.

Fig. 27

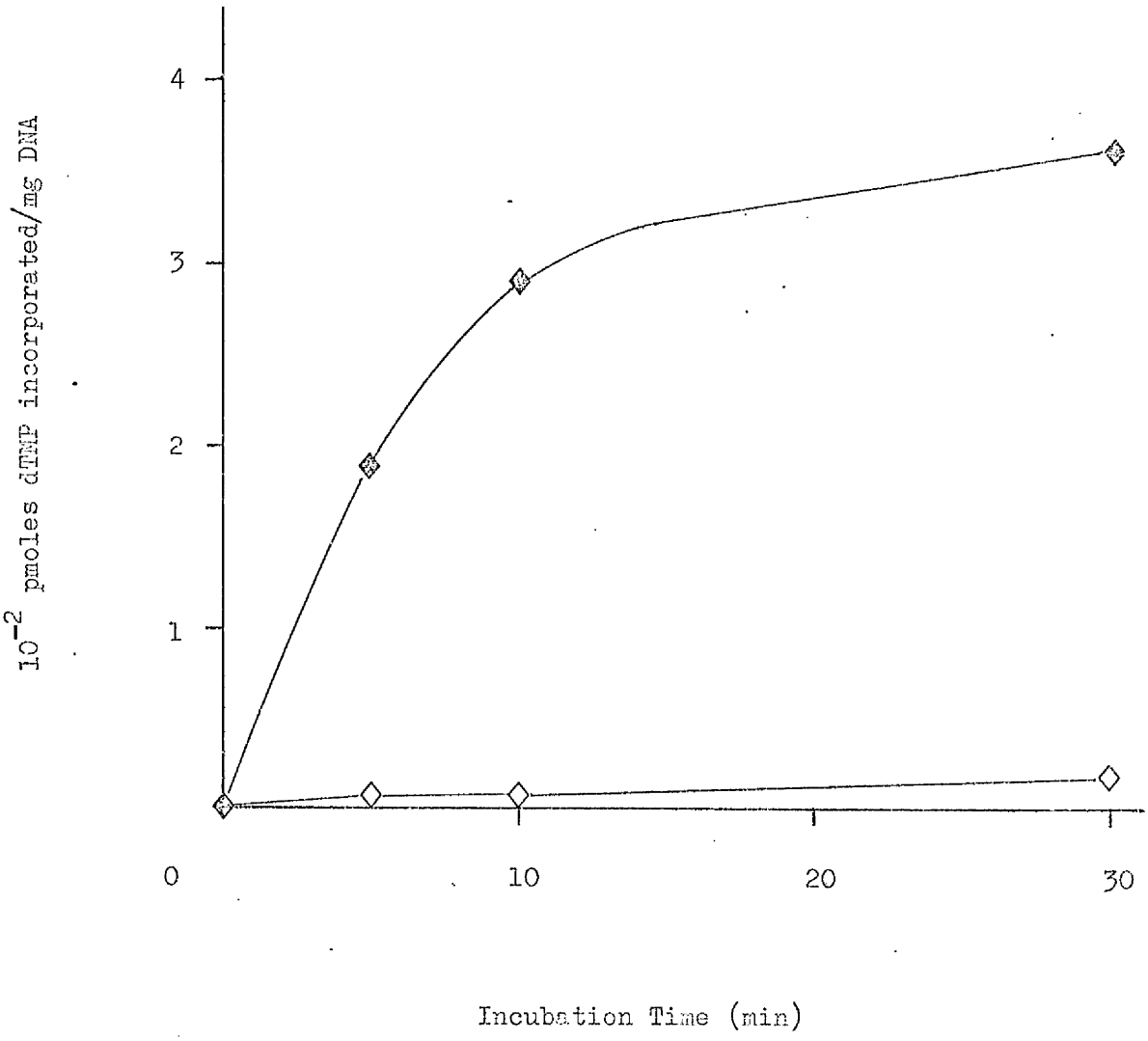
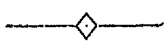

10^{-2} pmoles dTMP incorporated/mg DNA
4
3
2
1
0
10
20
30
Incubation Time (min)

Fig. 28

Recovery of endogenous DNA synthetic ability by nuclei from S-phase cells after extraction by 0.4M KCl

Equal batches of nuclei (N3) from S-phase cells are treated as follows and the endogenous DNA synthetic capacities are measured as before.

dialysed against 100 volumes of Buffer A for 4x1h - centrifuged at 800g for 10 min - washed with 10 volumes of Buffer A - resuspended in 1 volume of Buffer A.



dialysed against 100 volumes of Buffer A + 0.4M KCl for 2x1h - centrifuged at 800g for 10 min - washed with 10 volumes of Buffer A + 0.4M KCl - washed with 10 volume of Buffer A - resuspended in 1 volume of Buffer A.

dialysed against 100 volumes of Buffer A + 0.4M KCl for 2x1h - continued dialysis against 100 volumes of Buffer A for 2x1h (to allow rebinding of extracted material) - centrifuged at 800 g for 10 min - washed wi 10 volumes of Buffer A - resuspended in 1 volume of Buffer A.

The variation is a result of 3 separate experiments.

Fig. 28

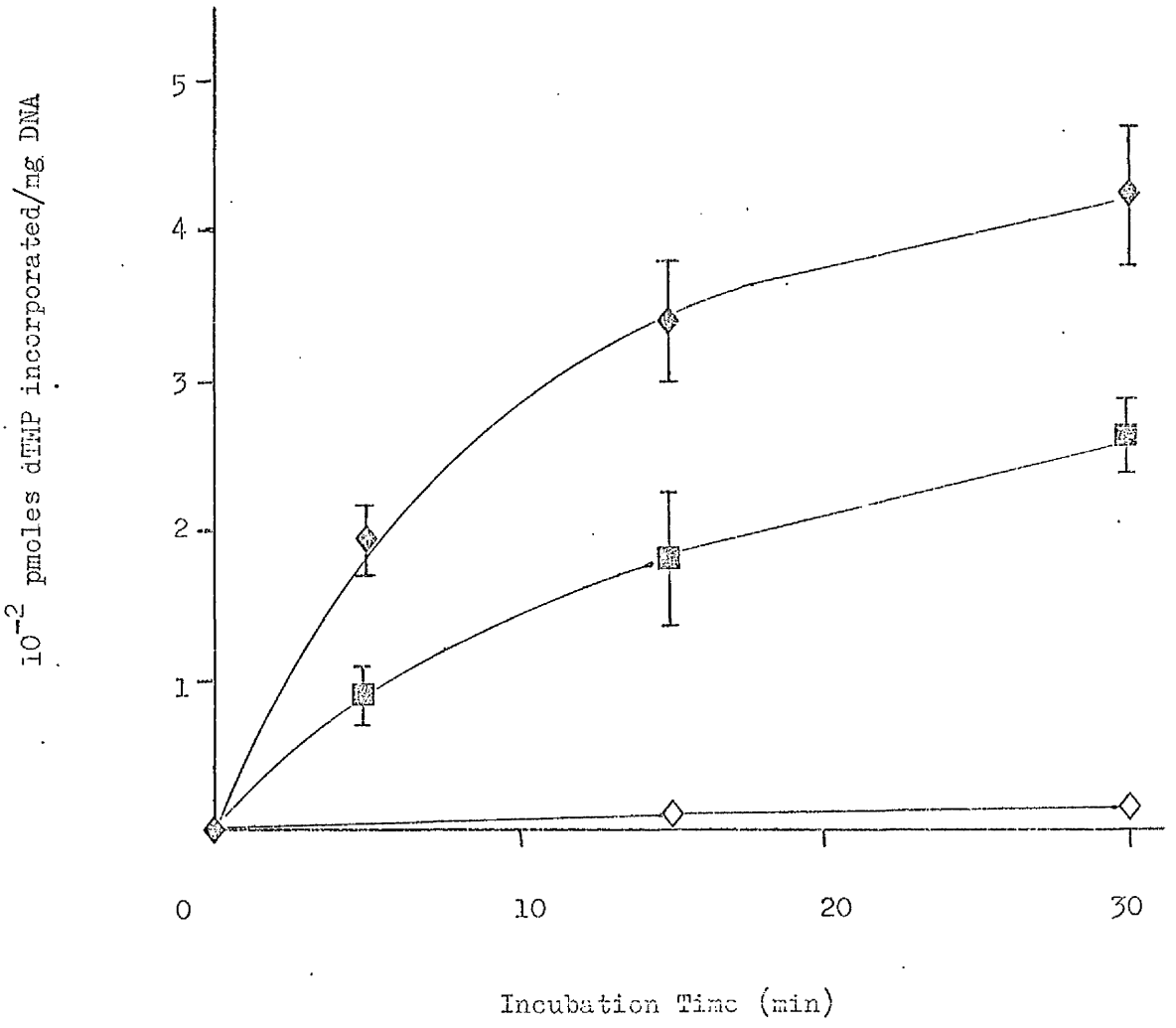
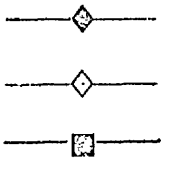

10^{-2} pmoles dTMP incorporated/mg DNA
5
4
3
2
1
0
10
20
30
Incubation Time (min)

Fig. 29

Characterisation on alkaline sucrose gradients of the product of endogenous DNA synthesis recovered by nuclei from S-phase cells after extraction by 0.4M KCl.

Nuclei (W3) from S-phase cells are treated in a similar manner to that described in the legend to Fig. 28 and the products of endogenous DNA synthesis analysed on alkaline sucrose gradients as previously described (see legend to Fig. 25).



as for nuclear preparations treated as described in the legend to Fig. 28, but represent [^{3}H]-labelled acid-insoluble material present in gradient fractions in each case.

Fig. 29

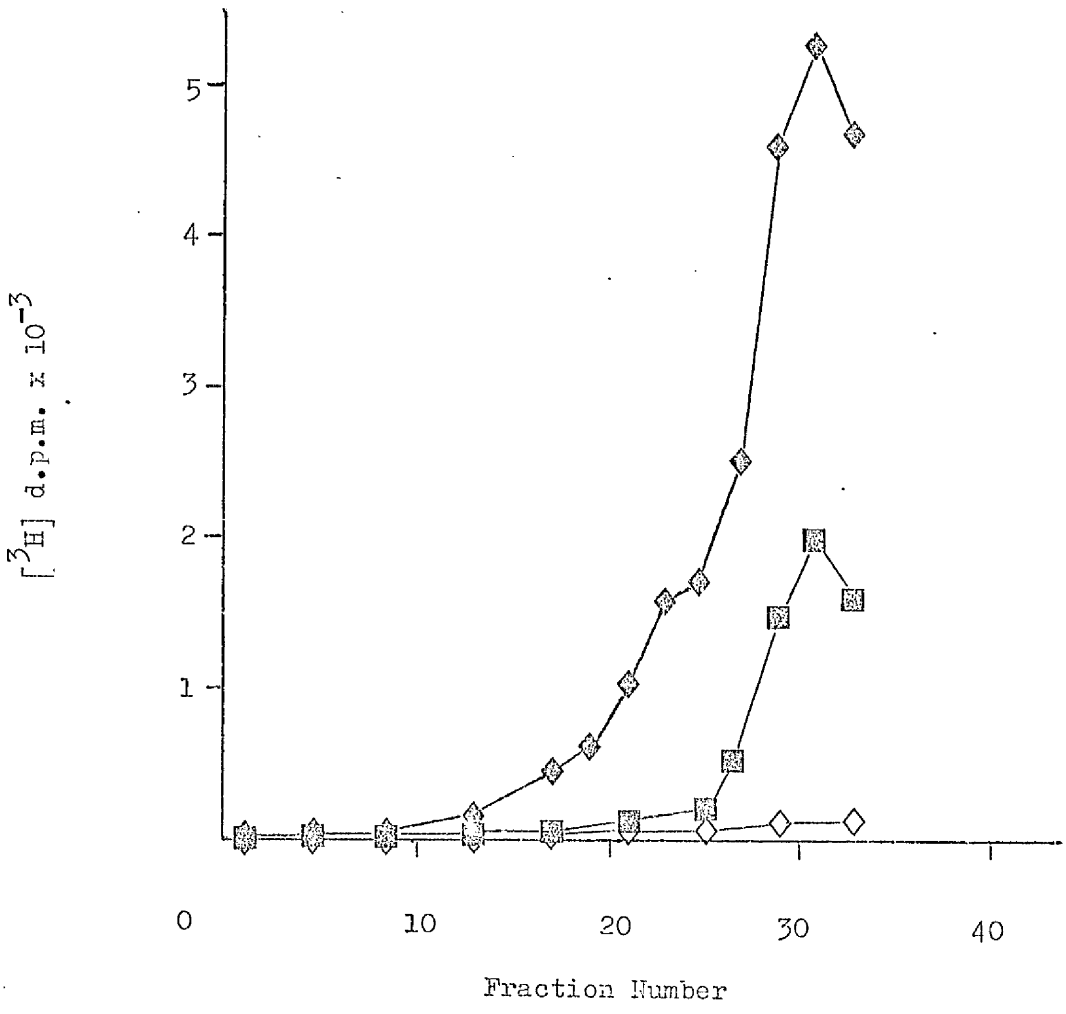
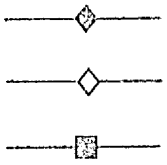


Fig. 30

Effect of exhaustive 0.4M KCl extraction on the ability of nuclei from S-phase cells to recover endogenous DNA synthetic capacity

The procedure described in the legend to Fig. 28 is repeated with the additional nuclear treatment described below. The endogenous DNA synthetic abilities are measured as before.

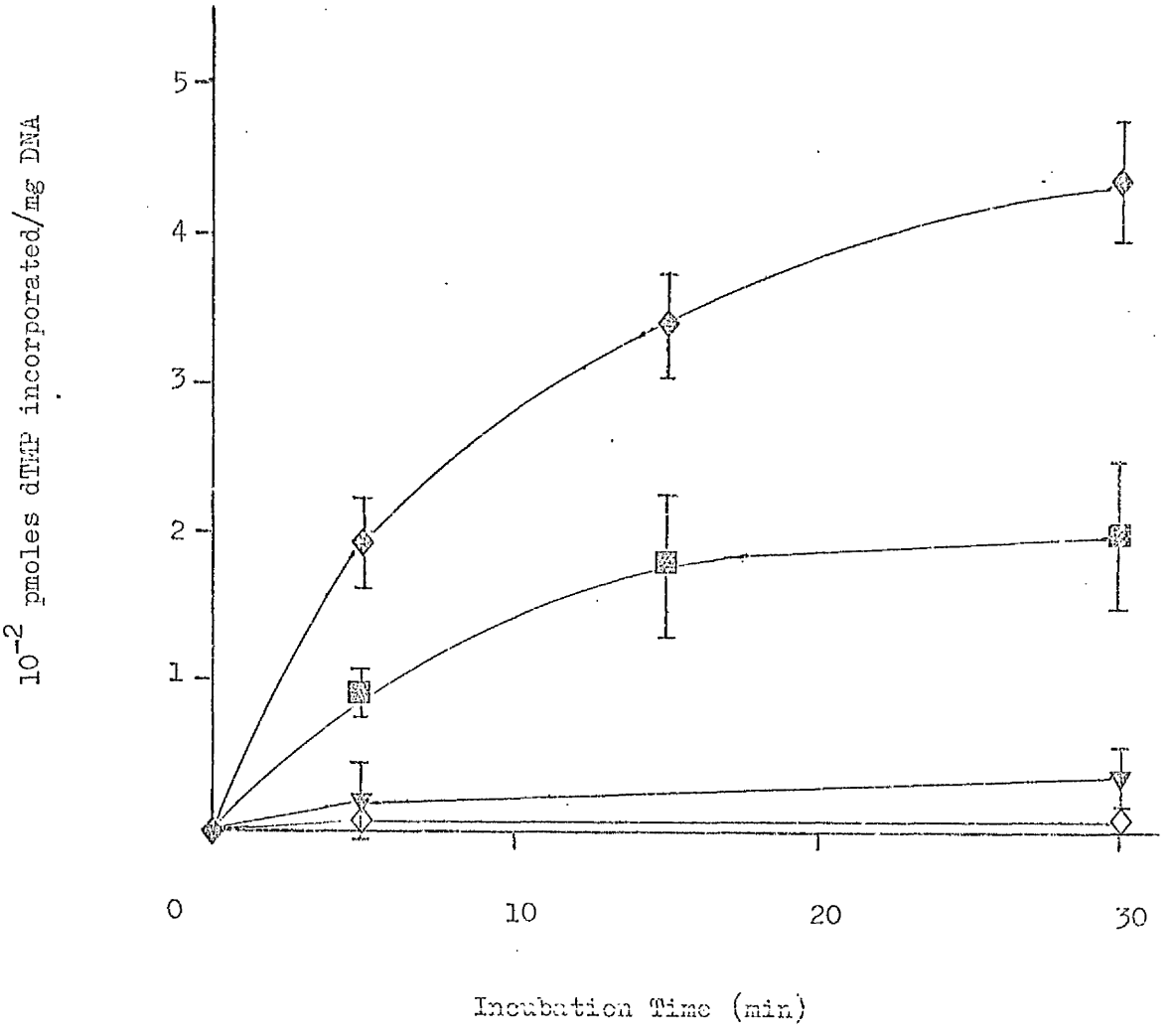


as for Fig. 28

dialysed against 100 volumes of Buffer A + 0.4M KCl for 2x1h - centrifuged at 800 g for 10 min - washed with 10 volumes of Buffer A + 0.4M KCl - resuspended in the original 0.4M KCl extract - dialysed against 100 volumes of Buffer A for 2x1h - centrifuged at 800 g for 10 min - washed with 10 volumes of Buffer A - resuspended in 1 volume of Buffer A

The variation is a result of 2 separate experiments

Fig. 30


10^{-2} pmoles dTMP incorporated/mg DNA
5
4
3
2
1
0
10
20
30
Incubation Time (min)

12.2 Effect of other extracting agents on nuclear DNA synthesis

It has been reported by Ove et al. (1973) that extraction of nuclei from regenerating rat liver with 0.2M potassium phosphate does not reduce their endogenous DNA synthetic ability, but that it does result in extraction of excess nuclear DNA polymerase activity not involved in the endogenous reaction. Extraction of nuclei from S-phase L929 cells with 0.2M potassium phosphate, however, appreciably reduces their DNA synthetic activity measurable after 5 min incubation (Table 13).

A report by Lynch & Lieberman (1973) demonstrated that Brij 58, a non-ionic detergent, at a concentration of 5%, in the presence of 0.05M magnesium acetate buffer pH 7.4 (hereafter referred to as Brij/Mg) extracts DNA polymerase activity from nuclei of normal and regenerating rat liver. The DNA polymerase activity extractable by Brij/Mg was considered not to be involved in the endogenous DNA synthesising reaction because the two reactions show differing inhibition by ara CTP. The DNA polymerase activity extractable by high salt after Brij/Mg extraction showed similar inhibition by ara CTP as did the endogenous reaction.

Extraction of nuclei from S-phase L929 cells with Brij/Mg results in retention of 70-90% of their endogenous DNA synthetic ability (Table 13).

Table 13 also shows the effect on the endogenous reaction of extraction by 0.1M KCl. As can be seen, the endogenous incorporation is reduced to 60-75% of control level by 0.1M KCl extraction. Continued extraction by 0.2M KCl does not result in any further reduction in endogenous activity.

Table 13

Effect of extraction by various agents on the endogenous DNA synthetic ability of S-phase cells

Nuclei (N3) from S-phase cells are extracted (Extraction Method III, Methods, section 3.7) with the agents shown below and the percentage retention of endogenous DNA synthesising ability determined after 5 min incubation using Assay System B.

Extracting agent	Endogenous DNA synthetic ability % of control
None	100
0.2M potassium phosphate	9 -11 (2 experiments)
5% Brij 58/50mM magnesium acetate	70 -90 (3 experiments)
0.1M KCl	60 -75 (2 experiments)
0.2M KCl after 0.1M KCl	60 -70 (2 experiments)

13. DNA POLYMERASE ACTIVITY EXTRACTED FROM NUCLEI BY VARIOUS EXTRACTING AGENTS

The fact that DNA polymerase is stimulated on extraction from nuclei by 0.4M KCl (see Table 3) makes it almost impossible to compare, with any accuracy, the proportion of the total nuclear DNA polymerase activity extractable by any of the extracting agents used in the previous section. However the DNA template preference of the DNA polymerase activity assayable in the extracts and the extracted nuclear pellets (later fully extracted by 0.4M KCl) may give a clue to the nature of the DNA polymerase species present within the respective extracts.

Table 14 shows the DNA polymerase activity, as assayed by native and denatured DNA templates, extracted from nuclei of S-phase cells using the various extracting agents employed in the previous section. Several interesting facts can be deduced from the results in Table 14. 0.2M potassium phosphate appears to act in a similar way to 0.4M KCl in that its inhibitory effect on endogenous nuclear DNA synthesis (see Table 13) seems to be a result of complete extraction of DNA polymerase activity from the nuclei. (No residual activity was detected when the extracted nuclear pellet was assayed.)

From the template preference (i.e. native DNA) of the DNA polymerase activity extracted by Brij/Mg, it suggests removal of low M.W. nuclear enzyme. This is confirmed by fractionation of the Brij/Mg extract by gel filtration on Sephadex G-200 (Fig. 31) which shows the presence of a major DNA polymerase peak eluting later than the haemoglobin marker ($< 65,000$).

Another interesting finding (Table 14) is that 0.1M KCl removes substantial DNA polymerase activity with a preference for

Table 14

DNA polymerase activity extracted from nuclei from S-phase cells by various extracting agents

Nuclei (N3) from S-phase cells are extracted (Extraction Method III, Methods, section 3.7) with the agents shown below and the resulting extracts assayed for DNA polymerase activity after dialysis against 100 volumes of Buffer A for 2x1h to remove extracting agent.

Extracting agent	DNA polymerase activity in the extract (10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample)	
	native DNA	denatured DNA
A 0.4M KCl	23.7	33.2
B 0.2M potassium phosphate	23.2	30.1
C 5% Brij 58/50mM magnesium acetate	10.8	0.5
D 0.1M KCl	3.4	7.4
E 0.2M KCl after 0.1M KCl	1.8	0.2

A, B and C were carried out using equal aliquots of one batch of nuclei; D and E using a second different batch of nuclei.

E - nuclei are extracted exhaustively with 0.1M KCl and then the extraction repeated with 0.2M KCl.

Fig. 31

Fractionation on Sephadex G-200 of the DNA polymerase activity present in a 5% Brij 58/50mM magnesium acetate extract of nuclei from S-phase cells

Nuclei (N3) from S-phase cells are extracted with 5% Brij 58/50mM magnesium acetate (Extraction Method I, Methods, section 3.7). The resulting extract is concentrated (Methods, section 3.6 ii) and a 1 ml sample fractionated on Sephadex G-200 as previously described. The fractions obtained are assayed for DNA polymerase activity

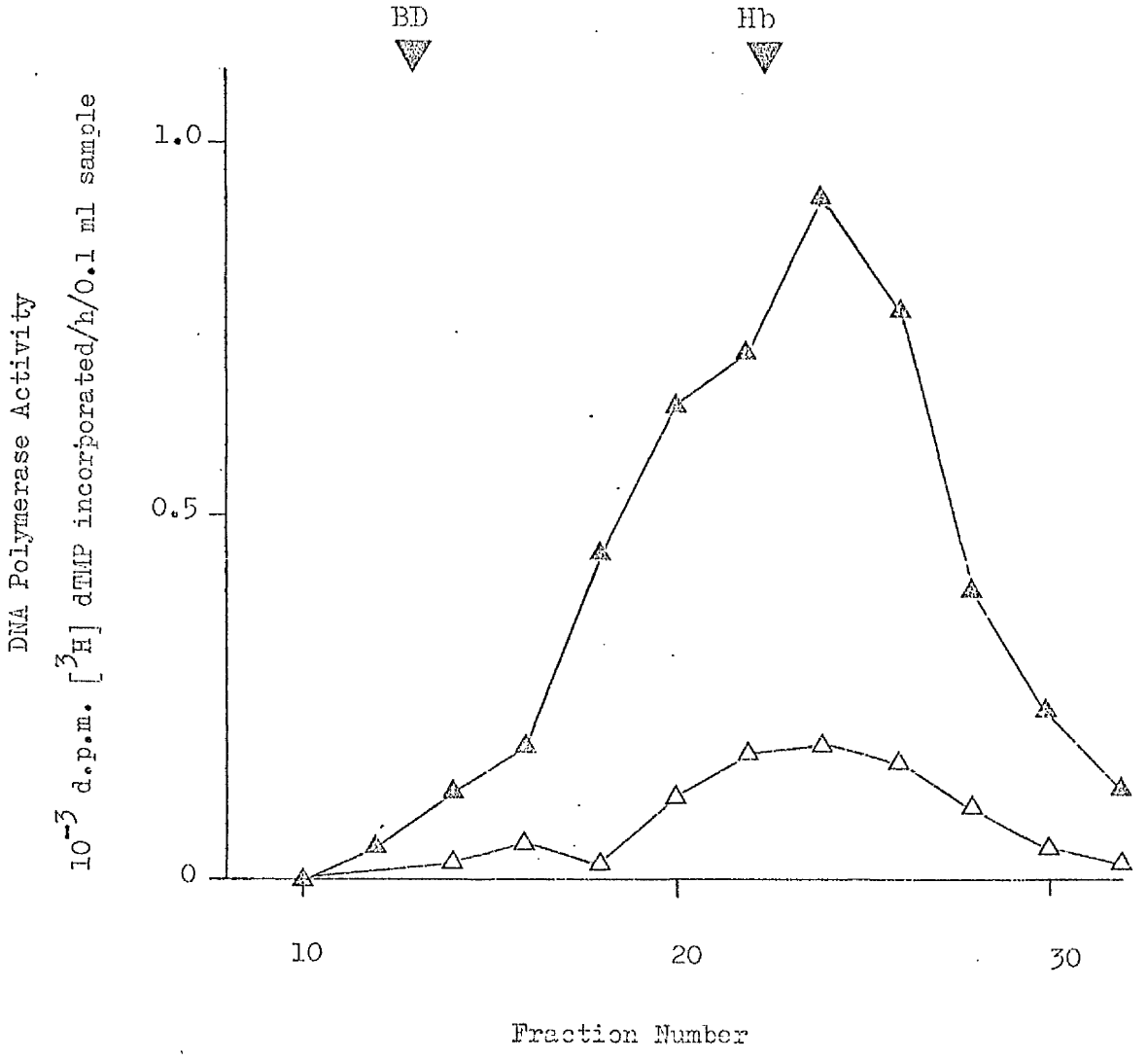
——△—— with native DNA

——△—— with denatured DNA

——△—— denotes coincident points

Markers are as for Fig. 2.

Fig. 31


BD
Hb
1.0
0.5
0
10
20
30
DNA Polymerase Activity
10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample
Fraction Number

denatured DNA, from nuclei of S-phase cells. 0.2M KCl extraction of the same nuclei, which have been exhaustively extracted with 0.1M KCl results in removal of DNA polymerase activity with a native DNA preference. This suggests that the bulk of peak I nuclear activity is being extracted by 0.1M KCl. Sucrose gradient analysis has shown that the activity extracted from nuclei with 0.1M KCl is predominantly the high molecular weight species i.e. peak I (T.R. Butt, personal communication).

14. EXTRACTION OF DNA POLYMERASE ACTIVITY FROM NUCLEI OF STATIONARY AND S-PHASE CELLS BY BRIJ/Mg

Lynch & Lieberman (1973) showed that the proportion of DNA polymerase activity extracted from nuclei by Brij/Mg differed depending on the source of the nuclei. A greater proportion was extracted from nuclei of normal rat liver than from nuclei isolated from regenerating tissue. This suggests that the DNA polymerase extractable by Brij/Mg represents a higher proportion of the total DNA polymerase activity of nuclei isolated from cells not active in DNA synthesis.

Table 15 shows that 70-85% of the total 0.4M KCl extractable DNA polymerase activity present in nuclei from stationary L929 cells is extractable by Brij/Mg whereas similar extraction of nuclei from cells in S-phase results in only 25-50% of the total DNA polymerase activity being extracted.

These observations show that Brij/Mg is having a similar effect on L929 nuclei in that a greater proportion of the total activity is extractable from nuclei of cells not actively replicating DNA. Taken together with the previous observations that Brij/Mg specifically extracts low M.W. enzyme and that extraction with Brij/Mg does not appreciably reduce the endogenous nuclear DNA synthesis,

Table 15

Extraction of nuclei from stationary and S-phase cells with 5% Brij 58/50mM magnesium acetate

Nuclei (N3) from stationary and S-phase cells are exhaustively extracted with 5% Brij 58/50mM magnesium acetate (5% Brij/Mg) using Extraction Method III, Methods, section 3.7. The resulting nuclear pellets are further extracted with 0.4M KCl (Extraction Method I, Methods, section 3.7) in the same total volume as the first extraction. To the resulting extracts from both extractions is added 1 volume of the corresponding agent not used for the extraction and the samples are dialysed for 2x1h against 100 volumes of Buffer A + 0.15M KCl (As Brij 58 is not readily dialysable, it is added to the 0.4M KCl extracts to ensure an equivalent final assay concentration in both cases). The samples are assayed for DNA polymerase activity and the total activity present in the extracts determined. The results are expressed as the percentage of the total DNA polymerase activity extractable from the nuclei by 5% Brij/Mg.

Source of nuclei	% of total nuclear DNA polymerase activity extractable by 5% Brij/Mg	
	native DNA	denatured DNA
Stationary cells	70 - 85	75 - 80
S-phase cells	45 - 50	27 - 30

The variation is a result of 3 separate experiments

it appears as if this form of DNA polymerase (i.e. Brij/Mg extractable) is not the DNA polymerising activity responsible for DNA synthesis by isolated nuclei from S-phase cells.

15. DNA POLYMERASE SPECIES PRESENT IN NUCLEI WHICH STILL RETAIN ENDOGENOUS DNA SYNTHESISING ABILITY AFTER EXTRACTION

Extraction of nuclei from S-phase cells by Brij/Mg is shown to reduce the endogenous DNA synthesising ability of the nuclei by only 10-30% (Table 13). Further extraction of these nuclei by 0.4M KCl and fractionation of the resulting extract on Sephadex G-200 reveals that some low molecular weight DNA polymerase is still present within the nuclei along with peak I activity (Fig. 32a). No conclusions, therefore, can be made as to which of the DNA polymerase species still present is responsible for endogenous nuclear DNA synthesis although it appears as if the low M.W. enzyme is present in nuclei from S-phase cells in at least two forms. One of the forms is extractable by Brij/Mg and represents nearly all of the DNA polymerase activity present in nuclei from stationary phase cells. The other form is extractable by 0.4M KCl and may represent a more tightly bound form of the low molecular weight enzyme peculiar to nuclei from DNA synthesising cells.

Fractionation on Sephadex G-200 of the DNA polymerase activity present in a 0.4M KCl extract of nuclei from S-phase cells previously extracted with 0.1M KCl, reveals that both high and low molecular weight enzymes are still present (Fig. 32b). Again no conclusion could be drawn about the activity involved in nuclear DNA synthesis.

However, when nuclei from S-phase cells were extracted with 0.2M KCl and the subsequent 0.4M KCl extract fractionated on

Fig. 32

Fractionation on Sephadex G-200 of the DNA polymerase activity present in a 0.4M KCl extract of nuclei from S-phase cells which have previously been extracted with various agents

Equal batches of nuclei (N3) from S-phase cells are exhaustively extracted with the agents shown below (Extraction Method III, Methods, section 3.7). The resultant nuclear pellets are further extracted with 0.4M KCl (Extraction Method I, Methods, section 3.7) and the extracts obtained concentrated (Methods, section 3.6 ii) and a 1 ml sample fractionated on Sephadex G-200 as before. The resulting fractions are assayed for DNA polymerase activity.

(a) 5% Brij 58/50mM magnesium acetate

(b) 0.1M KCl

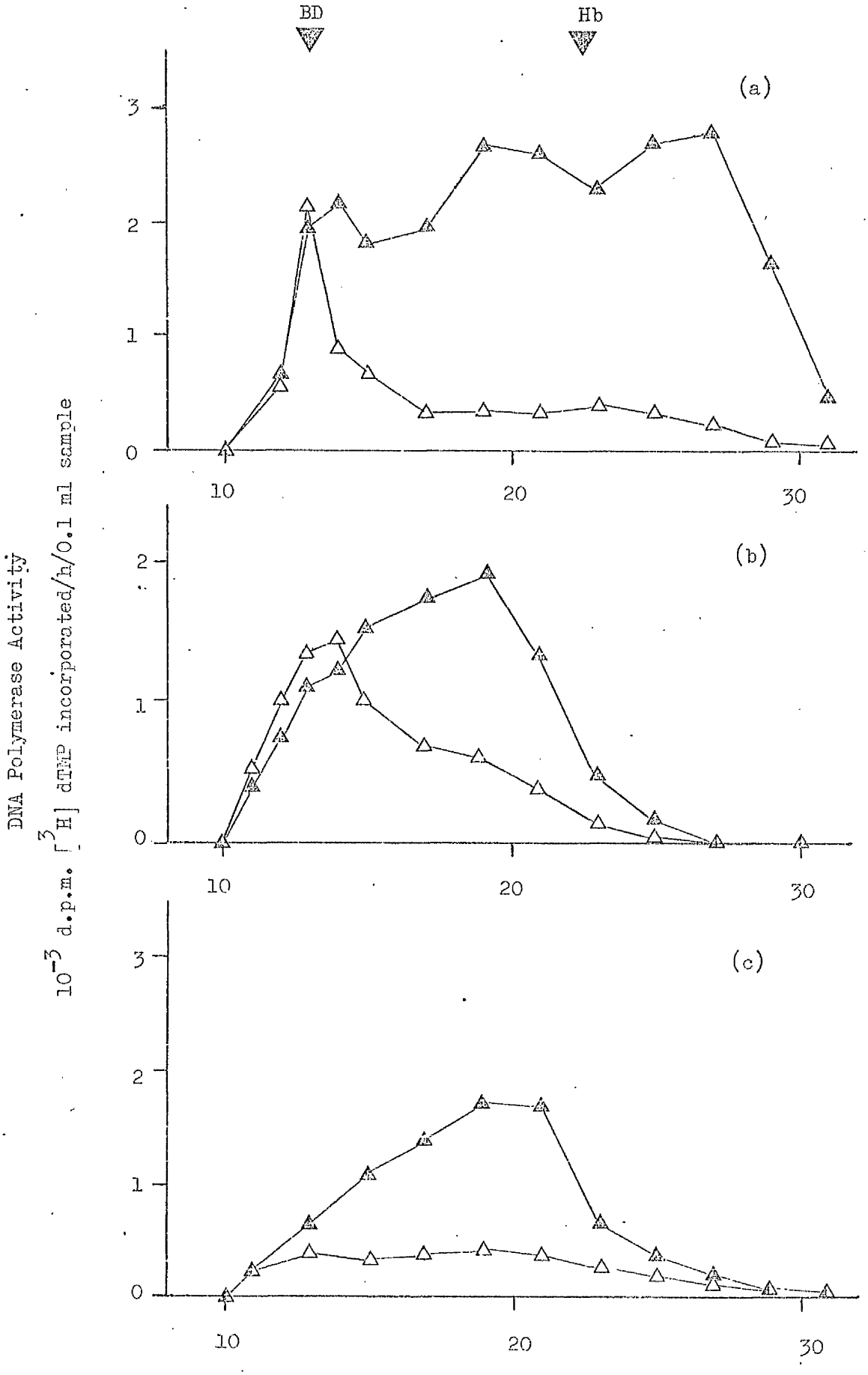
(c) 0.2M KCl

——▲—— DNA polymerase activity with native DNA

- - -△- - - with denatured DNA

Markers are as for Fig. 2.

Fig. 32


BD
Hb
(a)
(b)
(c)
3
2
1
0
10
20
30
DNA Polymerase Activity
10^{-3} d.p.m. [^{3}H] dTMP incorporated/h/0.1 ml sample
Fraction Number

Sephadex G-200, only low molecular weight enzyme was evident (Fig 32c). As 0.2M KCl extracted nuclei still retain appreciable DNA synthetic capacity (Table 13), the finding of only low molecular weight enzyme within these nuclei points to a role in the endogenous reaction for this species.

These results, which conflict with the inhibitor comparison studies (Results, section 11), which support a role for peak I activity in the endogenous reaction, must be interpreted with caution. KCl, which is capable of dissociating protein from chromatin, can render the DNA liable to damage (e.g. single-strand breaks) due to harsh treatment, which can lead to artefactual nuclear DNA synthesis. It has been shown that 0.3M KCl extracted nuclei from stationary L929 cells, although lacking most of their original DNA polymerase activity, are capable of substantial endogenous incorporation when treated with pancreatic DNase during incubation (R.L.P.Adams, personal communication). This suggests that any DNA polymerase species present within nuclei is capable of using the endogenous DNA template when it is sufficiently primed by natural or artificial means.

V DISCUSSION

1. INTRODUCTION

The purpose of this discussion is to correlate the results obtained in the previous chapter with similar work in other systems, in an effort to elucidate whether a particular DNA polymerase species is implicated in the process of DNA replication in L929 cells.

It is obvious that the process of DNA replication requires, among many activities, an enzyme capable of polymerising deoxynucleotidyl residues in a particular order dictated by a pre-existing DNA template (DNA polymerase activity).

Such enzymes are widespread in nature, but only one purified species, DNA polymerase III from *E. coli* has been shown to be involved directly in the replication of DNA. The unequivocal proof of involvement is derived from biochemical and genetical analysis of mutants deficient in the DNA replication process, an approach which at present is not applicable to mammalian systems. Other more biochemical approaches have therefore to be adopted.

DNA polymerising activity is present in L929 cells and characterisation of the product synthesised by crude cell fractions, using added DNA as template, suggests that the activity present is capable of polymerising deoxynucleotidyl residues from triphosphate precursors in a manner consistent with a replicative function (Lindsay, J.G., Ph.D. Thesis, University of Glasgow, 1969). However the lesson from *E. coli*, that an enzyme activity demonstrable in crude extracts may not be the only one of its kind in the cell, prompted a search for other DNA polymerising activities present in L929 cells.

2. MULTIPLE DNA POLYMERASE SPECIES OF L929 CELLS

The first suggestion that there may be more than one type

of DNA polymerase in L929 cells resulted from the finding that activity in soluble and nuclear fractions differed in the type of DNA best used as template (Lindsay & Adams, 1968). DNA polymerase activity from supernatant preparations preferred DNA which had been heat-denatured whereas nuclear preparations exhibited a preference for native DNA.

It was first thought that differential nuclease contamination in the crude cell fractions could be responsible for the observed template preferences but further fractionation of the supernatant preparation and extracts of nuclei revealed that the ability to use denatured DNA was associated with a species of high M.W. ($>$ 200,000 daltons) found in both supernatant and nuclear fractions, whereas native DNA was the preferred template of a smaller species which exhibited peaks of approximate M.W. 35,000; 70,000; 140,000 daltons and were found only in nuclear extracts (Results, section 2.1 and 2.2; Adams et al., 1973).

Several groups have shown the existence of at least two species of DNA polymerase activity, when attempts were made to identify all the species present (excluding the mitochondrial enzyme). Molecular weights and DNA template preference, when looked at, are similar to those for L929 cells (see Table 2, Introduction).

Possible explanations for the observed heterogeneity of L929 cell DNA polymerase

At least four explanations are available which would explain the observed findings. They are:-

(a) The small species is the *in vivo* enzyme and the isolation procedure leads to aggregation or association with other cellular

material creating an artefactual large species with altered DNA template requirements.

(b) The large species is the *in vivo* enzyme and the nuclear extraction procedure results in release of smaller active fragments not normally existing *in vivo*.

(c) The enzymes are structurally related and are readily inter-converted *in vivo* in response to changes in DNA metabolism e.g. replication, repair etc. The species distribution that is found on isolation will depend on the reactions being carried out by the cell prior to isolation.

(d) The enzymes are distinct and unrelated species involved in different aspects of DNA metabolism as in (c).

(a) and (b)

There is some evidence to suggest that the low ionic strength of the isolation buffer used leads to aggregation of the smaller nuclear DNA polymerase species. Peaks of DNA polymerase activity elute from Sephadex G-200 at positions corresponding to a monomer-dimer-tetramer association when extracts of nuclei are fractionated (Fig. 4). This was also found by Wicha & Stockdale (1972) for the small DNA polymerase species of chick embryo muscle under low ionic strength conditions. Further evidence in L929 cells stems from the finding that when a sample containing a lower protein concentration is applied to Sephadex G-200, a different elution pattern is observed, most of the activity eluting at the region of molecular weight 35,000 (Adams et al., 1973).

If the high molecular weight species is a result of further aggregation, or the low molecular weight species a result of artefactual dissociation of the larger species on salt extraction of nuclei, then it would be expected in both cases that the large species should release active low molecular weight enzyme on treatment with high salt solutions. This is not found in L929 cells, where the large species present in supernatant fractions dramatically loses activity without production of active small enzyme, when fractionated in the presence of 0.5M KCl (Fig. 9). Fractionation in high salt is essential, as simple treatment of the enzyme with 0.5M KCl followed by removal of salt by dialysis does not cause loss of activity. This suggests dissociation and separation of a factor necessary for activity. A search for this putative factor and elucidation of its role in the activity of high molecular weight DNA polymerase is under investigation.

Haines et al. (1971) reported that the high molecular weight soluble DNA polymerase activity in rat liver is susceptible to fractionation in high salt concentrations. No conversion to low molecular weight enzyme, which they find to be unaffected by high salt fractionation, is detectable. Other workers, although not reporting overall recoveries, find substantial high molecular weight enzyme after high salt fractionation (Bellair, 1968; Furlong & Gresham, 1971). Recently Hecht (1973a,b) in mouse testis and Lazarus & Kitron (1973) in baby hamster kidney cells, succeeded in releasing DNA polymerase activity similar in size to the low molecular weight species, by fractionating supernatant preparations on sucrose gradients in the presence of salt (Hecht used 0.125M $(NH_4)_2SO_4$ and Lazarus & Kitron used 0.45M NaCl). Low salt control gradients revealed only activity of high molecular weight prior to

salt treatment. Their results could be explained by release of nuclear-type activity from association with ribosomal material (Baril et al., 1971) or nuclear fragments. In neither case was the soluble fraction centrifuged sufficiently during preparation to remove such material. This may be the explanation for the results of Lazarus & Kitron, who still retain substantial DNA polymerase activity of high molecular weight after high salt treatment. Hecht's results, however, are harder to reconcile with this theory, as complete conversion to smaller enzyme is achieved. It is possible that the high molecular weight enzyme is inactivated due to the high salt fractionation as is found for the L929 enzyme.

(c)

The high molecular weight enzyme of L929 cells may still contain the smaller enzyme as a sub-unit, but for some reason not detectable on dissociation from it by salt. An interaction involving lipid however may be resistant to salt dissociation. Treatment of the high molecular weight enzyme with phospholipase, sodium deoxycholate or Triton X-100 does not result in low molecular weight enzyme being produced (Results, section 5.2 and 8). RNase treatment also has no effect. The foregoing results weigh heavily against specific association with lipid or RNA. Furthermore, association of DNA polymerase activity from L929 nuclei with DNA is not detectable at the salt concentration of the fractionation buffer used (Henderson, N.A.L., Ph.D. Thesis, University of Glasgow, 1972).

No activity capable of facilitating an *in vivo* conversion of the large DNA polymerase species to an enzyme of lower molecular weight is detectable in certain cell fractions from rapidly growing L929 cells (Results, section 5.1). Byrnes et al., (1974a) reported

an ATP-dependent dissociation of a large soluble species of DNA polymerase from rabbit erythroid hyperplastic bone-marrow but the dissociation product was still of high molecular weight.

(d)

There is no positive evidence that the two species represent distinct and unrelated enzymes and in fact immunological studies suggest that they may have antigenic sites in common (Chang & Bollum, 1972c).

Conclusions

Although most of the preceeding evidence shows no positive evidence for the low M.W. enzyme existing as a sub-unit of the high M.W. species, it cannot be ruled out that the large species contains a highly activated form of the low M.W. enzyme. On dissociation by any of the aforementioned treatments, detectable low M.W. activity would not be found.

3. HIGH MOLECULAR WEIGHT NUCLEAR DNA POLYMERASE FROM L929 CELLS

Nuclei from L929 cells contain a DNA polymerase activity indistinguishable from the soluble enzyme in size and DNA template preference (Fig. 4). There is an increased amount of this activity associated with nuclei from cells actively synthesising DNA (Fig. 14).

A high M.W. species of DNA polymerase has been described and in some cases partially purified from nuclei from a variety of rapidly growing cells e.g. HeLa cells (Weissbach et al., 1971); KB cells (Sedwick et al., 1972); regenerating rat liver (Wallace et al., 1971; Baril et al., 1973). It is similar in size and DNA template preference to the enzyme found in supernatant fractions from the same source. The two enzymes elute at the same salt

concentration from ion-exchange columns and are both susceptible to inhibition by sulphydryl group inhibitors (Adams et al., 1973; Weissbach et al., 1971; Sedwick et al., 1972). It therefore appears that the large enzyme, on extraction from nuclei, is very similar to the soluble species and may in fact be identical to it. There is some evidence that it might be slightly modified with respect to DNA template usage. Sedwick et al. (1972) report that the nuclear species is more efficient with a gapped DNA template than the corresponding enzyme from soluble fractions. Better use of homopolymer templates (i.e. poly dA:dT_{10}) was reported for a large tightly bound nuclear species from cultured hamster fibroblasts (Furlong et al., 1973). A large species isolated from nuclei of DAB induced tumours in rat liver appears to have reverse transcriptase activity (Chiu et al., 1973). However this may represent a species peculiar to this system.

Possible explanations for the observed intracellular distribution of high M.W. DNA polymerase in L929 cells

Five explanations which could account for the observed findings are as follows:-

(a) Soluble enzyme arises by leakage of high M.W. enzyme from nuclei.

(b) High M.W. nuclear enzyme is a result of contamination of nuclei by soluble enzyme.

(c) The soluble enzyme is modified by a factor synthesised in S-phase cells enabling binding to nuclei.

(d) DNA or chromatin is modified during S-phase enabling soluble

enzyme (unmodified) to bind.

(e) The high M.W. nuclear species represents a distinct and unrelated enzyme from the soluble enzyme and is synthesised specifically in response to cell proliferation.

(a)

It is somewhat paradoxical that the high M.W. DNA polymerase, which seems to demonstrate the correct biological correlation with nuclear DNA synthesis in vivo, should be consistently recovered in cytoplasmic fractions. It leaves the method of cell fractionation open to suspicion. The intracellular integrity and in vivo nucleocytoplasmic interactions could be completely disrupted on cell breakage giving rise to artefactual distribution of cellular material. It is possible therefore that the presence of DNA polymerase activity in supernatant fractions of cell homogenates may represent leakage from the nucleus especially using aqueous isolation techniques. This was originally thought to be the case, and use of non-aqueous solvents and incorporation of nuclear membrane stabilisers (e.g. Ca^{2+}) into aqueous isolation media increased the proportion of activity retained by nuclear preparations (Keir, 1965). However high losses of activity resulted and no definite conclusions about intracellular distribution could be made.

Byrnes et al. (1974b) claim that the cytoplasmic location of DNA polymerase activity is not artefactual or trivial by revealing the presence of the enzyme in the rabbit reticulocyte, which is an anucleate cell.

(b)

It has been evident for some years now that increased levels of high M.W. soluble DNA polymerase activity are associated with cell proliferation (Iwamura et al., 1968; Ove et al., 1969; Chang & Bollum, 1972d; Chang et al., 1973). It is possible that the high M.W. nuclear species found by several groups, especially in rapidly growing systems, is a result of cytoplasmic contamination. The results in the present work with L929 cells show that DNA polymerase activity is not exchangeable between cytoplasmic and nuclear fractions during the isolation procedure (Table 6).

Several groups find no evidence for a high M.W. nuclear species. Whether it is found seems to depend on the ionic strength of the isolation medium used and the treatment employed for further nuclear purification. Wallace et al. (1971) showed that slight differences in the contents of isolation media, not accountable by ionic strength alone, had a dramatic effect on the amount of high M.W. DNA polymerase recovered in nuclear preparations from regenerating rat liver. The main difference appears to be a higher concentration of polyamines in the isolation media resulting in loss of high M.W. enzyme. Brewer & Rusch (1966) reported that an S-phase specific increase in DNA polymerase activity associated with the nuclei of the slime mould Physarum polycephalum, was not measurable with an added DNA template unless the polyamine, spermine was present. Therefore it appears as if polyamines may release DNA polymerase from nuclear binding. Treatment involving the Blobel & Potter (1966) method of nuclear purification, or a modification thereof (i.e. involving hypertonic sucrose), results in nuclei lacking high M.W. enzyme.

Coincidentally, similar nuclear purification procedures also lead to loss of correlation between nuclear DNA synthesis and the DNA synthetic capacity of the cells of origin (Hyodo & Ono, 1970a; Kaufman et al., 1972), thereby throwing suspicion on this method of nuclear preparation when studying the intracellular distribution of DNA polymerase.

(c) and (d)

There is evidence for a positive control on the binding of high M.W. enzyme to nuclei of L929 cells during S-phase. The enzyme present in a 0.4M KCl extract of nuclei from S-phase cells is able to rebind to the extracted nuclei, whereas binding of enzyme in the supernatant fraction to extracted nuclei under the same ionic strength conditions, is not found (Results, section 9.1 and 9.2). The enzyme in extracts of S-phase is able to bind equally well to nuclei from stationary cells, but only after 0.4M KCl extraction (Results, section 9.3). Unextracted nuclei from stationary cells, which contain low levels of the high M.W. enzyme, are unable to bind enzyme from S-phase nuclear extracts (Fig 19).

The foregoing findings suggest that two steps are necessary before high M.W. enzyme can bind to nuclei. Firstly a block which prevents binding of enzyme to nuclei from stationary cells must be removed (achieved experimentally by 0.4M KCl extraction). The inability of nuclei from stationary cells to bind the high M.W. enzyme may be analogous to the situation in sea urchin embryos. Fansler & Loeb (1972) showed that DNA polymerase activity in early embryos fluctuates between nucleus and cytoplasm, being found in much lower amounts in nuclei isolated from mitotic or G-2 embryos. (There is no G-1 phase in sea urchin embryos.) They interpret this

as being due to the condensation of chromosomal material for mitosis. As the chromosomes begin to unwind after mitosis, DNA polymerase attachment points on the DNA or chromatin become available. The DNA of stationary L929 cells may also be present as a compacted structure preventing enzyme attachment. The potential enzyme binding sites possibly become unmasked as the chromatin slowly unwinds during G-1 phase. Secondly, a factor necessary for binding must be present, as in 0.4M KCl extracts of S-phase nuclei. It is not known whether this factor remains associated with the large nuclear enzyme (implying a modified form of the soluble species) or is merely present in nuclear extracts of S-phase cells.

(e)

There is little positive evidence to suggest that the high M.W. nuclear DNA polymerase species is a distinct enzyme unrelated to the soluble species. A large enzyme isolated from nuclei of DAB-induced tumour cells in rats, is reported to exhibit substantial incorporation with ribohomopolymer templates (Chiu et al., 1973). In contrast, the high M.W. soluble enzyme from calf thymus has negligible reverse transcriptase activity (Chang & Bollum, 1972a).

Conclusions

From the evidence available, it appears as if the high M.W. DNA polymerase enzyme of L929 cells partitions itself between nucleus and cytoplasm depending on the presence of a binding factor probably synthesised during periods of DNA synthesis.

Evidence from other sources which supports a physiological role for high M.W. nuclear DNA polymerase are the reports which reveal differences between the properties of the nuclear-located

and cytoplasmic enzyme (Sedwick et al., 1972; Furlong et al., 1973).

4. THE NATURE OF DNA SYNTHESIS *IN VITRO* USING ISOLATED L929 NUCLEI

Several reports have demonstrated that isolated eukaryotic nuclei may be useful in studying the mechanism of DNA replication.

Results, section 10 shows that nuclei from L929 cells are capable of DNA synthesis in the absence of added DNA as template. The basic requirements are similar to those for DNA polymerase assays except that the optimum salt concentration is higher and the reaction is stimulated by ATP. A greater initial rate of synthesis is exhibited by nuclei isolated from cells in S-phase compared to nuclei from stationary cells (Fig. 24). The product of nuclear DNA synthesis is demonstrated by alkaline sucrose gradient centrifugation to be exclusively in small pieces compared to bulk nuclear DNA (Fig. 25). This suggests synthesis of fragments similar to those found *in vivo* in prokaryotic systems i.e. Okazaki fragments. However, no evidence for *in vitro* initiation of such fragments is found, and lack of radioactive label associated with bulk DNA suggests that ligation of the small fragments to bulk DNA is not occurring (Fig. 25). It therefore appears as if the L929 nuclear DNA synthesis involves extension and possible completion of Okazaki-type fragments already initiated *in vivo*.

Similar observations have been reported by other workers using isolated nuclear systems from various sources.

Nuclear DNA synthesis appears to be S-phase specific (Friedman & Mueller, 1968; Lynch et al., 1970), except when nuclei are treated with hypertonic sucrose solutions (Kaufman et al., 1972; Hyodo & Ono, 1970a; Ove et al., 1971). Further studies by the same workers support the results with L929 cells, that the product of *in vitro* DNA synthesis by nuclei is at least partly in small

fragments (Kidwell & Mueller, 1969; Friedman, 1973; Hershey & Taylor, 1974). In addition, the latter two reports provide evidence which suggest that one of the DNA strands is synthesised continuously.

The absence of *in vitro* ligation found for L929 nuclei may suggest that mammalian ligase is not retained by nuclei during the isolation procedures employed. Evidence for ligation on addition of cytoplasm to HeLa cell nuclei has been reported (Kidwell & Mueller, 1969).

Other evidence to suggest that the *in vitro* reaction is a continuation of the process taking place in the nuclei prior to isolation, has been reported. When cells are pre-treated with bromodeoxyuridine immediately prior to nuclear isolation, then the *in vitro* product of nuclear DNA synthesis is of hybrid density (Lynch et al., 1970; Hershey et al., 1973a). This suggests direct continuation by nuclei *in vitro*, at the *in vivo* sites.

Lynch et al., (1970) also showed by autoradiography that the same percentage of nuclei from regenerating rat liver are incorporating *in vitro* as were actively replicating *in vivo*. Control experiments, labelled *in vivo* and *in vitro* confirmed that the same nuclei were involved in the two reactions.

Incorporation by isolated nuclei therefore appears to represent a continuation of *in vivo* replication and therefore they probably contain the enzymes, including the DNA polymerising enzyme necessary for synthesis of Okazaki pieces.

The inability to maintain the initial rate of nuclear DNA synthesis, a finding confirmed by most investigators, appears in L929 cells to be a result of loss of an essential factor caused by incubation at 37°C (Table 11). Hershey et al. (1973a) came to a similar conclusion with HeLa nuclei based on the fitting of

experimental time course points to a theoretical exponential decay curve. Further work by the same group (Hershey et al., 1973b), investigating the effect of inhibiting cellular synthesis of RNA, DNA and protein on the DNA synthetic activity of isolated S-phase nuclei, suggested a requirement for an unstable protein necessary for the maintenance of DNA replicase sites which are established in S-phase nuclei.

5. ROLE OF DNA POLYMERASE SPECIES IN DNA SYNTHESIS BY ISOLATED L929 NUCLEI

The following observations imply a role for the high M.W. DNA polymerase enzyme in nuclear DNA synthesis.

(a) Association of high M.W. enzyme with nuclei shows S-phase specificity, as does DNA synthesis by isolated nuclei.

(b) Hypertonic sucrose treatment of nuclei which leads to loss of the high M.W. enzyme, also leads to loss of the S-phase specificity of nuclear DNA synthesis.

(c) DNA synthesis by L929 nuclei is inhibited by NEM, a sulphydryl group inhibitor, Triton X-100 and araCTP. The high M.W. nuclear enzyme is similarly inhibited by these substances whereas the low M.W. enzyme is only slightly inhibited by NEM and not at all by Triton X-100 or ara CTP (Results, section 11).

A role for low M.W. enzyme in nuclear DNA synthesis is suggested by the following observations:-

(a) Brij/Mg extracts virtually all of the DNA polymerase activity present in nuclei isolated from stationary L929 cells

(Table 15). However, after similar extraction of S-phase nuclei, which results in retention of nuclear DNA synthesis, a substantial amount of low M.W. enzyme is retained by the nuclei, suggesting an S-phase specific form of low M.W. enzyme (Fig. 32a).

(b) 0.2M KCl extracted nuclei are still capable of *in vitro* DNA synthesis (Table 13). Fractionation of the DNA polymerase activity remaining in such nuclei reveals only low M.W. enzyme (Fig. 32c)

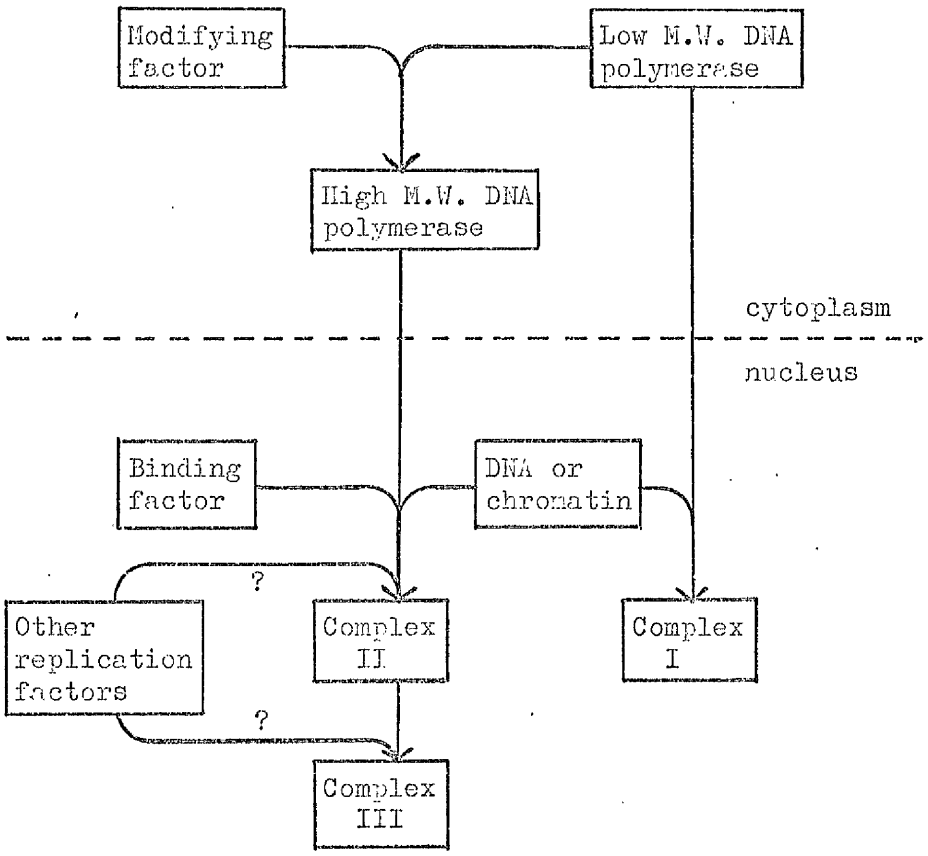
(c) Chiu et al. (1973) find an S-phase specific increase in a tightly-bound low M.W. DNA polymerase species.

This apparent discrepancy, i.e. both nuclear DNA polymerase species being implicated in nuclear DNA synthesis, is possibly explained by the following model which speculates on the possible *in vivo* forms of DNA polymerase based on evidence presented in the Results chapter. The model is described in schematic form in Fig. 33.

It is proposed that the low M.W. enzyme is responsible for the catalytic activity of all the DNA polymerising species identified. It has a high affinity for DNA and is rapidly transported to the nucleus (after synthesis in the cytoplasm) where it is potentially available for gap-filling functions requiring DNA complementing activity, e.g. repair, recombination, perhaps even Okazaki fragment completion during replication in a similar manner to *E. coli* DNA polymerase I (Okazaki et al., 1971). However it is not involved in the bulk of DNA replication. It is this form which is extractable from nuclei by Brij/Mg, hence its almost complete removal from nuclei of stationary L929 cells (Table 15).

Fig. 33

Model describing the proposed intracellular forms of L929 cell DNA polymerase



Complex I - complex of DNA or chromatin with low M.W. DNA polymerase - involved in gap-filling functions e.g. repair etc. - dissociable by 5% Brij 58/50mM magnesium acetate (see text).

Complex II- weak complex of DNA or chromatin with high M.W. DNA polymerase - possibly binding of enzyme to replicon or Okazaki fragment initiation sites when they become available as S-phase progresses - dissociable by salt (>0.1M KCl) (see text).

Complex III- tight complex of DNA or chromatin with DNA polymerase (high or low?) involved in Okazaki fragment extension and/or completion - dissociable by salt (>0.2M KCl) (see text).

In actively dividing cells, a modifying factor is present which binds to the low M.W. enzyme resulting in production of high molecular weight enzyme which has a preference for denatured DNA as *in vitro* template and a low affinity for DNA, hence its recovery in cytoplasmic fractions on isolation in aqueous media. This predicts that low M.W. enzyme should be dissociable from its association with modifying factor. Fractionation in high salt (0.5M KCl) results in loss of high M.W. activity (Fig. 9) but as indicated in the inset to Fig. 9, unless fractionated under the high salt conditions reassociation occurs on reduction of ionic strength. Only a very small proportion of the total low M.W. enzyme appears to be present in this form hence the inability to detect active enzyme at the expected region on the column profile after fractionation of the high M.W. enzyme in 0.5M KCl (Fig. 9). This therefore suggests that, on association with the modifying factor, there is an enhancement of the catalytic activity of the low M.W. enzyme as measured on *in vitro* DNA templates, particularly when denatured. It is this enhanced DNA polymerase activity which is comparatively unstable and susceptible to the inhibitors described in Results, section 11. The modifying factor probably contains a sulphydryl group(s) necessary for its function, hence its severe inhibition by NEM.

AraCTP probably affects the enhanced DNA synthesis reaction carried out by the high M.W. enzyme rather than directly inhibiting the modifying factor. Complementation of long single-stranded regions, the type of synthesis characteristic of high M.W. DNA polymerase and probably of the *in vitro* nuclear DNA synthesis, is more likely to be susceptible to araCTP than the short gap-filling reaction carried out *in vitro* by the low M.W. enzyme (see Sedwick

et al., 1972).

Evidence to support just such an inhibitory action for ara CTP has been reported recently by Masker & Hanawalt (1974). They showed that in toluenised-bacterial cells, DNA replication was strongly inhibited by araCTP whereas u-v stimulated repair, which involves synthesis of short stretches of DNA, was relatively unaffected.

As the modifying factor stimulates DNA polymerase activity with a denatured DNA template, it could be similar in nature to the gene 32 protein of T4 phage which stimulates the activity of T4 DNA polymerase 5-10 fold with a single-stranded DNA template (Huberman et al., 1971). This stimulation is probably a result of favourable template alignment by gene 32 protein.

Another analogous situation is the apparent modification of DNA polymerase III of E. coli to DNA polymerase III star which is then able to use long single-stranded templates (Wickner et al., 1973c).

Returning to eukaryotic systems, the high M.W. DNA polymerase enzyme, formed by binding of the modifying factor to low M.W. enzyme, has little affinity for nuclei and thus may represent a precursor form of the actual replicating enzyme created in readiness for DNA replication.

Binding of the high M.W. enzyme to the nucleus is S-phase specific and appears to depend on two pre-requisites:-

(1) availability of nuclear binding (or initiation) sites normally masked in stationary cells.

(2) presence of a binding factor probably synthesised during S-phase.

Certain results from the present work suggest that satisfaction of one of the above requirements is not sufficient for binding to occur. S-phase nuclei contain both binding-sites and binding factor and hence high M.W. enzyme is found attached to nuclei. When extracted with 0.4M KCl, both stationary and S-phase nuclei are equally capable of binding high M.W. enzyme from 0.4M KCl extracts of S-phase nuclei (Fig. 19). This binding is not found for high M.W. enzyme in a S-phase supernatant fraction (Fig. 18), suggesting that the binding factor is present in the 0.4M KCl nuclear extracts of S-phase cells. However binding of DNA polymerase from such extracts to unextracted nuclei from stationary cells is not observed (Fig. 19). This suggests that such sites are normally masked in stationary cells probably becoming available when cells are induced to proliferate.

This nuclear binding factor may serve a similar role to copolymerase III star, a protein factor purified from extracts of ØX 174 infected E. coli. This factor is involved in initiation of DNA synthesis, on long single-stranded DNA templates by a modified form of DNA polymerase III (i.e. DNA polymerase III star) (Wickner & Kornberg, 1973). Copolymerase III star is not required to sustain synthesis as only initiation, and not elongation is susceptible to antibody raised against it.

The bound form of high M.W. DNA polymerase may remain associated with binding factor on extraction as differences in template use between the soluble and the nuclear enzyme have been reported (Sedwick et al., 1972; Furlong et al., 1973).

High M.W. DNA polymerase is removed from nuclei by, for example, 0.1-0.2M KCl in the case of L929 cells (Table 14; Fig. 32c), and as such nuclei are still capable of endogenous incorporation

(Table 13), a further more tightly-bound form of DNA polymerase must be involved in this reaction.

Other enzymes which react with nucleic acids have been shown to have weak and tight binding transitions which appear to be temperature-dependent (may involve helix opening). Qβ phage RNA polymerase has been shown to form a weak salt-dissociable complex with Qβ RNA at $0^{o}C$ and a tight non-dissociable complex at $37^{o}C$ (Silverman & August, 1970). E. coli RNA polymerase also forms a stable holoenzyme-DNA complex, which requires incubation to a temperature above $17.5^{o}C$, to be formed (Hinkel & Chamberlin, 1970; Zillig et al., 1970). Rat liver DNA methylase exhibits a similar temperature-dependent binding transition (Drahovsky & Morris, 1971).

If the replicating activity represents a tightly bound form of the nuclear high M.W. enzyme, then two deductions can be made. Firstly, the proportion of the total low M.W. enzyme bound up in the tightly bound replicating complex must be greater than that in the high M.W. species of supernatant fraction as low M.W. enzyme is detectable on salt dissociation (Fig. 32a). Secondly, the modifying factor as present in the tightly-bound complex must either be inactivated or not extractable by 0.4M KCl otherwise it would give rise to high M.W. enzyme in 0.4M KCl extracts of 0.2M KCl extracted nuclei, which is not observed.

6. FURTHER WORK

The model described in Fig. 33, proposes how certain factors synthesised in response to cell proliferation can alter the size, catalytic activity and intracellular location of DNA polymerase activity.

A search for the modifying factor which can presumably be separated from the large M.W. DNA polymerase enzyme by fractionation in high salt, is underway. The ability to greatly enhance the

activity of low M.W. activity particularly with a denatured DNA template would provide an assay for its detection.

The nuclear binding factor, which appears to be present in S-phase nuclear extracts, could be assayed by its ability to induce binding of high M.W. supernatant enzyme to extracted nuclei (or perhaps even DNA).

Purification of such factors and elucidation of their relationship to DNA polymerase should provide a new approach to the problem of DNA replication and its control in eukaryotic systems.

VI SUMMARY

SUMMARY

1. L929 cultured mouse fibroblasts contain multiple species of DNA polymerase. High-speed supernatant preparations contain one species as revealed by Sephadex G-200 gel filtration. It has a molecular weight in excess of 200,000 daltons and has a preference for denatured DNA as template. An 0.4M KCl extract of nuclei exhibits multiple peaks of DNA polymerase activity on Sephadex G-200. One of the species (peak I) is similar in size and DNA template preference to the soluble enzyme. Three other species (peaks II, III and IV) are usually found, the major peak (peak III) having a M.W. of approximately 70,000 with the other two species often eluting as shoulders of this central peak. Size considerations suggest a monomer - dimer-tetramer relationship but this has not been firmly established. The smaller nuclear species have a preference for native DNA as template.

2. Previous studies had suggested that deoxyribonucleoside 5'-diphosphates may be nearer, than the triphosphates, to the immediate substrate for the nuclear DNA polymerase. Any conclusions were always complicated by the presence of nucleoside diphosphokinase activity. Fractionation of nuclear extracts shows that for nuclear peak I activity, incorporation of diphosphates, even in the presence of a phosphate donor, is not demonstrable due to the absence of nucleoside diphosphokinase activity.

3. An activity capable of stimulating DNA polymerase by providing RNA primers with 3'OH ends is not detectable in

soluble or nuclear fractions of L929 cells.

4. An activity capable of converting soluble DNA polymerase into smaller active fragments typical of the smaller nuclear species is not detectable in supernatant or extracted nuclei. However nuclear extracts were not tested as they already contained substantial nuclear species.

5. Centrifugation and agarose gel filtration studies suggested that the soluble enzyme was very large ($> 0.5 \times 10^6$ daltons). Using chemical and enzymic treatment of the soluble high M.W. activity, attempts were made to release smaller DNA polymerase species resembling the low M.W. nuclear species. The attempts were unsuccessful, but do not rule out production of a low M.W. species of dramatically reduced activity. The soluble enzyme is sensitive to salt dissociation and to the ionic detergent, sodium deoxycholate suggesting a lipoprotein complex. Association with low density lipoprotein such as smooth membrane was not demonstrable.

6. Responses of L929 cell DNA polymerases to growth changes, suggest that the soluble DNA polymerase activity becomes associated with the nucleus when cells are synthesising DNA (S-phase cells). Attempts to elucidate the nuclear binding site revealed that the soluble enzyme is unable to bind to native or denatured DNA. Peak I nuclear activity present in 0.4M KCl extracts of nuclei from S-phase cells is, however, able to rebind to the nuclei after reconstitution in low salt. Soluble activity is unable to bind under the same conditions suggesting that S-phase nuclear extract contains a factor essential for binding. This was confirmed by showing that peak I activity in S-phase extracts was able to

bind to extracted nuclei regardless of the growth state of the cell of origin.

7. Isolated nuclei from L929 cells are capable of DNA synthesis in the absence of added DNA. This endogenous synthesis reflects the in vivo DNA synthetic capacity of the cells of origin. The endogenous reaction differs from the DNA polymerase reaction in having a higher optimum salt concentration and by being stimulated by ATP. The DNA product, as opposed to the template DNA, is in small pieces similar to those found in vivo, i.e. Okazaki fragments. This confirms previous findings that nuclei are extending Okazaki pieces in vitro. In vitro, S-phase nuclei stop DNA synthesis after 10-20 min of incubation and attempts to discover the cause of this premature cessation, led to the implication of a thermolabile or thermodiffusible factor rather than exhaustion of an added assay constituent or completion of available incorporation sites.

8. Comparison of the effects of various inhibitors on nuclear DNA synthesis and on the activity of DNA polymerases within the nuclei, implies that a reaction similar to that carried out by peak I is taking place in the isolated nuclei.

9. Extraction of all DNA polymerase activity from nuclei causes complete loss of their ability to make DNA. Attempts to reactivate the nuclei were only partially successful. When the nuclei are centrifuged and washed in high salt solutions, their capacity for reactivation is dramatically reduced.

10. Use of alternative extracting agents leads to removal of specific DNA polymerase species from nuclei. 5% Brij 58 in conjunction with 50 mM magnesium acetate removes only low M.W.

DNA polymerase from nuclei. A proportionately higher level is extractable by Brij/Mg from nuclei of stationary phase cells compared to nuclei from S-phase cells. Brij-extracted S-phase nuclei still retain 70-90% of their DNA synthetic capacity suggesting that the activity removed with Brij is not involved in nuclear DNA synthesis. Extraction with low concentrations of KCl (0.1M and 0.2M) has very little effect on endogenous nuclear DNA synthesis and appear specifically to remove peak I activity from the nuclei. Investigation of the residual DNA polymerase species within nuclei after the afore-mentioned extractions reveals that nuclei containing only a species eluting in the region of 70,000 on Sephadex G-200, are still capable of DNA synthesis. This suggests a role for this species in the extension of Okazaki fragments.

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