THE SYNTHESIS OF DNA IN TUMOUR CELLS

by

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CONTENTS.

ABBREVIATIONS.

The abbreviations used were those recommended by the Biochem.J. in their Instructions to Authors. In addition the following were also used :-

BSA	Bovine serum albumin
Buffer 1	0.02M-tris-HCl, pH7.5
Buffer 2	0.02M-tris-HCl, pH7.5, 5mM-2-mercaptoethanol
Buffer 3	0.02M-tris-HCl, pH7.5, 5mM-2-mercaptoethanol, 1mM-EDTA
Buffer 4A	0.02M-tris-HCl, pH7.5, 5mM-2-mercaptoethanol, 1mM-EDTA, 0.15M-KCl
Buffer 4B	0.04M-tris-HCl, pH7.5, 5mM-2-mercaptoethanol, 1mM-EDTA, 0.15M-KCl.
Buffer 5	0.02M-tris-HCl, pH7.5, 5mM-2-mercaptoethanol, 1mM-EDTA, 0.15M-KCl, 4mM-$MgSO_4$.
DNase	Deoxyribonuclease (EC 3.1.4.5.)
DNA polymerase	DNA nucleotidyltransferase (EC 2.7.7.7.)
PBS	Phosphate buffered saline (7.7mM-Na_2HPO_4, 1.5mM-KH_2PO_4, 0.14M-NaCl)
PCA	Perchloric acid
SSC	Standard saline citrate (0.15M-NaCl, 0.015M-sodium citrate, pH7.0)
$\frac{1}{10}$ SSC	0.015M-NaCl, 0.0015M sodium citrate, pH7.0.
TCA	Trichloroacetic acid.

INTRODUCTION

The most exciting aspect of the postulated model of the structure of DNA (Watson & Crick, 1953) was that it suggested how DNA can produce an exact replica of itself to be passed on to a daughter cell.

Since the late 1950s an increasing amount of research has been carried out to elucidate the exact processes which occur during DNA replication, but the picture is still not complete.

I D N A

I A Structure of Native DNA.

DNA is a high molecular weight polymeric macromolecule which exists in its native form as a double-stranded helical structure, with a backbone of repeating units of phosphate-β-2-deoxyD-ribose linked by 3',5'-phosphodiester bonds. Each carbon 1 of the ribose moiety is covalently bound to either a purine (adenine or guanine) or to a pyrimidine (cytosine or thymine) base. Occasionally other bases (5-methyl cytosine, 5-hydroxymethyl cytosine, 5-hydroxymethyl uracil, 6-methylaminopurine) can replace those mentioned in small quantities (Davidson, 1965).

Chargaff (1955b) analysed the base ratios from many DNA species and found equivalence between adenine and thymine, and between guanine and cytosine. X-ray diffraction studies on crystalline fibres of alkali metal salts of DNA (Wilkins, Stokes & Wilson, 1953) led Watson & Crick (1953) to postulate the structure of native DNA as consisting of two right-handed helical polynucleotide chains of opposite polarity,

Nearest neighbour frequency analysis of the DNA bases has since confirmed that the chains are of opposite polarity (Josse, Kaiser &Kornberg, 1961). The bases are on the inside of the molecule so that there is hydrogen bonding between the pyrimidine bases of the one strand and the purine bases of the other. The planes of the aromatic rings of the bases are at right angles to the common axis of the helices, and are stacked on top of each other. The dipole moments between the successive base-pairs account for the stability of DNA (Devoe & Tinoco, 1962). There are 10 base pairs per turn of the double helix structure, with 34Å in height per turn. The DNA molecule has two grooves, one shallow, 12Å across between the hydrogen bonded strands, and one deep, 24Å between the non bonded adjacent strands.

I B Structure of denatured DNA.

The ordered structure of the native rod-like polymers of DNA can be destroyed in the process known as denaturation. The separation of the two strands and the disruption of the base stacking forces are accompanied by increases in ultraviolet absorption at 260 mμ. Other methods of detection include optical rotatory dispersion, circular dichroism, and viscometry, as well as physical methods of separation of native and denatured DNA. Denaturation can be brought about by high temperatures, extremes of pH, removal of ions, or by the addition of various organic reagents (alcohols, glycols, amides, aliphatic and heterocyclic amines, substituted ureas, phenols, sulphoxides) or certain anions ($CCl_3CO_2^-$, CNS^-, ClO_4^-). The structure of this DNA can vary between completely isolated single strands of randomly coiled DNA, which is irreversibly denatured, to DNA that is almost native. Partial renaturation usually occurs after withdrawal of the denaturing agent,

but the extent depends largely on the method of denaturation and on the type of DNA e.g. whether or not it has covalent cross-links (Becker, Zimmerman & Geiduschek, 1964). Gentle denaturation by the removal of ions during dialysis can be almost completely reversed by the addition of ions (Lin & Chargaff, 1966). More detailed analyses of the structure of intermediates between native and denatured DNA have been made using various techniques, as listed by Paleček (1968). Intermediates which are not detectable by ultraviolet absorption, but that exist at temperatures below melting temperature are thought to have areas of strand separation without disruption of the base stacking.

I 0 In vivo function and structure

It is now well established that the sequence of bases in DNA contains coded genetic information for the synthesis of the cell's proteins (Sinsheimer, 1957).

Different forms of DNA exist in different forms of life. In DNA viruses it is contained within the protein capsid in a contracted form. Molecular weights vary from 10^6 to 2×10^8 daltons in different viruses. The DNA can occur as single-or double-stranded, and either linear or circular, though all single stranded DNA so far found is circular. Bacterial DNA exists in one (though occasionally up to four) nucleoid in the centre of the cell, and there is $2\text{-}25 \times 10^{-6}$ µg./cell. Cairns (1963a and b) isolated whole circular DNA of molecular weight 2.8×10^9 daltons from Escherichia coli. This supports the theory that, like viruses, the DNA of bacteria may exist in one molecule of DNA per chromosome.

In the eucaryotic cell DNA is contained in chromosomes within a distinct nucleus. The chemical constituents of chromosomes include DNA, RNA, proteins (basic and acidic) and divalent ions. The physical structure is still, however, in doubt. X-ray analysis by Wilkins (1956) has shown that the basic proteins, protamines and histones are bound, probably electrostatically, to the shallow groove of the DNA double helix. Models of chromosome structure have been suggested by Freese (1958) and Taylor (1963) and theories on the chromatid folding by DuPraw (1966).

In addition to the nucleus, DNA is present in the mitochondria and other cytoplasmic organelles (Granick & Gibor, 1967). Electron microscopy of mitochondrial DNA from mouse fibroblast cells by Nass (1966) shows it to be circular with two to six DNA rings in one mitochondrion. They appear to be attached in places to the mitochondrial membrane. Differences between mitochondrial DNA of normal mammalian cells and of leukaemic leucocytes have been shown by Clayton et al.(1968)

I D *In vivo* metabolism

Incorporation of labelled precursors into DNA *in vivo* was first investigated in 1942 by von Euler and von Hevesy. Since then this technique has been widely used for studying the mechanisms of DNA synthesis *in vivo*.

In bacteria DNA synthesis occurs throughout the cell cycle (Abbo & Pardee, 1960), though in higher organisms DNA synthesis occupies only a small portion of the cell cycle. In mammalian tissue it takes place shortly before mitosis.

DNA synthesis in Esch.coli was shown to take place by semiconservative replication in the classic experiment by Meselson and Stahl (1958). Further insight to the problem was produced by Cairns (1963a and b) in his autoradiographs of the replication of the Esch. coli chromosome. The daughter rings were made by unidirectional semiconservative replication and remained attached to the starting point until completion. DNA synthesis took place at a fork at the rate of 33 μ/min. To allow unwinding of the strands at the necessary speed for synthesis (10,000 rev./min.) he postulated a swivel at the starting point. Helmstetter (1968) claims multiple replication forks in the one chromosome.

In higher organisms replication takes place at a number of different points on the chromosome as different rates (Stubblefield & Mueller, 1962; Taylor 1963). Coleman and Shigefumi (1968) have isolated a replicating fork in mammalian replicating DNA, and electron micrographs show it to have 2.3×10^7 daltons/fork and the daughter strands to be completely native. DuPraw (1966) postulated that the replication of DNA in human chromosomes takes place at a rate of 2μ/min.

Recently the nature of newly synthesized DNA has been investigate by various groups. Kidson (1966) isolated rapidly labelled bacterial DNA by countercurrent distribution, and found it to be more denatured than the bulk DNA.. Okazaki and his associates, and Oishi have shown by short pulse labelling that newly synthesized DNA can be separated from the bulk DNA on hydroxyapatite and on CsCl gradients. It was shown to be single stranded by susceptibility to exonuclease I

degradation and of low molecular weight by alkaline sucrose gradients. A second intermediate of higher molecular weight was detected by longer pulse labelling, which was essentially double-stranded although it had a greater affinity for nitrocellulose powder than the bulk DNA (Sakabe & Okazaki, 1966; Okazaki et al., 1968; Oishi, 1968a, b & c). Rosenberg and Cavalieri (1968) suggest, however, that this low molecular weight, partly denatured DNA may occur during cell lysis. Lysis produces shear in the DNA which lowers the melting temperature.

Similar work on newly synthesized DNA has been performed in mammalian systems. Paoletti, Dutheillet-Lamonthezie, Jeanteur & Obranovitch (1967) working with ascites cells found that the newly synthesized DNA was of low molecular weight and had a higher affinity for MAK than the bulk DNA although its bouyant density on CsCl and Cs_2SO_4 was the same as that of the bulk DNA. They suggested that its structure was intermediary between native and denatured DNA. Lieberman and his colleagues (Tsukada, Moriyama, Lynch & Lieberman, 1968) have performed similar studies with newly synthesized DNA from regenerating rat liver and found it to be low molecular weight, but increasing with length of pulse. Unlike the bacterial systems it was double-stranded although its susceptibility to exonuclease I was slightly greater than the bulk DNA. In contrast to this Nasavi (1967) has detected short segments of single stranded DNA in lymphocyte chromosomes by studies with fluorescein labelled nucleotide antisera.

II Properties of DNA polymerase *in vitro*

An alternative approach to the understanding of DNA synthesis is its attempted reconstruction *in vitro*. This has been undertaken using the enzyme DNA polymerase, and will be described in this section along with a survey of the occurrence and properties of the enzyme.

II A Distribution

DNA nucleotidyltransferase activity (EC 2.7.7.7.), which shall be referred to throughout as DNA polymerase, was first reported in 1956 in *Esch. coli* by Kornberg's group (Kornberg, Lehman & Simms, 1956). Since then it has been found in a multitude of other organisms: *Bacillus subtilis* (Okazaki & Kornberg, 1964); *Alcaligenes faecalis* (Hori, Fujiki & Takagi, 1966), regenerating rat liver (Bollum & Potter, 1957); calf thymus (Bollum, 1960a); various mammalian organs (Bollum, 1959a), Ehrlich ascites-tumour cells (Davidson, Smellie, Keir & McArdle, 1958); Landschütz ascites-tumour cells (Shepherd & Keir, 1966); Walker 256 fibroma (Furlong & Williams, 1965); L-cells (Gold & Helleiner, 1964); *Physarum polycephalum* (Brewer & Rusch, 1966); sea urchin embryos (Loeb, Maela & Ruby, 1967). In addition to these, induction of DNA polymerase after phage infection has been found: T2 (Aposhian & Kornberg, 1962); T4 (Goulian, Lucas & Kornberg, 1968); T5 (Steuart, Anand & Beesman, 1968a). Infection of mammalian tissues by certain viruses has also resulted in the induction of new DNA polymerases:- Shope fibroma (Chang & Hodes, 1968); herpes simplex, pseudorabies, poxvirus, polyoma, vaccinia and adenovirus (reviewed by Keir, 1968).

II B Bacterial DNA Polymerase

DNA polymerase has been very extensively studied in Esch. coli over the last twelve years by Kornberg's group, and their latest publication illustrates their near completion of solving the problems that this bacterial enzyme preparation has posed (Englund et al., 1968).

The enzyme catalyses the polymerisation of the four deoxyribonucleotides in the presence of DNA primer from the corresponding deoxyribonucleoside triphosphates with the stoichiometric release of inorganic pyrophosphate. The sequence of bases in the product is complementary to the sequence in the DNA primer or template (Kornberg 1961). There is a requirement for magnesium ions; when manganese ions are substituted a slight incorporation of ribonucleotides instead of deoxyribonucleotides is observed (Berg, Fancer & Chamberlin, 1963). The enzyme has been purified 5,000 fold to a homogeneous form (Richardson, Schildkraut, Aposhian & Kornberg, 1964). An amino acid analysis has been performed and the molecular weight is 109,000. The enzyme is composed of a single polypeptide chain, with a methionine residue at its amino terminal, one sulphydral group and one disulphide-bond (Englund et al., 1968). The conclusion that the enzyme is a single polypeptide chain makes the possibility of different subunits unlikely, though it does not rule out the existence of more than one polymerase species. Cavalieri & Carroll's (1968) claims of subunits are based on rather unconvincing evidence especially since the molecular weights of the subunits are variable at different stages of their purification. In connection with this Lezius, Hennig, Menzel & Nets (1967

have separated two polymerase species which differ only in ability to start *de novo* poly d(A-T) synthesis. Hord *et al.* (1966) have separated two polymerases from *Alcaligenes faecalis*. One is more active with, and has a higher affinity for denatured DNA, and the other is more active with and has a higher affinity for native DNA.

The *Esch. coli* DNA polymerase has one DNA binding site (Englund *et al.*, 1968). It has an absolute requirement for a DNA primer but can use single or double stranded DNA equally effectively. Purified enzyme preparations require the primer to be partially denatured with endonuclease to produce 3'- hydroxyl and 5'- phosphoryl-terminal groups. Synthetic poly d(A-T) is more effective primer, and *de novo* synthesis of poly d(A-T) will take place in the absence of primer in the presence of dTTP and dATP after a lag phase (Richardson, Schildkraut, Aposhian & Kornberg, 1964). Circular single-stranded DNA can also serve as a template for *Esch. coli* DNA polymerase; this suggests that the enzyme catalyses *de novo* synthesis of the product strand, so that it utilises the DNA as a template and not as a primer (Mitra *et al.*, 1967). However, subsequent work by Goulian (1968) has shown that the *Esch. coli* supernatant extract, that was necessary for *de novo* synthesis of product strands with circular Mf3 template, contained small oligonucleotides which acted as initiators. So there is still considerable doubt as to whether the enzyme can initiate new DNA strands which have no covalent linkage with the DNA template (Englund *et al.*, 1968).

The product of the polymerase reaction is, as previously mentioned, of complementary base sequence to the primer, by nearest

neighbour frequency analyses. The extent of the replication of the primer can exceed duplication of the primer by as much as 5-fold. The product, however, differs from the primer in that it is very readily renatured after denaturation and electron micrographs have revealed it to be a highly branched molecule (Schildkraut, Richardson & Kornberg, 1964). However, the enzyme can perform complete repair, with no branchings, of DNA which was partially degraded with exonuclease III (Richardson, Inman & Kornberg, 1964). With circular M13 as primer the product after 1 replication is unbranched, but, if the reaction is allowed to continue, branching will occur, thereafter (Mitra et al., 1967) although at a reduced rate of polymerisation. The most critical test of all to examine the fidelity of copying by polymerase is to assay the product for the biological activity of the primer. This has recently been performed with 100% success (Goulian & Kornberg, 1967; Goulian, Kornberg & Sinsheimer, 1967). The primer was circular, single stranded ϕX174 DNA, and a complementary (-) strand was synthesized substituting bromouracil for thymine so that the product could be separated from the primer by equilibrum sedimentation on a CsCl gradient. In addition to Esch.coli DNA polymerase the polynucleotide joining enzyme, or ligase, was required to join the terminals of the product to form a closed circle. The product (-) circle was infectious, and moreover, by a repeat of the previous process, was used as a primer for the synthesis of synthetic (+) circles of the same specific infectivity as the original phage DNA.

The other substrates for the enzyme are the deoxyribonucleoside triphosphates although Cairns & Denhardt (1968) have recently suggested that they may not be the immediate precursors *in vivo*. This, however, contradicts all the *in vitro* findings and kinetic evidence has shown there to be a binding site on the enzyme for the γ phosphate of the triphosphates (Beyersmann & Schramm, 1968). Englund *et al.* have shown that with *Esch. coli* polymerase there is one binding site for which all four triphosphates compete.

The *Esch. coli* DNA polymerase has other enzyme activities associated with it which remain in constant ratio of activity after purification, and these are shown in Table 1 (Englund *et al.*, 1968). In addition the purified polymerase contains significant polynucleotide ligase activity (Goulian & Kornberg, 1967). The hydrolysis by exonuclease II (from the 3' end) (Lehman & Richardson, 1964) and the pyrophosphorolysi take place at a common active site (Beyersmann & Schramm, 1968). Since there is only one DNA binding site the hydrolysis by exonuclease VI (from the 5' end) (Klett, Cerami & Reich, 1968) and the polymerisation probably also take place at the same site (Englund *et al.*, 1968). Lehman (1967) has reviewed the role that nucleases may possibly play in DNA synthesis with the conclusion that in spite of the circumstantial evidence of their increased presence during DNA synthesis their function can only be postulated. The exonuclease may function to "undo" mistakes made by the polymerase. Exonuclease II activity has been found also in partially purified DNA polymerase from *Micrococcus lysodeikticus* (Zimmerman, 1966). *Bacillus subtilis*, however,

TABLE 1

Reactions of Esch. coli DNA polymerase

1. Binding	Polymerase + dXTP	$\rightleftharpoons$	Polymerase.dXTP
	Polymerase + DNA	$\rightleftharpoons$	Polymerase.DNA
2. Polymerisation	$(DNA)_n$ + dXTP	$\rightleftharpoons$	$(DNA)_{n+1}$ + PP_i
3. Pyrophosphate exchange	$(DNA)_n$ + dXTP + PP_i^*	$\rightleftharpoons$	$(DNA)_n$ + dXTP* + PP_i
4. Pyrophosphorolysis	$(DNA)_n$ + PP_i	$\rightleftharpoons$	$(DNA)_{n-1}$ + dXTP
5. Exonuclease (from 3' and 5' ends)	$(DNA)_n$ + H_2O	$\longrightarrow$	$(DNA)_{n-1}$ + dXMP

although similar to Esch. coli DNA polymerase in all other respects does not appear to possess the exonuclease activity (Okazaki & Kornberg, 1964).

II C Phage induced DNA polymerase in bacteria.

Phage specified DNA polymerases have been highly purified from Esch. coli infected by T2 (Aposhian & Kornberg, 1962), T4 (Coulien et al., 1968) T5 (Steuart et al., 1968a). These enzymes all utilise denatured DNA primers in preference to native, and the product of the T4 and T5 is covalently bound to the primer forming a loop where the molecule doubles back along itself. Synthesis of DNA with T5 polymerase is stimulated markedly by the addition of DNase digests especially when the digested DNA is homologous to the primer. The product of the T5 polymerase has slight biological activity (Steuart, Anand & Bessman, 1968b).

Evidence that the T4 polymerase is coded for by the T4 genome was produced by De Waard, Paul & Lehman (1965) who found a temperature sensitive polymerase induced by infection with a temperature sensitive phage.

Each of the phage induced enzymes contains associated exonuclease activity and is free of endonuclease though this is less certain in the case of T2 polymerase. Nossel (1969) has reported the isolation of a protein induced after infection by T4 with a mutation in gene 43 (the polymerase gene) which lacks polymerase activity but still maintains exonuclease activity.

II D DNA polymerase from mammalian systems

1) Calf thymus: a) Replicative enzyme

DNA polymerase has been well characterised in bacterial systems, but investigation of the mammalian enzyme has lagged far behind. This is primarily because most mammalian systems are not amenable to large-scale purification procedures.

The most extensively studied mammalian DNA polymerase is the calf thymus enzyme which has been purified 200-fold from the crude cytoplasmic extract by Yoneda & Bollum (1965). The enzyme is endonuclease free, but it contains traces of exonuclease activity (Bollum, 1963b; Yoneda & Bollum, 1965). The enzyme has, like the bacterial enzyme, pyrophosphatase activity (Bollum, 1960a). Its molecular weight, as determined by gel filtration, is 1.1×10^5 (Bollum, 1966a), though its sedimentation coefficient by analytical ultracentrifugation is $^{12.2}$S (Yoneda & Bollum, 1965).

The enzyme utilises all four deoxyribonucleoside triphosphates and requires magnesium ions and a DNA primer (Bollum, 1960a). Denatured DNA is a much better primer than native, although the ratio of the priming abilities varies with the DNA (Bollum, 1959b). This probably reflects either the extent of denatured DNA in the supposedly native DNA, or its ease of denaturation.

The product of synthesis with a single-stranded primer is double-stranded DNA representing 100% replication (Bollum, 1963a; Bollum, 1963b). Density gradient analysis of the product of polyadenylate replication, however, showed there to be a low density intermediate containing all the primer at only 50% replication (Bollum, 1966b). The product has the same ratio of bases (Bollum, 1960b)

and the same dinucleotide frequency (Bollum, 1963a) as the primer, as determined by the method of Josse et al. (1961).

Bollum has also been concerned with the nature of the primer for DNA polymerase. The reaction rate is greatly increased by the presence of small oligonucleotide "initiators" in addition to the high molecular weight template. In the presence of the initiator the product DNA (+ initiator) is separable from the template DNA. In the absence of an initiator the reaction is slower and the product is covalently bound to the template (Bollum, 1964; Bollum 1968).

II D ib) Terminal addition DNA polymerase.

As well as the replicative DNA polymerase, which has the properties described above, calf thymus contains another very similar enzyme, known as the terminal addition DNA nucleotidyltransferase. This enzyme was first found in the nucleus by Krakow, Kammen and Canellakis (1961) and was partially purified (Krakow, Goutsogeorgopoulos & Canellakis, 1962; Gottesman & Canellakis, 1966). It catalyses the addition of deoxyribonucleotides onto the 3'OH ends of a DNA chain from the corresponding triphosphates in the presence of magnesium ions, a sulphydryl compound and a DNA primer. Only one of the four deoxyribonucleoside triphosphates is required and there is inhibition of incorporation in the presence of the other three triphosphates. The enzyme can also incorporate one ribonucleotide per DNA molecule from the corresponding ribonucleoside triphosphate. Wang (1968) has isolated the nuclear enzyme from non-histone chromatin proteins of calf thymus and it is similar to the Canellakis enzyme.

Yoneda & Bollum (1965) have separated cytoplasmic calf thymus terminal addition polymerase from the replicative polymerase. It differs from the nuclear terminal addition enzyme in being able to utilise oligodeoxyribonucleotides as primer (Bollum, 1962). These require to have at least three phosphate groups although polymerisation can occur in the absence of an initiator after a lag period (Kato, Gonçalves, Hartz & Bollum, 1967). The enzyme can incorporate small amounts of ribonucleotides but differs from the nuclear enzyme since they are incorporated internally in the polymer chain. The incorporation of a ribonucleotide inhibits further action by the nuclear enzyme, and so they are only incorporated onto the ends (Kato et al., 1967).

The cytoplasmic enzyme polymerises dATP at an approximately 5-fold greater rate than the other triphosphates, whereas the nuclear enzyme polymerises all four at an equal rate (Kato et al., 1967; Gottesman & Canellakis, 1966).

Hayes and his colleagues have found that the cytoplasmic terminal addition enzyme is able to make copolymers as well as heteropolymers. In homopolymer formation dATP incorporation exceeds that of the other substrates, but in heteropolymer formation dGTP is preferentially incorporated (Ratcliff, Hoard, Ott & Hayes, 1967; Ratcliff et al., 1968).

The function of the terminal addition enzyme *in vivo* is still unknown, although Kelz (1965) has suggested that it may be a subunit of the replicative enzyme. There is as yet no evidence to support this apart from its low molecular weight of 57,000 (Bollum, 1966a).

II D ii) Regenerating rat liver.

An enzyme from rat liver incorporating deoxyribonucleotides into DNA was first described by Bollum & Potter (1958). The enzyme has since been partially purified from soluble cell fractions (Mantsavinos, 1964; Mantsavinos & Munson, 1966). It differs from the calf thymus enzyme since it utilises native DNA in preference to denatured DNA, and resembles the Esch. coli enzyme in preferring polyd(A-T) as primer to native DNA.

DNA polymerase has been purified 250-fold from the non-histone chromatin protein of rat liver (Patel, Howk & Wang, 1967). It prefers native DNA primer and is similar to the enzyme from regenerating rat liver.

The relative occurence of native and denatured DNA primed polymerases was examined at different stages of liver growth by Mukundan, Devi & Sarkar (1963). They found that the ratio of denatured primed to native primed polymerase increased with the age of the rat and that in regenerating liver the reverse was true. They suggested that some factor, possibly DNase, is responsible for converting native DNA into an efficient primer. Inhibition of polymerase by extracts of normal liver was shown by Gray et al. (1960), and further studies by Smellie (1963) showed the inhibiting factor to be a DNase. In more purified fractions the DNase stimulated the reaction. This was confirmed in Walker 256 carcinosarcoma by Furlong (1966). Further evidence by de Recondo (1966) has confirmed the presence of an activating factor in rat liver which is not an endonuclease, but has similarities to Esch. coli exonuclease III.

II D iii) L - cells

Gold & Hollelner (1964) have partially purified DNA polymerase from soluble cell extracts of L-cells (mouse fibroblasts) in culture. The enzyme is primed by denatured DNA at a 10-fold greater efficiency than by native DNA. Lindsay & Adams (1968) have reported a nuclear polymerase in L-cells which preferentially utilises native DNA, and a cytoplasmic enzyme which preferentially utilises denatured DNA.

II D iv) Mouse embryo

Binnie & Fox (1966) reported nuclear and cytoplasmic polymerases from mouse embryo cells which differ in response to pH changes, magnesium and manganese ions, and to native and denatured DNA primers.

II E Mammalian Tumour cells.

i) Ascites cells

For the past few years Keir and his colleagues in Glasgow have been working with DNA polymerase from Ehrlich and Landschütz ascites-tumour cells. The Landschütz enzyme was partially purified and found to be very similar to the calf thymus enzyme (Shepherd & Keir, 1966). It has a requirement for DNA primer, preferably denatured (Keir, Binnie & Smellie, 1962). As purification proceeds the preference increases (Shepherd & Keir, 1966), probably owing to the removal of DNase I which increases the priming activity of the DNA by producing 3'-hydroxyl and 5'-phosphate terminals (Keir, 1962). The enzyme requires magnesium ions, which can be substituted, in part, by manganese ions. Calcium and zinc ions are powerful inhibitors of the enzyme, and EDTA stimulates the reaction 5-10 fold, presumably by

effectively removing traces of these ions (Keir & Shepherd, 1965). The enzyme is protected, completely or partially, by the thiol containing compounds 2-mercaptoethanol and glutathione from inhibition by the thiol group inhibitors p-hydroxymercuri-benzoate and carzinomycin (2-methylene-3-oxocyclopentane carboxylic acid), though they enhance inhibition by iodoacetamide. The enzyme therefore differs from the Esch. coli polymerase which contains a sulphydryl group which can be modified by iodoacetic acid, or mercuric ions without inhibition of enzyme activity (Englund et al., 1968)

The product of the reaction has the same dinucleotide frequency as the primer, as analysed by the method of Josse et al., (1961). However, it bands coincident with denatured rather than native DNA on CsCl density gradients (Shepherd & Keir, 1966).

DNA polymerase from Landschütz ascites-tumour cells contains very little terminal addition polymerase amounting to only 2% of the replicative enzyme (Shepherd, 1965).

II Eii) Walker 256 carcinosarcoma

Other tumour systems whose DNA polymerases have been studied are the Walker 256 carcinosarcoma (Furlong & Williams, 1965), the Flexner-Jobling carcinoma (Bollum & Potter, 1958) and the Novikoff hepatoma (Furlong, 1960). They are similar to the other mammalian enzymes.

Recently Rothschild, Halpern & Smith (1968) have found there to be a terminal addition DNA polymerase in the cytoplasm of Walker 256, and the replicative enzyme to be in the nuclear fraction.

II F DNA polymerase from other systems

i) Sea urchin embryos

Sea urchin embryo DNA polymerase has been studied by Mazia. It is located in the nuclei, associated with the chromosomes, during DNA synthesis, though it appears in the cytoplasm at other times (Mazia, 1963). The enzyme is detectable in the nucleus in the absence of exogenous DNA, but is more active with added native DNA (Mazia & Hinegardner, 1963). It utilises native DNA at an 8.6 fold greater efficiency than denatured DNA (Loeb, Mazia & Ruby, 1967)

II Fii) Physarum polycephalum

DNA polymerase activity in the nuclei of the synchronously dividing Physarum polycephalum slime mould has been studied by Brewer & Rusch (1965, 1966).

II G Mitochondria

Recently there have been several reports of DNA polymerase in mitochondria. Winterberger (1966) reported it in yeast, and since then it has been found in rat liver mitochondria (Parsons & Simpson, 1967; Noubert, Oberdisse, Schneider & Reinsch, 1967), and in Physarum polycephalum mitochondria (Brewer, DeVries & Rusch, 1967). The enzyme has been partially purified from rat liver mitochondria (Kalf & Ch'ih, 1968; Meyer & Simpson, 1968), and requires the four deoxyribonucleoside triphosphates and magnesium ions. It is perhaps significant that it resembles the bacterial polymerase in preferentially utilising native DNA primer. Its most striking characteristic is its very marked preference for native mitochondrial DNA rather than DNA from other sources (Kalf & Ch'ih, 1968).

II H Virus induced DNA polymerase in mammalian cells.

Polymerases induced in mammalian cells after virus infection are less well characterised than phage induced enzymes, and have recently been reviewed by Kelz (1968).

II I Differences in DNA polymerases from various sources.

From this survey of the properties of DNA polymerase from various systems it can be seen that there is good evidence for real differences in the polymerases that cannot be explained by interfering activities in impure enzyme preparations.

The most significant difference is that of template requirement. Bollum (1966b) has suggested that the polymerase primed by denatured DNA may lack an "initiation site" (present in the Esch. coli polymerase) permitting it to initiate synthesis on double stranded DNA. He suggested that the polymerisation and initiation sites are on different subunits which purification has separated in the case of the calf thymus enzyme but not in the case of the bacterial enzyme. Erhan (1968) postulated that DNA polymerase contains various subunits including a "wedge" which facilitates unwinding of the helix. The possibility of the bacterial enzyme being constructed in a similar fashion must now be re-evaluated in the light of the finding that it is a single polypeptide chain (Englund et al., 1968).

Reported molecular weights of DNA polymerase are very similar: Esch. coli 109,000; T4 112,000; T5 96,000; calf thymus 110,000; so it may well be that they are each composed of a single polypeptide chain (Englund et al., 1968; Goulian et al., 1968; Steuart et al., 1968a;

Bollum 1966c). On the other hand the amino acid composition of T4 polymerase and Esch. coli polymerase differ quite considerably. The T4 polymerase has 15 half-cystine residues compared with three for Esch. coli . Whereas, when these sulphydryl groups are blocked the T4 and ascites-tumour cell enzymes lose activity the Esch. coli enzyme does not. This may represent structural differences between the enzymes permitting the existence of subunits in the T4 and other polymerases.

The association of other enzyme activities with polymerase preparations is not yet well enough established in most enzyme preparations to draw conclusions regarding differences between polymerases from different sources.

III The Role of DNA Polymerase in vivo

A The in vivo site and state of polymerase

Dillon (1963) found that all the DNA polymerase of Esch. coli was complexed to the DNA, and Kadoya, Mitsui & Takagi (1964) showed that there was a deoxyribonucleoprotein containing DNA and RNA polymerases. In the eucaryotic cell the situation is further complicated by the apparent existence of the enzyme in the nucleus and the cytoplasm; though it now seems quite well established that polymerase actively engaged in DNA synthesis is complexed in the nucleus, whereas the cytoplasmic polymerase represents either newly synthesized, or "already used" enzyme (Littlefield, McGovern & Margeson, 1963; Gold & Hollelner, 1964; Mazia, 1963). There is also evidence that at least some of the polymerase in the cytoplasm is an artifact of its isolation through leakage of the soluble enzyme from the nucleus (Smith & Keir, 1963; Main & Cole, 1964).

III B Control of DNA replication and the effect of template availability on the in vivo action of DNA polymerase

Very little is understood about the control of DNA synthesis. Experiments in transplanting nuclei into cells at different stages in the cell cycle have shown that cytoplasmic factors can initiate synthesis (Prescott & Goldstein, 1967). There are theories that the levels of DNA precursors may act in the control of synthesis since their levels and the levels of the enzymes required for their syntheses increase during growth, tissue regeneration and tumour growth (Schneider & Brownell, 1957; Baserga, 1965). Schmidt (1966) has reviewed the relationship between the enzymes involved in dTTP synthesis and DNA replication.

The state of the DNA template is likely to affect its replication since its usual environment of histones in the higher organisms and its double stranded form are inhibitory to most mammalian polymerases (Bazill & Philpot, 1963; Schwimmer & Bonner 1965). Crude preparations of DNA isolated from Esch. coli cells during thymine starvation have a diminished template activity for DNA polymerase, but if these DNA preparations are purified their activity is normal (Schaiberger, Giegel & Sallman, 1967). DNA synthesis in isolated nuclei of Physarum polycephalum occurs in the absence of exogenous DNA, but only if they are isolated during the period of DNA synthesis (Brewer & Rusch, 1965). Synthesis increases if native DNA and spermine are added suggesting that synthesis depends on the availability of the template (Brewer & Rusch, 1966). More recently, the same workers have produced evidence of a heat-sensitive substance which initiates mitosis and is necessary for DNA synthesis (Brewer &

Rusch, 1968). Buttin & Kornberg (1966) showed limited DNA synthesis in the absence of exogenous DNA using exogenous deoxyribonucleoside triphosphates and Esch. coli cells treated with tris-EDTA or tris-Mn^{++}. The tris-EDTA treatment was thought to release endonuclease I which would activate the DNA since synthesis was not detected in endonuclease-deficient mutants.

Cairns and Denhardt (1968) have demonstrated the existence of a KON and OO sensitive reaction in Esch. coli which is necessary for Esch. coli DNA synthesis, but not for the replication of the single-stranded DNA of ϕX174. This suggests an energy requiring reaction which converts the DNA into a state containing an accessible template - possibly the unwinding of the DNA.

Phillips (1968) has reported a protein from calf thymus which denatures DNA and is not an endonuclease.

III C Models and mechanisms of DNA synthesis.

Since Cairns (1963) showed that in vivo DNA synthesis takes place in both strands simultaneously and unidirectionally there has been much speculation on the mechanisms involved. DNA polymerase synthesises DNA in vitro in the 5'→3' direction i.e. 5'→ 3' along the product strand, but 3'→ 5' along the template; there is no evidence to show that synthesis can occur in the reverse direction. Attempts to demonstrate 3'→ 5' synthesis using 3'-dTTP in the calf thymus terminal addition reaction, proved unsuccessful (Adams & Kelr, 1966). The discovery of Okazaki's newly synthesised oligonucleotides (Introduction I D), and of the enzyme polynucleotide ligase has since provided a satisfactory answer to the problem.

Polynucleotide ligase will repair a single break in one strand of a double-stranded DNA by joining a 5'-phosphate terminal to a 3'-OH terminal giving a 3', 5'-phosphodiester bond. The enzyme was first detected in Esch. coli by Gellert (1967) and utilizes NAD^+ as a cofactor. It has since been found in T4 and T7 infected Esch. coli and has been purified 200-fold from rabbit bone marrow, but in these systems the cofactor is ATP. However they resemble the Esch. coli ligase since in all cases an adenylate-enzyme intermediate is formed (Little, Zimmerman, Oshinsky & Gellert, 1967; Becker, Lyn, Geffer & Hurwitz, 1967; Lindahl & Edelman, 1968). A second intermediate, a DNA-adenylate compound, has been identified (Olivera, Hall & Lehman, 1968).

The requirement for the ligase in DNA synthesis has been demonstrated by Fareed & Richardson (1967) using T4 mutants which induce a temperature sensitive ligase. Okazaki's group have showed with the same mutant that there is accumulation of short newly synthesised DNA strands at high temperatures (Sugimoto, Okazaki & Okazaki, 1968).

The discovery of polynucleotide ligase and the Okazaki fragments led to postulations of a model for in vivo DNA synthesis shown in Fig.1 where the thick line represents newly synthesised DNA (Mitra et al., 1967). Synthesis occurs along one strand in the usual 5'→ 3' direction, and in the other strand by "reverse-direction" of synthesis of oligonucleotides, which, when completed, are joined by the ligase. In this mechanism employment for the 400 molecules of DNA polymerase per cell of Esch. coli could be visualised (Richardson, Schildkraut, Aposhian & Kornberg, 1964).

FIGURE 1

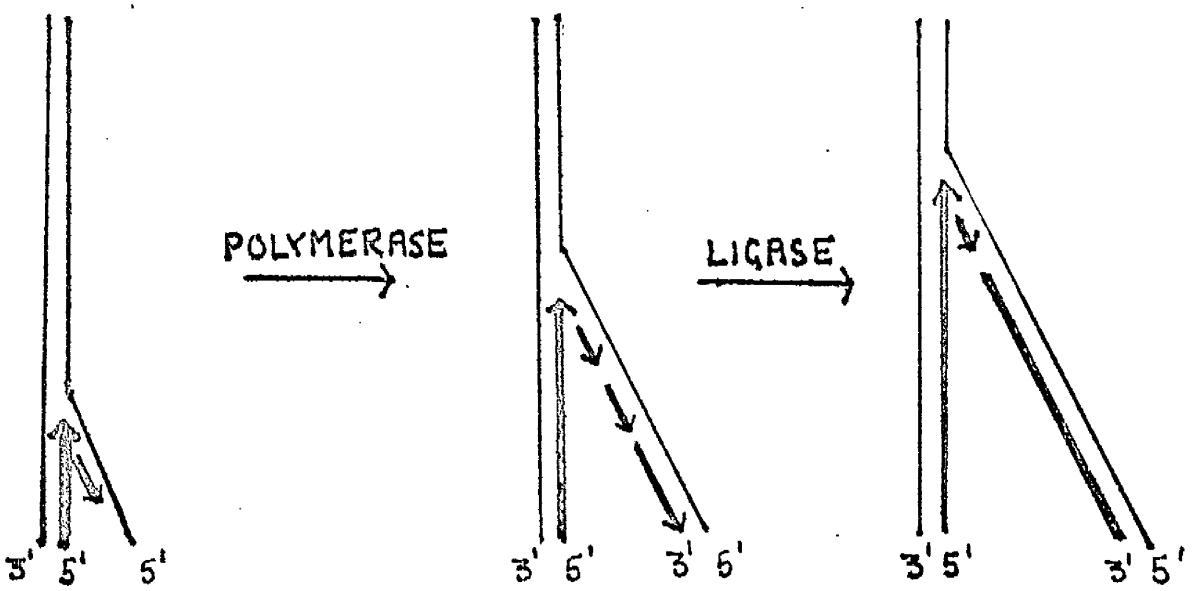
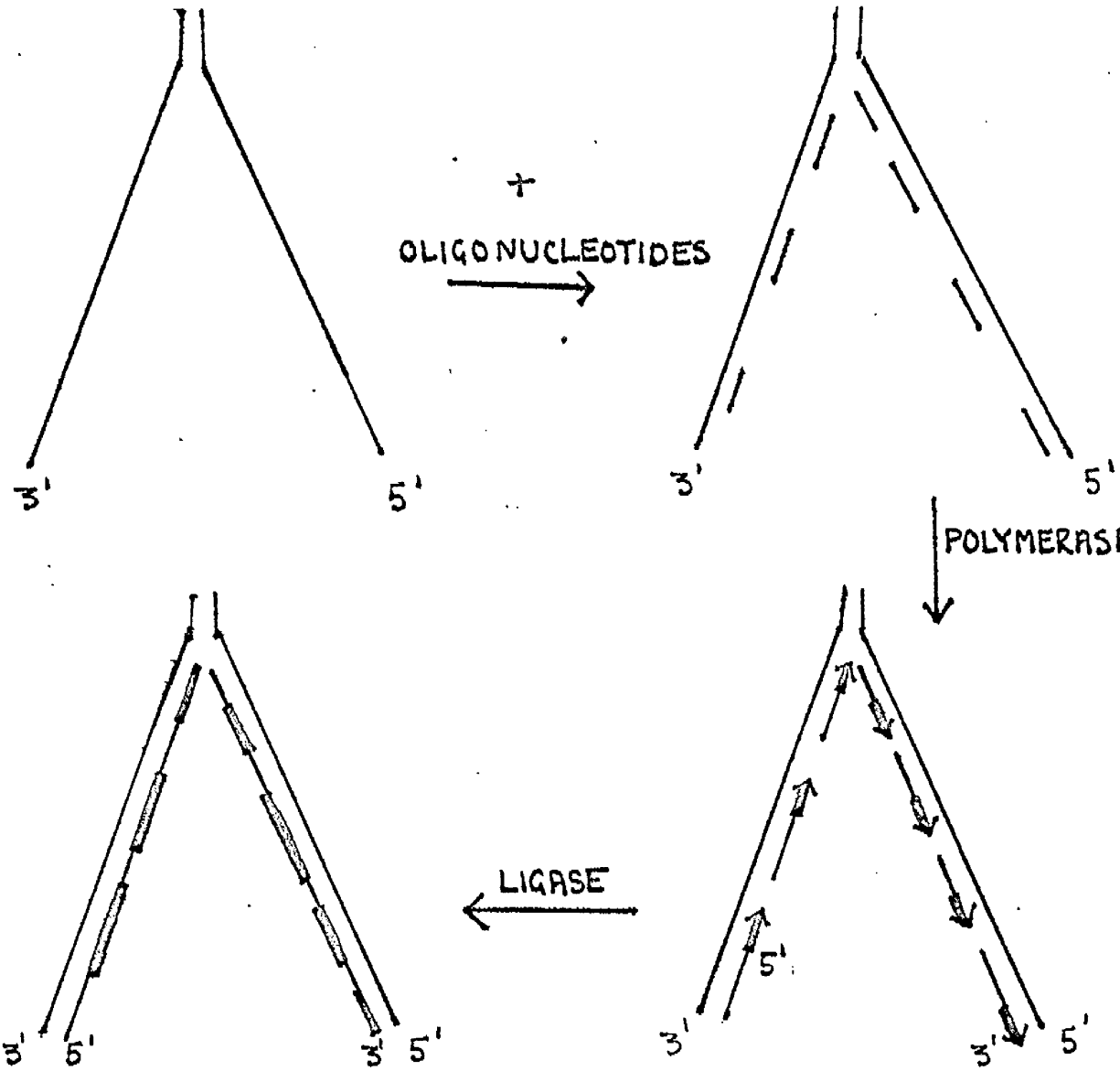


FIGURE 2



SEE TEXT FOR EXPLANATION

The rate of DNA synthesis would be increased and make the figure of 10^3 nucleotides polymerised/min./molecule of enzyme for the *in vitro* synthesis by the *Esch. coli* polymerase (Englund *et al.*, 1968) compatible with the *in vivo* figure of 10^5 base-pairs/min. (Donhoeffer & Gierer, 1963; Cairns 1963; Cooper & Helmstetter, 1968).

The objection to this mechanism is that it is becoming increasingly doubtful that any DNA polymerase can initiate new strands, since the presence of oligonucleotide initiators, or of an endonuclease, can account for any apparent uninitiated synthesis previously reported (Englund *et al.*, 1968). Studies on the enhancement of DNA synthesis by T5 DNA polymerase in the presence of oligonucleotides had led Steuart *et al.* (1968b) to propose that oligonucleotides formed by breakdown of the host cell DNA, or of T5 DNA itself, act as initiators for DNA synthesis (Fig. 2). After they performed their function the initiators could subsequently be removed by exonuclease if their sequence were not entirely complementary to the template DNA. The problem of how the DNA becomes denatured has already been discussed.

In these models it is possible that more than one species of polymerase could be involved, thus explaining reports of two or more polymerase species in the cell (Cavalieri & Carroll, 1968; Lezius *et al.*, 1967; Mori *et al.*, 1966; Lindsay & Adams, 1968; Kinnie & Fox, 1966; Chang & Hodes, 1967, 1968). In such a scheme, a function for the terminal addition enzyme could be envisaged, e.g. it could participate in the synthesis of the oligonucleotide initiators.

Englund et al. (1968) have produced a modified model that requires neither oligonucleotides nor the DNA to be predenatured (Fig. 3.) It is possible that this mechanism occurs in bacterial DNA synthesis and the previous one in other systems.

Speyer (1965) showed that polymerase participates in the selection of the correct base, but Freese & Freese (1967) suggest with some genetic evidence that the polymerase specificity lies in the rejection of incoming triphosphates which do not form the correct base-pair. Hall & Lehman (1968) have demonstrated *in vitro* that misincorporation of dTTP (instead of dCTP) along a polydG template occurs with a greater frequency when a mutant DNA polymerase is used. However, there was no difference between the mutant and wild-type polymerases when dCTP was misincorporated into poly d(A-T) in place of dTTP. Substitution of magnesium ions by manganese ions greatly increases misincorporation which could explain how manganese ions can induce mutations in Esch. coli and T4 (Demerec & Hanson, 1951; Orgel & Orgel, 1965). In opposition to the models in Figs. 1-3 is the genetic evidence of Kubitschek of a master strand in the parent DNA which directs synthesis of both daughter strands. He suggests that the active site of the polymerase accommodates an incoming triphosphate base-pair and the replicating base-pair in the parent strands (Kubitschek, 1966; Kubitschek & Henderson, 1966). This is not supported by the finding of only one triphosphate binding site in Esch. coli DNA polymerase (Englund et al., 1968). Jehle (1967) has postulated an ingenious model in which there is a master strand (Fig 4).

FIGURE 3

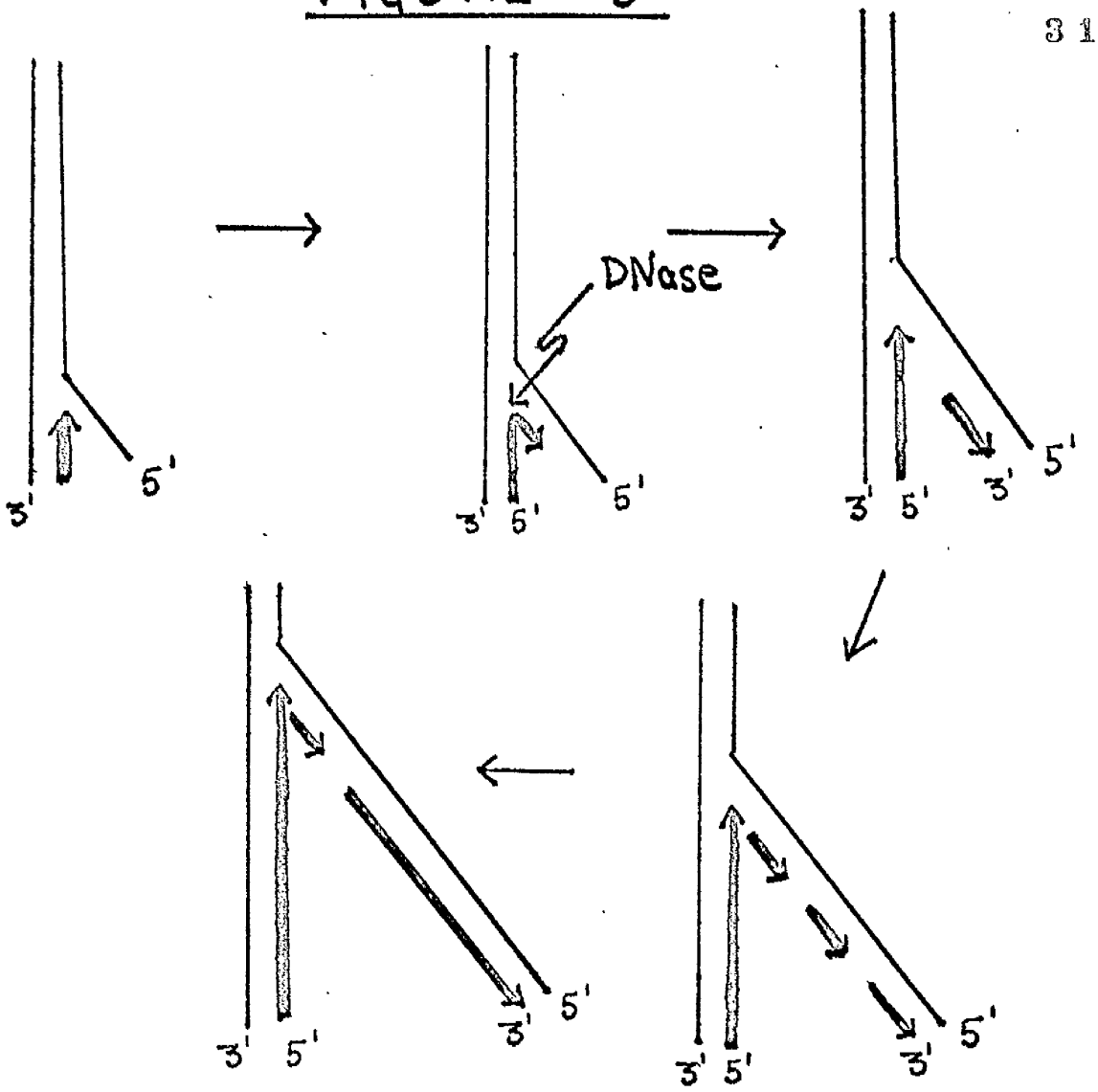
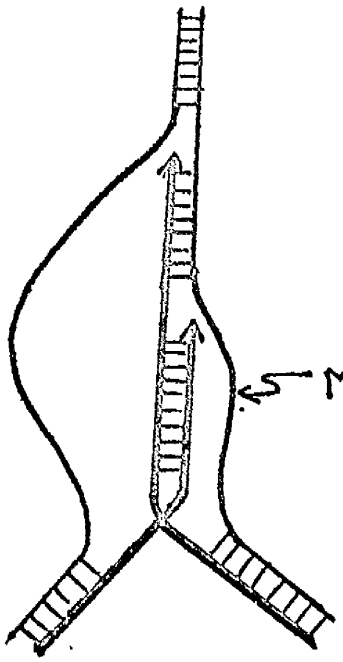


FIGURE 4



SEE TEXT FOR EXPLANATION

A mutation in the master strand would be transmitted to both daughter strands, whereas one in the other parental strand would not be passed on.

III D Concluding remarks and aim of the project.

A complete understanding of the mechanisms involved in DNA synthesis has still not been achieved, inspite of the many recent advances, and the exact role of DNA polymerase and its *in vivo* mechanisms is still the subject for much conjecture. The bacterial and phage polymerases, although well characterised compared with the mammalian polymerases, still present unsolved questions concerning their *in vivo* mechanisms of action and require further research, including genetic and kinetic studies, for fuller understanding. The mechanisms of DNA synthesis in mammalian systems are of necessity different in at least a few respects from these simpler forms of life and their polymerases therefore merit separate study.

Furthermore, various mammalian polymerases have been shown to have some different characteristics, and tumour cell polymerase may present yet further differences. Since the loss of control of growth of a mammalian cell is inheritable it implies a change in the genetic information content or in the expression of this information. Studies of the mechanism and control of DNA synthesis reveal quantitative differences in the levels of enzymes involved in DNA synthesis but these may be a consequence rather than a cause of malignancy, and studies on the qualitative differences in enzyme mechanisms may well be more fruitful. The obvious importance of DNA polymerase in the synthesis of DNA and the current lack of definitive information on its physical and kinetic properties were the starting point for the present work. Further studies

were therefore carried out on one of the established mammalian polymerase systems, namely, the DNA polymerase of Landschütz ascites-tumour cells.

EXPERIMENTAL

I MATERIALS

A Chemicals

$(NH_4)_2SO_4$ (special enzyme grade) was purchased from Mann Research Laboratories, New York, and CsCl (optical grade) from the Harshaw Chemical Co., Cleveland, Ohio. Other inorganic chemicals were ANALAR grade, wherever possible, and purchased from BDH Biochemicals, Poole, Dorset. Tris base ("Trizma") was obtained from the Sigma (London) Chemical Co., London, dissolved in water and acidified with HCl to the required pH for use. Dextran 500, number average molecular weight = 180,000 was from Pharmacia, (G.B.) Ltd., London, and polyethylene glycol 6000 was BDH grade.

B Biochemicals

Nucleic acids and proteins

Salmon testes DNA was purchased from the Worthington Biochemical Corp., Freehold, New Jersey, ferritin from General Biochemicals, Chagrin Falls, Ohio, haemoglobin from Nutritional Biochemicals Corp., Cleveland, Ohio, bovine serum albumin from Armour Pharmaceutical Co. Ltd., Eastbourne, and pancreatic ribonuclease from Worthington Biochemical Corp., Freehold, New Jersey.

Deoxyribonucleoside 5'-triphosphates.

dATP, dCTP, dGTP and dTTP were purchased from Calbiochem, Los Angeles, California, and P-L Biochemicals Inc., Milwaukee, Wisconsin.

[α^{32}P]-dATP, [α^{32}P]-dGTP, [α^{32}P]-dCTP and [α^{32}P]-dABP were from International Chemical and Nuclear Corp., City of Industry, California, and [^{3}H-dATP] from Schwarz Bioresearch Inc., Orangeburg, New York.

The purity of the deoxyribonucleoside triphosphates was checked as in Methods F(ii).

IC Liquid scintillation materials.

1,4, di-2-(5-phenyl oxazolyl)-benzene, (POPOP), and 2,5-diphenyl oxazole (PPO) were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks, and Hyamine hydroxide, IM in methanol, from Nuclear Enterprises (G.B.) Ltd., Edinburgh.

ID Materials for chromatography

Whatman No.1 filter paper and DE-81 (DEAE-paper) were purchased from H. Reeve Angel and Co. Ltd., London, and Sephadex G-150 from Pharmacia (G.B.) Ltd., London.

IE Biological Materials

Landschütz ascites-tumour cells were kindly maintained by Dr. M.F.I. Berclay, by serial transplantation in 8-10 week old albino mice (Porton strain) from the Departmental Colony. Calf thymus glands were obtained from the slaughter house and immediately frozen and stored at -70°.

II METHODS

A Preparation and Characterisation of DNA

(i) Preparation of mammalian DNA

DNA was isolated from Landschütz ascites-tumour cells and from calf thymus by the method of Kay, Simmons and Dounce (1952). The DNA was then put through the deproteinisation procedure described by Chargaff (1955a). One volume of chloroform-amyl alcohol (10:3, v/v) was added to the DNA dissolved in 10% NaCl. The mixture was emulsified, by rapid manual shaking, and then centrifuged at 10,000g for 15min. in the 6 x 50ml. angle rotor of the M.S.E. 18 centrifuge. Three layers were formed, the top being a salt solution of DNA, the middle a protein gel, and the bottom a chloroform protein solution. The DNA - containing layer was re-extracted as before until no further gel was formed at the interface of the two layers. The DNA was precipitated with an equal volume of absolute ethanol, washed with absolute ethanol and ether, dried in air, then redissolved in 0.09% NaCl to a concentration of 1.5 - 2mg./ml.

II A(ii) Preparation of $[^{32}P]$ -DNA from Escherichia coli

$[^{32}P]$ -DNA from Esch. coli was prepared by a modification of the method of Lehman (1960). The Esch.coli cells (approx.3g.), grown in 200ml. of glycerol-lactate medium containing $[^{32}P]$ -orthophosphate (0.2 µg. at 50 c/µg) were sedimented by centrifugation for 20 min. in the 6 x 250 ml. rotor of the M.S.E. 18 centrifuge at 12,000 g. They were washed in SSC, recentrifuged, and resuspended in 100 ml. of SSC. 1g. of sodium dodecyl sulphate was dissolved in the solution which was then incubated at 60° for 10min. $NaClO_4$ was added to 1M, and the

DNA extracted with an equal volume of chloroform-isoamyl alcohol (25:1, v/v). The interphase formed between the two layers was re-extracted by resuspending in SSC, and treating with sodium dodecyl sulphate and chloroform-amyl alcohol as before. If a further significant interphase was formed it was re-extracted repeatedly as before until it was negligible. The DNA was precipited from the pooled top layers of every extraction by 2 vol. of absolute ethanol and re-dissolved in $\frac{1}{10}$SSC. Pancreatic ribonuclease was added to 20 µg./ml. and the solution incubated at 37° for 30 min. The DNA was then extracted twice by adding 1 vol. of 90% (v/v) redistilled phenol in SSC. Two ethanol precipitations were performed and the DNA was dissolved in 0.02 M-KCl and dialysed against 0.02 M-KCl for 16hr. Acid-soluble backgrounds were approx. 2%.

II A(iii) Denaturation of DNA

DNA at 1 to 1.5 mg./ml. was heated for 10 min. at 100° and then rapidly cooled to 0°. Where possible the DNA was diluted up to five fold with water to ensure maximal denaturation (Cavalieri, Rosoff & Rosenberg, 1956).

II A(iv) Characterisation of the DNA

Sedimentation velocity analyses of the DNA solutions were performed on the Spinco Model E ultracentrifuge using U.V. optics [see Methods II D(i)(b)] and the sedimentation coefficients ($S_{20,w}$) ranged from 17-23s, the lower values being for the more extensively purified DNA. Approximate values of the molecular weight of the DNA were calculated from its sedimentation rate in sucrose density gradients

and it was found to be heterogeneous and of low molecular weight (Discussion II).

CsCl equilibrium ultracentrifugation of the DNA on analytical and preparative ultracentrifuges [Methods II D(i)(c) and II D(ii)(b)] showed the Landschütz ascites DNA to be heterogeneous since broad overlapping peaks were produced when native and heat denatured DNA were sedimented together.

"Native" calf thymus DNA prepared by the method of Kay et al. (1952) was found to contain denatured DNA at a level of 2.5% (w/w) of the total DNA (Johnson & Leskowski, 1968).

The protein content of the DNA as determined by the method of Lowry, Rosebrough, Farr & Randall (1951) was <2%(w/w).

II B Preparation of DNA Polymerase

The preparation of DNA polymerase up to $(NH_4)_2SO_4$ fractionation was, with minor variations, performed as described by Shepherd & Keir (1966). All operations were carried out at 0-4°.

(1) Harvesting of cells

Landschütz ascites-tumour cells were removed aseptically from mice innoculated intraperitoneally 6-9 days previously with 0.2ml. of ascites-tumour fluid. The cells were withdrawn into 2-5 vol. of PBS and washed in PBS and finally sedimented at 500g. The sedimented cells were either used immediately, or stored at -70° for up to 1 year.

II B(ii) Cell Disruption.

(a) By homogenisation

The cells were suspended in 5 to 15 vol. of Buffer 3 and disrupted in a Potter-Elvehjem homogeniser. 2 to 3 passes at low speed (600 rev./min. for 10 sec.), with a mechanically driven teflon pestle (0.1mm. clearance), were sufficient for previously frozen cells, but more were necessary for fresh cells (2000 rev./min. for 20 sec.) and for concentrated cell suspensions. Disruption of the cells was followed microscopically using crystal violet stain [1% (w/v) in 0.1M-citric acid]. The liberated nuclei clumped together in large masses visible to the naked eye. Solid KCl was then added to the suspension to 0.15M.

II B(ii)

(b) By sonication

The cells were suspended in Buffer 4A and sonicated in the Dawe Soniprobe at full power (approx. 7 amps) for two 15 sec. bursts. This was sufficient to disrupt the cells and nuclei. Shorter times of sonication ruptured the cell walls only.

II B(iii) Supernatant fraction

High speed supernatant fractions of the disrupted cells were prepared by centrifugation of the disrupted preparations at 105,000g for 1 hr. on the rotor no. 50 of the Spinco Model L preparative ultracentrifuge.

II B(iv) pH5 precipitation

0.2M-acetic acid was added dropwise to the 105,000g supernatant fluid at 0° at a pH meter, with constant stirring until the pH fell to 5.

The cloudy suspension was centrifuged at 700g for 10 min. The sediment was immediately suspended in Buffer 4A by two slow manual passes of a pestle through the mixture in a Potter-Elvehjem tube. If a concentrated solution >4mg. of protein/ml. was required a stronger buffer (Buffer 4B) was used and the pH of the final suspension was checked. The pH5 precipitate contained of denatured protein which could be removed by centrifugation at 600-700g.

II B(v) Ammonium sulphate fractionation

Saturated $(NH_4)_2SO_4$, 1mM-EDTA, 5mM-2-mercaptoethanol, at pH 7.5, was added dropwise from a burette into the enzyme solution to 25% saturation, with constant stirring. After 10min., the slight precipitate formed was removed by centrifugation for 10min. at 10,000g on the 8x50ml. angle head of the M.S.E. 18 centrifuge. To the supernatant solution was added further $(NH_4)_2SO_4$ to 45% saturation and the precipitate sedimented by centrifugation as before. This precipitate was dissolved in Buffer 4A and dialysed for 4hr. against Buffer 4A at 4° to remove the $(NH_4)_2SO_4$.

II B(vi) Gel Filtration

Sephadex G-150 was allowed to swell for at least 3 days in distilled water at 0°, and in 0.01M-HCl and 0.1M-NaOH for 1 day each, and finally in Buffer 4A for 2 days. The Sephadex was poured as a slurry into a glass column (LKB 105cm. x 3.2cm.). The apparatus used for gel filtration was the LKB 4900A Recychrom System. The inlet tygon tubing passed through the LKB 4912A peristaltic pump so that the sample was pumped upwards through the column. The outlet tube from the top of the column was coupled to the flow-cell of the LKB 4700A

Uvichord which recorded the E_{260} of the elysate. Fractions (7-10ml.) were collected on a Stälproduktor fraction collector at a rate of two fractions/hr. The enzyme solution, 10-20ml. at a protein concentration of 20mg./ml., was applied to the column at a rate of 10-15ml./hr., and was eluted with de-aerated Buffer 4B containing BSA at 1mg./ml. The column was run at 4° for 30hr., and the fractions stored in ice and assayed as soon as possible for DNA polymerase, DNase and protein. The fractions containing optimal polymerase and minimal DNase were pooled, and concentrated by precipitation at pH5, or preferably by the Diaflo ultrafilter using the XM50 membrane. This concentrated the solution under a pressure of 60lb./in.^{2} (from a nitrogen cylinder) by forcing molecules <50,000 molecular weight to pass through the membrane, and retaining the macromolecules. This method of concentration is very gentle and has the advantage of maintaining constant ionic concentrations.

II B(vii) Storage of DNA polymerase

The enzyme was stored in Buffer 4A or Buffer 4B, and latterly, glycerol was present at 20-30% (v/v). Over short periods (<2 days) the polymerase was kept in a Dewar flask packed in ice, but for longer periods it was stored in 0.1-1.0ml. portions in vials at -70°. Fractions containing glycerol were stored at -10° to -20° at which temperatures freezing was barely apparent.

II C Enzyme Assays

(i) DNA polymerase

The enzyme was assayed by measuring incorporation of deoxyribonucleoside monophosphate residues from the corresponding radioactive deoxyribonucleoside 5'-triphosphates into acid-precipitable material,

in the presence of template DNA. The assay is a slight modification of that described by Shepherd & Keir (1966). Each assay contained the following components in 0.25ml.: 1.5 μmoles of $MgSO_4$, 6-7 μmoles of tris-HCl, pH 7.5, 15 μmoles of KCl, 0.1 μmole of EDTA, pH 7.5, 3 μmoles of 2-mercaptoethanol, 50m μmoles each of dATP, dGTP, dTTP, 30-50m μmoles of [α^{32}P]-dCTP (2 to 5 x 10^6 counts/min./μmole), 50-70 μg. of heat denatured DNA-template, and 100-500 μg. of protein containing DNA polymerase. Controls were performed substituting BSA for enzyme.

The standard incubation (1hr. at 37° in a shaking water bath) was carried out in 3ml. stoppered glass tubes, and was stopped by placing the tubes in ice. They could be stored at -10° overnight at this stage if necessary. 0.05ml. samples of the reaction mixture were withdrawn and dispensed onto Whatman No.1 filter paper discs (2.5 cm. diameter) previously impregnated with 0.05ml. of BSA at 2mg./ml. and then dried. The discs were plunged into a beaker containing approx. 15ml./disc of ice cold 5% TCA., 2 to 5mM-sodium pyrophosphate. The discs were washed 4 times in TCA-pyrophosphate solution (10min./wash), then dried by washing with ethanol then ether.

On occasion the labelled triphosphate was [α^{32}P] -dCTP, [α^{32}P] -dGTP, [α^{32}P] -dATP or [^{3}H] -dATP. The determination of incorporated acid-insoluble radioactivity was as described in Methods II E(i) and (ii).

The specific activity of the DNA polymerase was expressed as nmoles of dTMP incorporated /mg. of protein/hr. One unit of enzyme

activity is defined as the incorporation of one millimicromole of labelled nucleotide in one hour under the optimal assay conditions.

Time curves of DNA polymerase activity were conveniently obtained by withdrawing 0.05ml. samples from individual assay tubes at the required times. The samples were dispensed onto filter discs, dipped into ethanol, dried with cold air from a hairdryer, and stored at -10° until the last sample was withdrawn. The discs were then processed simultaneously as above.

This method of immersing discs in ethanol and drying was adapted for applying volumes greater than 0.05ml. onto a disc in cases of suspected low levels of incorporation. Additional 0.05ml. samples were applied to the one disc, followed by alcohol precipitation and drying.

Heat stability studies were performed by prior incubation of the DNA polymerase in 0.1ml. of Buffer 4A at 45° for various times, and occasionally in the presence of DNA or glycerol, also. This was followed by the addition of the other components of the polymerase reaction system, and the subsequent assay of the remaining polymerase activity.

II C (ii) DNase

DNase was assayed under DNA polymerase reaction conditions by measurement of the hydrolysis of heat-denatured, radioactive DNA to give acid-soluble fragments.

The assay volume, incubation conditions, and assay components were the same as those of the DNA polymerase assay, omitting the deoxyribonucleoside triphosphates, and substituting 10 µg of heat

denatured Esch. coli [^{32}P] - DNA for the assay template-DNA of the polymerase assay. The reaction was carried out in a 15ml. conical centrifuge tube and stopped by immersing the tube in ice. 0.2ml. of BSA at 10 mg./ml. was added followed by 0.6ml. of 1N-PCA. After 10 min. in ice and 10 min. centrifugation at 800g the ^{32}P of the supernatant fraction was determined as described in Methods II E(i).

The activity of the enzyme was expressed as % or µg. of radioactive DNA rendered acid-soluble /hr./mg. of protein.

II D Ultracentrifugation Techniques.

(i) Analytical ultracentrifugation

The instrument employed for this technique was the Beckman-Spinco Model E analytical ultracentrifuge.

(a) Determination of $S_{20,w}$ using Schlieren optics.

A protein solution (0.6ml.) at 5-8mg/ml. was dispensed by syringe into the 12mm. 4° Epon cell of the AN-D rotor of the Spinco Model E ultracentrifuge, and centrifugation followed at 59,070 rev./min. (250,000g) for the time required for the sedimentation of the protein (usually less than 2hr.) Photographs were taken at suitable intervals using Schlieren optics and from these the $S_{20,w}$ of the protein was determined (Schachman, 1959).

II D (i)(b) Determination of $S_{20,w}$ using U.V. optics.

This technique is very similar to the above, but is routinely used for the strongly U.V.-absorbing nucleic acids. A DNA solution (E_{260} = 1.0) was sedimented in the same cell and rotor as above, at 44,770 rev./min. (50,000g) for 1hr., and photographs were taken every 4 min., from which the $S_{20,w}$ was determined. (Schachman, 1959).

II D (1)(c) Equilibrium centrifugation of DNA in CsCl density gradients.

CsCl was dissolved in the DNA solution (E_{260} = 0.2 to 0.7) and this solution was centrifuged, as above, at 44,770 rev./min. (50,000g) for 16hrs., after which a photograph was taken with the U.V. optics.

II D (2) Preparative Ultracentrifugation.

(a) Zonal centrifugation and determination of $S_{20,w}$ of enzymes on sucrose density gradients.

5-25% (w/v) sucrose linear gradients were prepared in 5/8 x 2in. cellulose nitrate tubes (Britten and Roberts, 1960). The sample (not more than 0.4ml.) was layered on top of the gradient and the tubes spun in the SW39 or SW50 rotor of the Spinco Model L ultracentrifuge for 3hr. at 50,000 rev/min. (204,000g). After centrifugation, the gradient was harvested as quickly as possible by puncturing the bottom of the tube with a no.16 hypodermic needle and collecting approx. 30 three-drop fractions under pressure. Alternatively, the gradients were harvested by upward displacement of the gradient by 40% (w/v) sucrose. The gradient was pumped through a flow-cell of the Spectronic 505 spectrophotometer and the E_{260} recorded. The output was then collected in fractions as before.

In these studies the enzyme was dissolved in water or in various aqueous solvents: Buffer 1, Buffer 2, Buffer 3, Buffer 4A or Buffer 5 (see Abbreviations). The samples applied to the gradient were DNA, or DNA polymerase preparations, or a mixture of both, for the purpose of investigating possible binding of the enzyme to DNA. The fractions obtained were assayed directly for DNA polymerase.

II D (11.)(b) Equilibrium centrifugation using CsCl density gradients.

This technique was used to investigate the product of the DNA polymerase reaction. The polymerase reaction was carried out in conditions designed to promote maximum replication of the template DNA, with 20 µg. of denatured DNA and 300-600 µg. of protein/assay. Incubation was at 37° for up to 5hr. and more enzyme and triphosphates were added at hourly intervals. CsCl solutions were made up as follows: 13.5g. of CsCl, 1ml. of 0.2M-tris-HCl, pH7.5, 9ml. of H_2O. 5ml. of solution were pipetted into a cellulose nitrate tube ($\frac{5}{8}$in. x 3in.) of the Griffin-Christ angle rotor no.60. On top of this was layered 0.25 ml., consisting of 0.2ml. of the polymerase reaction mixture and 40-50 µg. (in 0.05ml.) of native and/or heat denatured DNA. The tubes were filled to the top with liquid paraffin oil (sp.gr. 0.83-0.87). Centrifugation followed for 30-60hr. at 35,000 rev./min. (35,000g) at 15° (Flamm, Bond & Burr, 1966) in the Griffin-Christ Omega II preparative ultracentrifuge. After centrifugation, the gradient was immediately harvested by puncturing the bottom of the tube with a no.12 hypodermic needle and collecting approx. 100 one-drop fractions. Alternate fractions were diluted by adding 0.4ml. of H_2O and the E_{260} measured in 1cm. pathlength microcells of the Unicam SP500 spectrophotometer. The acid-insoluble radioactivity of 0.02ml. portions of the undiluted fractions was determined by precipitation onto filter paper discs as in the DNA polymerase assay [Methods II C(1)]. Densities of CsCl solutions were determined on the Abbé refractometer followed by extrapolation from a standard curve of refractive index against density in g./cm^3.

II E Instruments and Techniques used in Radioisotope Determinations

(i) Nuclear-Chicago gas flow counter.

Solutions of radioactive material (1.0ml. vol.) were applied directly onto a stainless steel planchettes. Neutralisation by alkali of the acid-soluble radioactivity was necessary in the case of material from DNase assays. The planchettes were dried by infrared heat and the radioactivity was determined on an automatic low-background Nuclear-Chicago gas flow counter, fitted with a Micromil end window. Dried filter paper discs containing precipitated radioactive material were placed on planchettes and counted directly. The efficiency of counting ^{32}P was taken as 50%.

II E(ii) Nuclear-Chicago Actigraph III

Chromatograms of ^{32}P - containing materials were scanned on the Nuclear-Chicago Model III Actigraph, and the results automatically recorded on a chart.

II E(iii) Liquid scintillation spectrometers.

Determination of $[^{3}H]$ - dATP incorporation into acid-insoluble material in DNA polymerase assays was on the Packard or Nuclear-Chicago liquid scintillation spectometers. The dried filter paper discs were placed in scintillation vials and 0.5ml. of Hyamine hydroxide added. The vials were heated at 60° for 10min. to solubilise the DNA, cooled, then 10ml. of toluene based scintillation fluid added (100g. of POPOP: 2g. of PPO: 473ml. of toluene). The efficiency of counting was approximately 20% on the Packard and 25% on the Nuclear-Chicago instrument, as determined by the channels ratio technique.

II F Other Analytical Methods

(i) Chromatography on sheets of DEAE-cellulose.

The products of the DNA polymerase and DNase reactions in their respective incubation media could be applied directly onto sheets of DEAE-cellulose (Whatman DE81), 28 x 11cm. (Furlong, 1967). 0.05ml. samples were spotted onto a line 5 cm. from the top and the chromatogram was developed for 1-2hr. in the descending direction using 0.75M - ammonium bicarbonate, pH 8.6, as solvent. After drying it was cut into strips (4cm. width) and scanned for ^{32}P in the Nuclear-Chicago Model III Actigraph. Rf values of DNA, oligomeric DNA and the mono-and tri-phosphates of the deoxyribonucleosides are given by Furlong (1967).

II F (ii) Isobutyric Acid paper Chromatography

Chromatography on Whatman No.1 paper of the deoxyribonucleoside triphosphates was performed by the method of Keir & Smellie (1959). The ascending chromatogram was developed overnight using as solvent isobutyric acid, ammonia (sp.gr. 0.880), 0.1M-EDTA, H_2O (100:4.2:1.6: 55.8, by vol.). Rf values of the mono-, di-, and triphosphates have been compiled by Grov. (1967).

II F(iii) Fractionation of DNA by Dextran-Polyethylene Glycol Two-Phase Systems.

This technique is described by Alberts (1967). The DNA was dialysed at 0° against 0.01M sodium phosphate, pH 6.8 until equilibrated, and 1 ml. portions of the DNA solution were then pipetted into cellulose nitrate centrifuge tubes (½in. x 2in.) and an appropriate volume of well-shaken phase system solution [13.3% dextran (w/w), 10% polyethylene glycol w/w)] was added. The volume of phase solution varied between 0.6 - 1.0 ml. and was chosen to give the optimal separation of native and denatured DNA. The tubes were sealed with parafilm and inverted

50 times, thoroughly mixing the layers. They were then centrifuged at 800g for 15 min., and the layers formed were separated by removing the upper with a Pasteur pipette, and the lower by puncturing the bottom of the tube. Great care was taken throughout the mixing, centrifugation and separation of layers to maintain a constant temperature of 1°.

The DNA in each layer was determined by absorption at 260 mμ but this was rather insensitive since the lower (dextran) layer gave high blanks. Other methods used were direct measurement of ^{32}P in the DNA where applicable, or by the micro-Burton method [Methods II F(v)].

II F(iv) Protein Estimations

These were routinely performed by the method of Lowry, Rosebrough, Farr & Randell (1951). The protein concentrations of DNA polymerase fractions dissolved in Buffer 4 were always determined in the presence of 0.1 ml. of Buffer 4 by comparison with a standard curve prepared with varying amounts of BSA in 0.1 ml. of Buffer 4.

II F(v) DNA Estimations

DNA was determined directly by ultraviolet absorption using the relationship that a solution of 42.5 μg. of DNA/ml. has an $E_{260} = 1.0$.

More accurate determinations were made by the Adams (1965) modification of the Burton method (1956). This modification scales down the volume, thereby increasing the sensitivity.

0.02ml. of 72% perchloric acid was added to the DNA sample in 0.2ml., and incubated for 30 min. at 70° in a stoppered tube. The sample was cooled and 0.25 ml. of the Burton reagent added (0.5g. of diphenylamine, 50ml. of glacial acetic acid, 0.5ml. of conc. H_2SO_4, to which 0.15 ml. of 1.6% acetaldehyde was added just before use). After

storing in the dark for 16hr. at room temperature any precipitate was removed by centrifugation, and the E_{600} determined on the Unicam SP500 spectrophotometer using microcells.

RESULTS

I Partial Purification of DNA polymerase and variations in standard preparative procedures.

Table 2 shows the range of results of the enzyme preparations. Although many such preparations were performed routinely, the specific activities of the fractions obtained often varied enormously for reasons that will be later explained. However, the purifications and recoveries obtained for the pH5 precipitate and ammonium sulphate precipitate were fairly constant. Purification and recovery tended to be higher the smaller the scale of preparation.

A State of ascites cells.

The number of days between innoculation of the mice and the harvesting of the cells was critical: after 6-7 days the DNA polymerase obtained had a high specific activity but there was a poor yield. The converse was true for the enzyme obtained from cells 8-10 days after innoculation. Sometimes the enzyme was prepared from cells frozen at -70° for up to 1 year and although some of the activity appeared to be lost by the freezing and thawing of the cells, no more appeared to be lost at -70°.

B Preparation of crude extract.

(i) Comparison of homogenisation and sonication

A 105,000g supernate (S_1) of ascites-tumour cells was prepared by homogenisation, and the sediment was resuspended in Buffer 4A. A 105,000g supernate (S_2) was prepared from this resuspended sediment by sonication. S_1 and S_2 were assayed for DNA polymerase and DNase. It was found that whereas S_1 had 90% of DNA polymerase of ($S_1 + S_2$), it had only 60% of the DNase.

TABLE 2

Preparation of DNA polymerase

Fraction	dTMP incorporated mμmoles/mg. of protein/hr.	Recovery %	Purification
105,000 g supernate	3-6		
pH 5 ppt	9-19	30-80	3-5
20-45% $(NH_4)_2SO_4$ ppt	15-25	10-70	4-6
Sephadex, pooled fractions	6-10	30-100% off column	2-3
Sephadex, optimum fraction	10-32		3-10

DNA polymerase was assayed by the standard procedure and the results shown are taken from 4-8 different enzyme preparations.

B (ii) Comparison of nuclear and cytoplasmic DNA polymerase.

Ascites-tumour cells were suspended in 0.01M-tris-HCl, pH7.5, 0.02M-$Mg(OAc)_2$ and sonicated for 20 sec. to break the cytoplasmic but not nuclear membranes. Sucrose was added to 0.25M giving the cytoplasmic fraction C and sedimented nuclei. The nuclei were resuspended in Buffer 4A and the mixture sonicated for 30sec. to break nuclear membranes, giving the nuclear fraction N. C and N were assayed for DNA polymerase. The ratio of cytoplasmic: nuclear enzyme was 57:1.

B (iii) Effect of temperature of preparation

105,000g supernatant fractions were prepared by homogenisation, duplicating the procedures exactly but varying the temperature of preparation. They were prepared (a) at 0 -4° and (b) at room temperature. However, both the centrifugations at 105,000g for 1 hour in the Spinco Model L were performed at 4°C. Both fractions were assayed for polymerase activity at varying protein concentrations. The results are shown in Fig.5. There was no difference between the preparations.

C The effect of protein concentration during pH5 precipitation.

A 105,000g supernatant fraction was prepared by sonication to give a solution of 15.3mg. of protein/ml. pH5 precipitates were made from (i) the undiluted 105,000g supernatant fraction (ii) a 1 in 4 dilution (3.82mg./ml.), using identical procedures, and dissolving the precipitates in Buffer 4A at approximately 25mg./ml. These preparations were then assayed for DNA polymerase (Table 3). The preparation using the concentrated protein solution produced a greater recovery of enzyme, a higher specific activity,

FIGURE 5

Comparison of protein concentration curves of DNA polymerase at 0^{o} and 20^{o}

Each assay (0.25 ml.) contained the following: Tris-HCl, pH 7.5, 6 μmoles; KCl, 15 μmoles; $MgSO_4$ 1.5 μmoles; 2-mercaptoethanol, 3.5 μmoles; EDTA, 0.1 μmole; DNA (heat denatured), 70 μg.; dATP, dGTP, dCTP, 50 mμmoles each; [α^{32}P]-dTTP (1.9×10^6 counts/min./μmole) 50 mμmoles. Incubations were for 1 hr. at 37^{o}. The protein concentrations are indicated opposite.

—— o —— Soluble extract prepared at 0^{o}.

—— ◎—— Soluble extract prepared at 20^{o}.

FIGURE 5

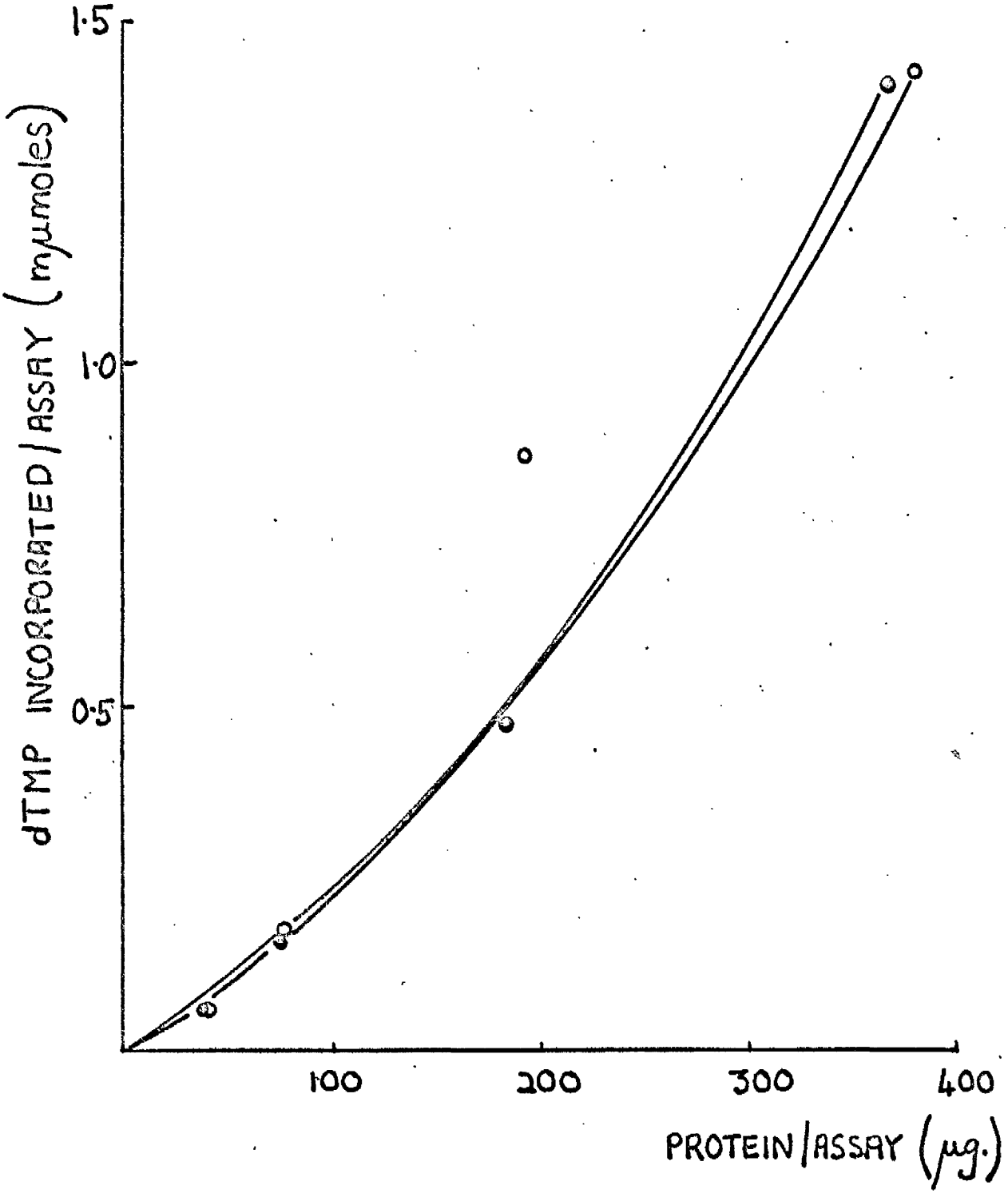

1·5
1·0
0·5
dTMP INCORPORATED / ASSAY (mμmoles)
100
200
300
400
PROTEIN / ASSAY (μg.)

TABLE 3

The effect of protein concentration during pH 5 precipitation of DNA Polymerase on the subsequent purification and recovery of activity

Protein concentration during pH 5 precipitation (mg./ml.)	dTMP incorporated (mμmoles/mg. of protein/hr.)	Recovery %	Purification
3.85	16.21	25.7	3.9
15.3	19.62	35.5	4.7

20 ml. of the same 105,000 g supernate fraction of DNA polymerase was used for pH 5 precipitate preparation at the two indicated protein concentrations. 0.5 N-acetic acid was added dropwise to pH 5.0 and the precipitate immediately centrifuged down and dissolved in Buffer 4A at approximately 2.5 mg./ml. The assay was performed by the standard procedure with 125 μg. of protein/assay.

but a greater proportion of enzyme in the supernatant fraction.

D Fractionation on Sephadex columns

This fractionation gave very varied results and for this reason the purification and recoveries were not recorded on Table 1. The main problems were to separate the DNA polymerase from DNase, and to prevent the great loss in DNA polymerase on the column which frequently occurred. In initial studies an ammonium sulphate fraction was applied to the column with about 15mg. of ferritin and 15mg. of haemoglobin to act as markers. The ferritin eluted with the void volume, the DNA polymerase just behind it and the haemoglobin and DNase then following in a coincident peak. The DNA polymerase containing fractions were pooled and concentrated by pH5 precipitation. There was a 35% recovery of the enzyme from the column. In attempts to improve the recovery some alterations were made. The fraction applied to the column was a 105,000g supernate prepared by sonication, and the eluting buffer used, Buffer 4B, was 0.04M in tris-HCl, pH7.5 (a doubling of the molarity previously used) and 1mg/ml. in BSA. There was a 100% recovery of polymerase from the column, probably owing to protection of the polymerase by BSA, and possibly also by DNA fragments present in the 105,000g fraction after sonication (Fig. 6). However, when the fractions containing the DNA polymerase were pooled and concentrated by pH5 precipitation 50% of the enzyme activity was lost. In addition, there was slight contamination by DNase arising from the overlapping of the DNase and DNA polymerase peaks. So whereas the 105,000g fraction when applied to the column resulted in greater enzyme recoveries than from an ammonium sulphate fraction there was more DNase to be removed, since this fraction, especially

FIGURE 6

Chromatography of DNA polymerase on Sephadex G-150

20 ml. of a supernatant fraction containing 386 mg. of protein/ml. were applied to the column and eluted with Buffer 4B. 7 ml. fractions were obtained and assayed for DNA polymerase as described in Fig. 5 (variations: 8 μmoles of tris-HCl, pH 7.5/assay; specific activity of [α^{32}P]-dTTP was 4.9 x 10^6 counts/min./μmole). Each DNase assay contained the same quantities of tris-HCl, pH 7.5, KCl, $MgSO_4$ 2-mercaptoethanol and EDTA as the DNA polymerase. In addition there was 10 μg. of heat denatured [^{32}P]-DNA (2.9 x 10^3 counts/min./μg.). Incubations were for 1 hr. at 37°.

——————— Protein

——o—— DNA polymerase activity

——⊙—— DNase activity

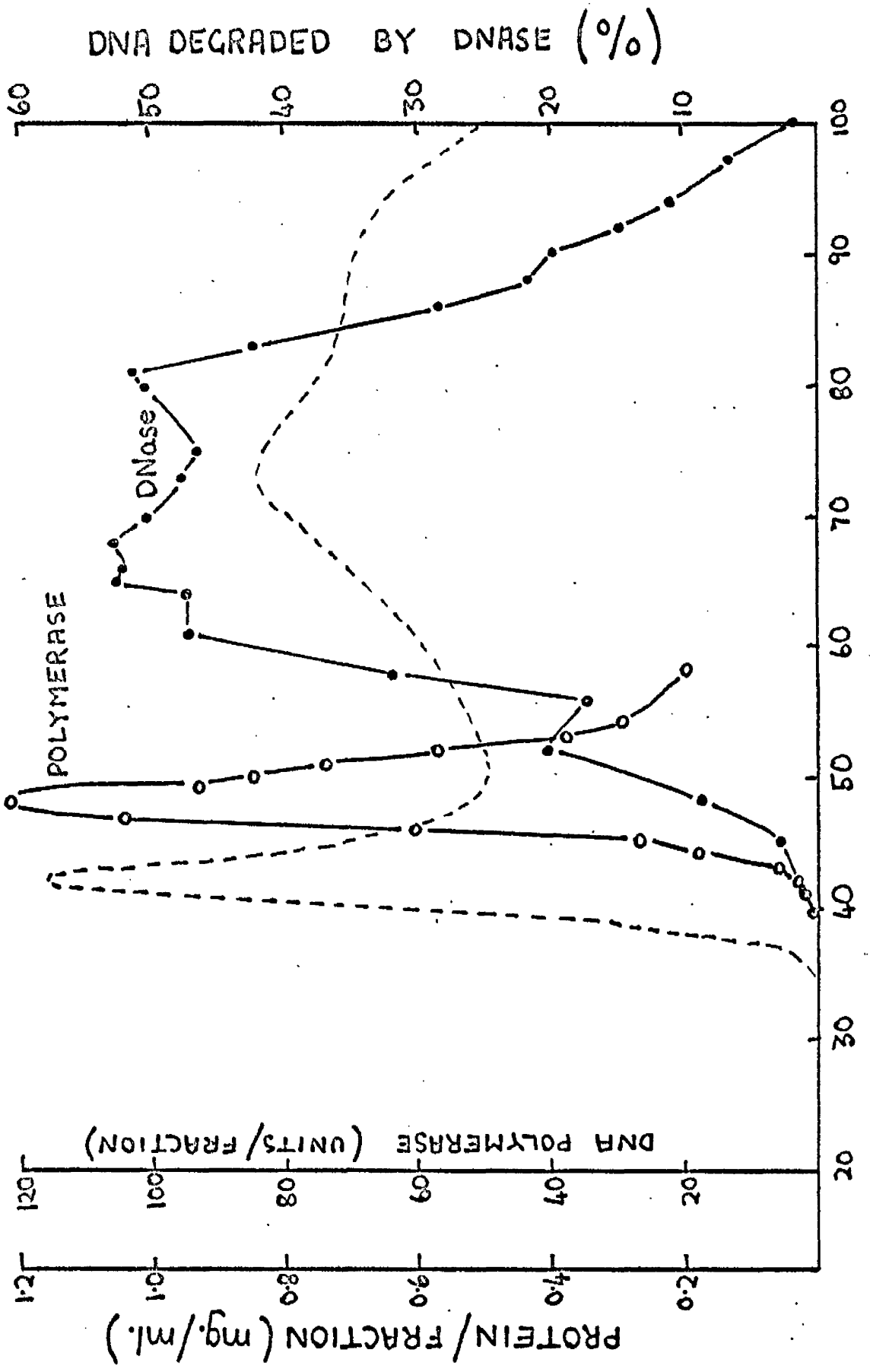

DNA DEGRADED BY DNASE (%)
60
50
40
30
20
10
DNase
POLYMERASE
PROTEIN / FRACTION (mg./ml.)
1·2
1·0
0·8
0·6
0·4
0·2
DNA POLYMERASE (UNITS/FRACTION)
120
100
80
60
40
20
20
30
40
50
60
70
80
90
100
FRACTION NUMBER (7 ml.)

FIGURE 6

prepared by sonication, contains much more of that enzyme than an ammonium sulphate precipitate. Later attempts using the Diaflo Ultrafilter to concentrate the DNA polymerase, instead of the harsh treatment of pH5 precipitation applied to the dilute purified enzyme preparation, resulted in no apparent loss of activity during the concentration process. Other enzyme preparations were applied to the column, a 105,000g supernatant fraction prepared by homogenisation, a pH5 precipitate fraction and an ammonium sulphate fraction and in all cases there was a slight overlap of the DNase peak with the polymerase peak. Some of the DNA polymerase previously prepared on the column was recycled in an attempt to attain complete separation, but there was still an overlap of the peaks. Although there was an almost negligible amount of DNase present, there was a large loss of polymerase activity during the recycling.

II Stability of DNA polymerase.

DNA polymerase from ascites-tumour cells is clearly a labile enzyme as has been shown by the losses in activity incurred during fractionation on SephadeX G-150. High temperatures, freezing and thawing, storage at temperatures > 0°, as well as many other factors known to denature proteins are harmful to the enzyme. These effects can be minimised by the presence of glycerol, DNA and protein as well as the conditions previously found to be optimal for storage of the enzyme (Methods Bvii).

II A Protection of DNA polymerase activity by glycerol.

(i) The effect of glycerol on storage of the enzyme.

Two enzyme batches were stored overnight at -10° in Buffer 4A, 25% (v/v) in glycerol, and control batches stored in the same buffer minus glycerol. The enzymes were (a) enzyme which had been recycled through Sephadex, (b) enzyme which had been fractionated once on Sephadex G-150 in the presence of ferritin, and so contained perhaps 0.5mg. of ferritin/ml. All four enzymes were assayed at various protein concentrations. There was no apparent difference in activity in the recycled enzyme if glycerol is present (Fig. 7A). However, ferritin appeared to inactivate the enzyme during the storage, or during the freezing and thawing, and glycerol protected the enzyme from this inactivation (Fig. 7B).

A (ii) Effect of glycerol on the time of enzyme incubation

DNA polymerase was assayed for varying times in the presence and absence of 10% glycerol (Fig. 8). There was no significant difference in the incorporation until after 60min. of incubation. Presumably the DNA polymerase begins to become inactivated after 60min. at 37°, and this inactivation is partially prevented by the glycerol.

A glycerol concentration curve, for 2hr. incubations, (Fig.9) showed an approximately 70% increase over the control in the presence of 20% glycerol(v/v).

FIGURE 7

Effect of glycerol on storage of DNA polymerase

The DNA polymerase assay conditions were as described for Fig. 5 (variations: 8 μmoles of tris-HCl, pH 7.5/assay, specific activity of [α^{32}P]-dTTP was 3.9 x 10^6 counts/min./μmole). Incubations were for 1 hr. at 37°, and protein concentrations were as indicated.

A The enzyme used had been twice fractionated on Sephadex G-150.

B The enzyme used had been fractionated on Sephadex G-150 in the presence of ferritin, and so contained approximately 0.5 mg. of ferritin/ml.

—— ⊙ —— DNA polymerase stored in 25% glycerol, and assayed in 10% glycerol.

—— ○ —— DNA polymerase stored and assayed in the absence of glycerol.

FIGURE 7

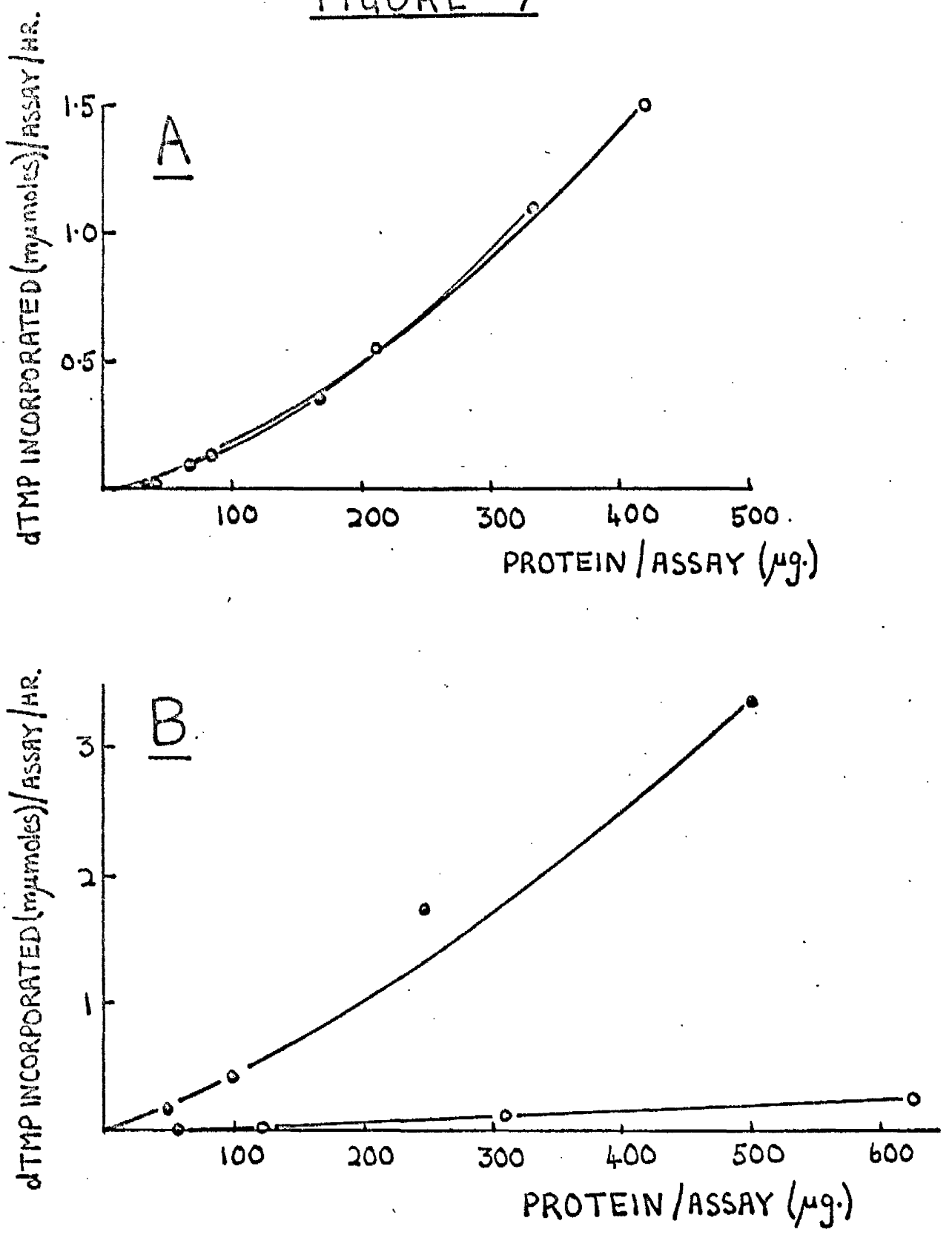

A
dTMP INCORPORATED (mμmoles)/ASSAY/HR.
1·5
1·0
0·5
100
200
300
400
500
PROTEIN/ASSAY (μg.)
B
dTMP INCORPORATED (mμmoles)/ASSAY/HR.
3
2
1
100
200
300
400
500
600
PROTEIN/ASSAY (μg.)

FIGURE 8

Effect on glycerol on the DNA polymerase time curve

The assay conditions were the same as in Fig. 7. The enzyme was that used in Fig. 7A.

—⊟— 334 µg. of enzyme fraction protein/assay, stored in 25% glycerol and assayed in 10% glycerol.

—□— 410 µg. of enzyme fraction protein/assay, stored and assayed in the absence of glycerol.

—⊙— 67 µg. of enzyme fraction protein/assay, stored in 25% glycerol and assayed in 10% glycerol.

—○— 84 µg. of enzyme fraction protein/assay, stored and assayed in the absence of glycerol.

FIGURE 9

Glycerol concentration curve

The assay conditions were the same as in Fig. 6. Specific activity of $[\alpha^{32}P]$-dTTP was 3.9×10^6 counts/min./µmole. The protein concentration was 150 µg./assay. Incubations were at 37° for 2 hr. Glycerol was present at the indicated concentrations.

FIGURE 8

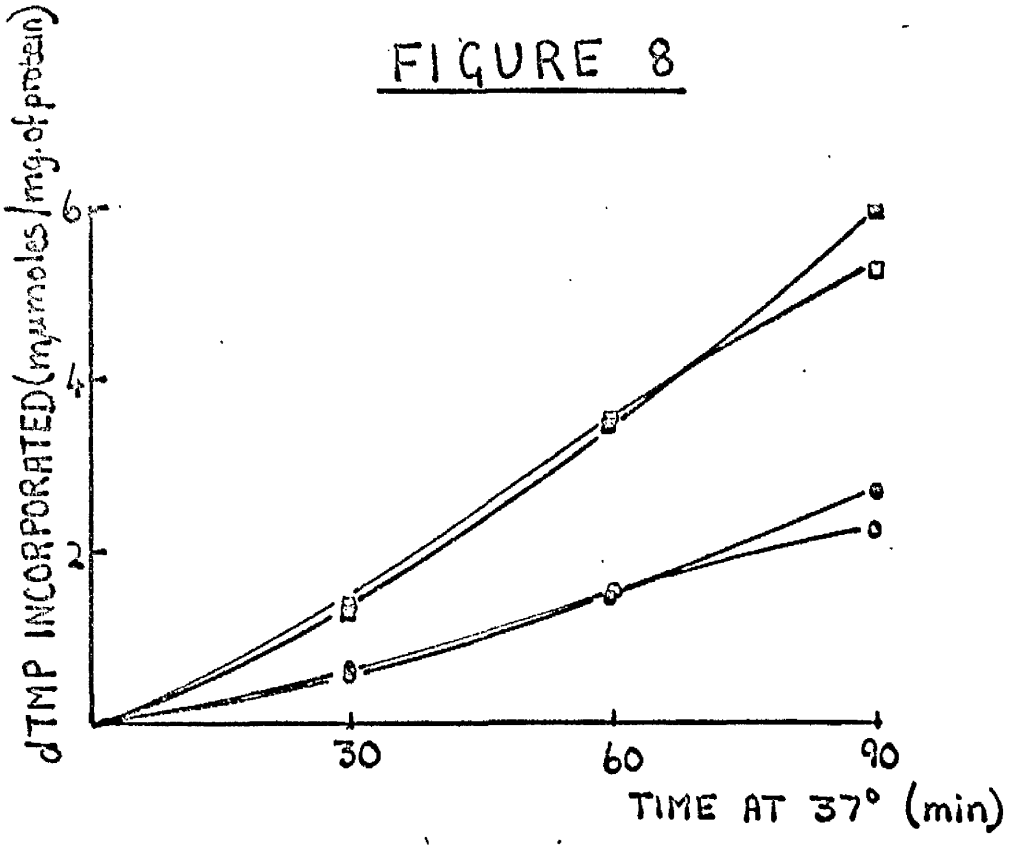
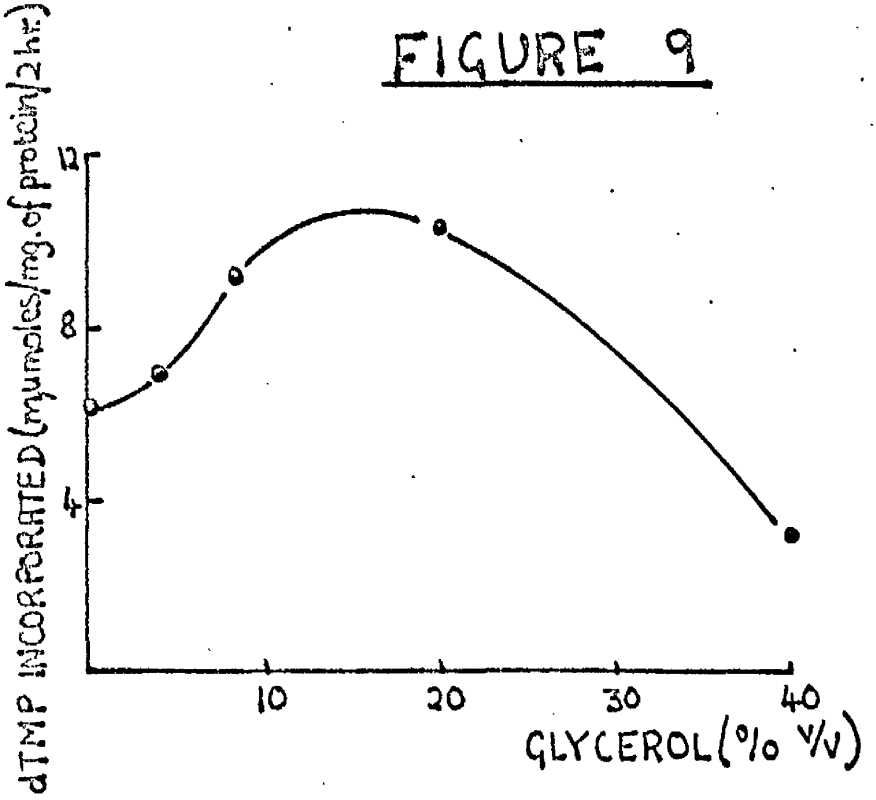


FIGURE 9



A (iii) Effect of glycerol on incubation of polymerase at 45°.

0.1ml. portions of DNA polymerase dissolved in (a) Buffer 4B, 40% glycerol, and (b) Buffer 4B alone, were incubated at 45° for periods up to 30min/. before undergoing the standard assay. The results (Fig. 10) show the marked protection of polymerase by 40% glycerol.

A (iv) The effect of glycerol on the loss of polymerase activity during freezing and thawing.

Solutions of DNA polymerase dissolved in (a) Buffer 4B, 40% glycerol, and (b) Buffer 4B alone at 2.55mg./ml. were successively frozen in Drikold-ethanol, and thawed in water at approximately 40°. After every freeze/thaw, 0.1ml. portions were removed and assayed under identical conditions. The results (Fig. 11) show that the polymerase activity remaining after 24freeze/thaws is twice as high in the presence of 40% glycerol.

B Protection by DNA

As already mentioned (Results ID) a 105,000g supernate prepared by sonication does not lose activity during Sephadex G-150 fractionation and this was attributed in part to the possible presence of DNA fragments bound to the enzyme. More conclusive evidence is the protective effect of DNA on polymerase incubated at 45° prior to the standard assay. The experimental conditions were similar to those described for glycerol (Results II A iii). The experiment was performed in duplicate using Sephadex G-150 purified enzyme (Fig. 12A) and an ammonium sulphate precipitate fraction (Fig. 12B). There was little difference in the behaviour of the two enzyme fractions, though there was possibly a greater effect with the more purified enzyme.

FIGURE 10

Effect of glycerol on denaturation of DNA polymerase by incubation at 45°

0.1 ml portions (255 µg. of protein) of enzyme dissolved in Buffer 4B in the presence or absence of 40% glycerol were incubated at 45° for the times shown and then assayed under the standard conditions. The specific activity of [α^{32}P]-dTTP was 6.5 x 10^6 counts/min./µmole.

—●— DNA polymerase incubated at 45° in 40% glycerol and assayed in 16% glycerol.

—○— DNA polymerase incubated and assayed in the absence of glycerol.

FIGURE 11

Effect of glycerol on denaturation of DNA polymerase by freezing and thawing

The enzyme preparations described in Fig. 10 were repeatedly quickly frozen and thawed, and assayed under the standard conditions. The specific activity of [α^{32}P]-dTTP was 8.6 x 10^6 counts/min./µmole.

—●— DNA polymerase in 40% glycerol during freeze/thaws and assayed in 16% glycerol.

—○— DNA polymerase in the absence of glycerol during freeze/thaws and assays.

FIGURE 10

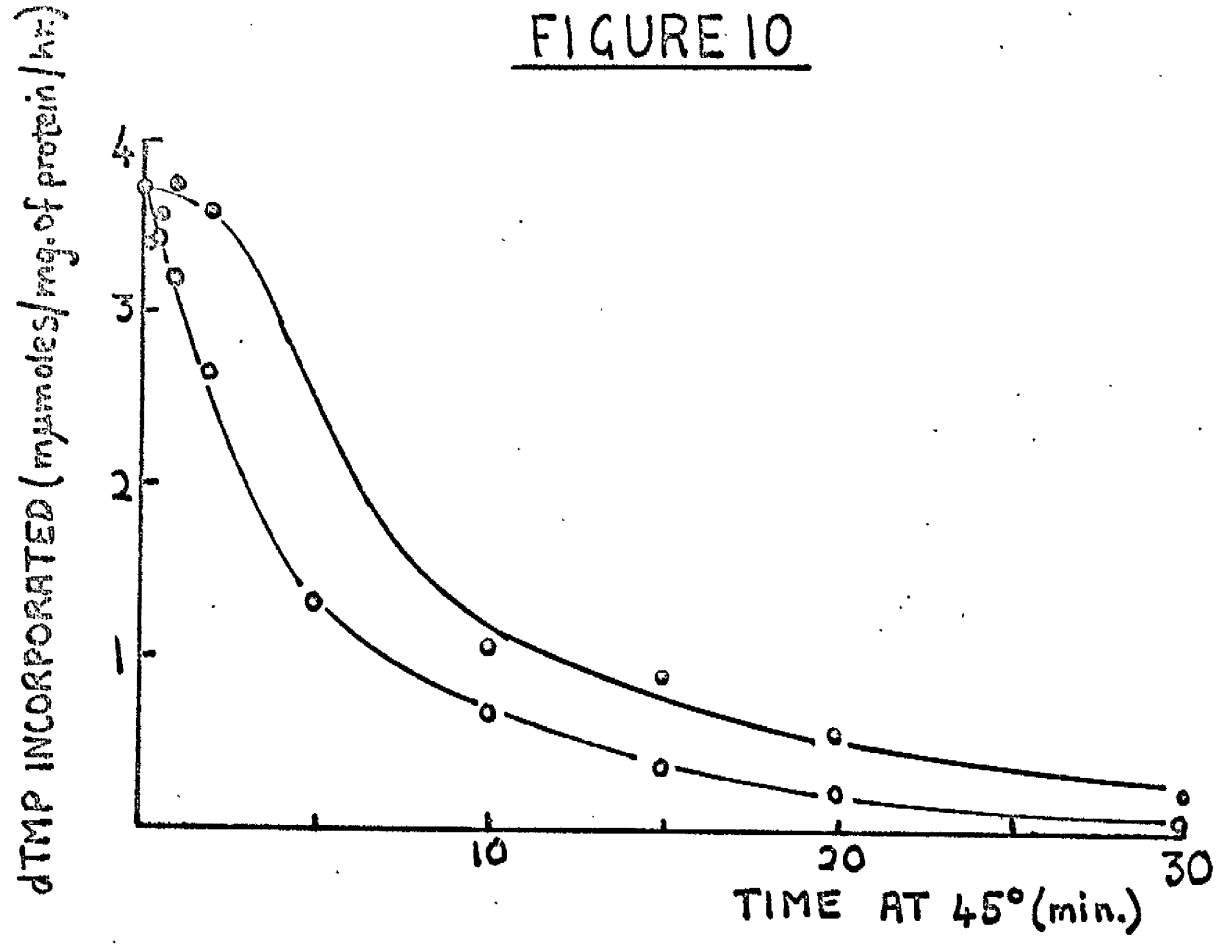


FIGURE 11

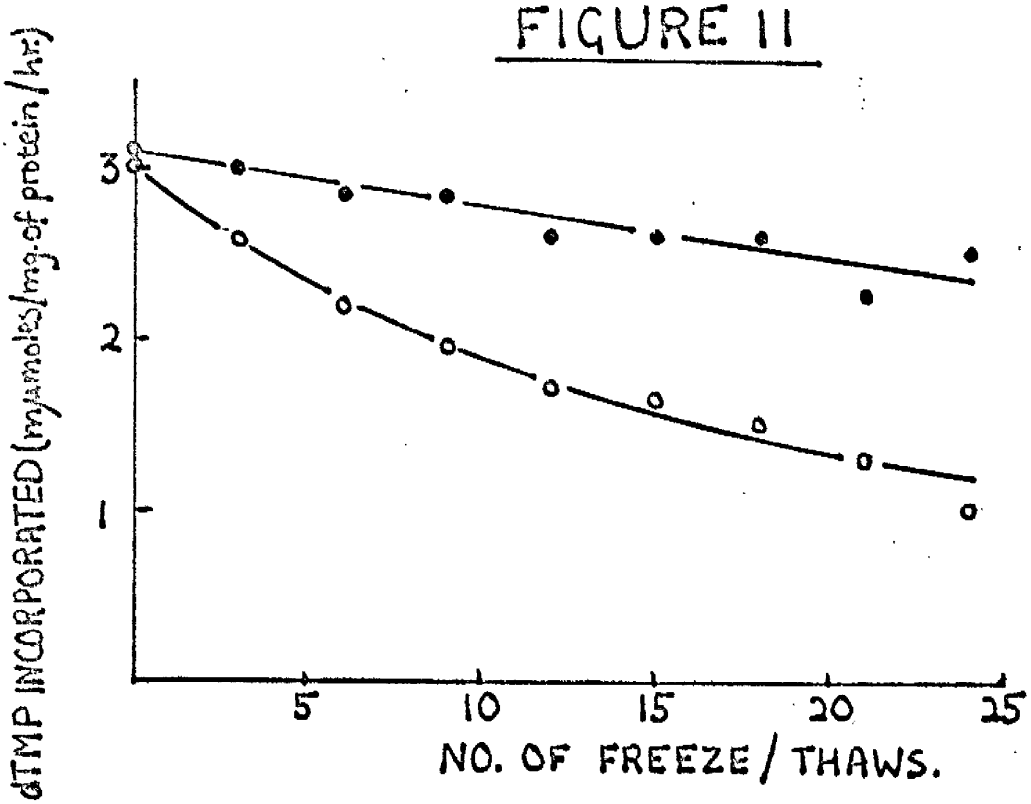


FIGURE 12

Effect of denatured DNA on denaturation of DNA polymerase by incubation at 45°

0.1 ml. portions of DNA polymerase in Buffer 4A were incubated at 45° in the presence or absence of 70 µg. of denatured DNA for the times shown. The remaining activity was assayed under the standard conditions; no more DNA was added to those already containing 70 µg. The specific activity of [α^{32}P]-dTTP was 3.9 x 10^6 counts/min./µmole.

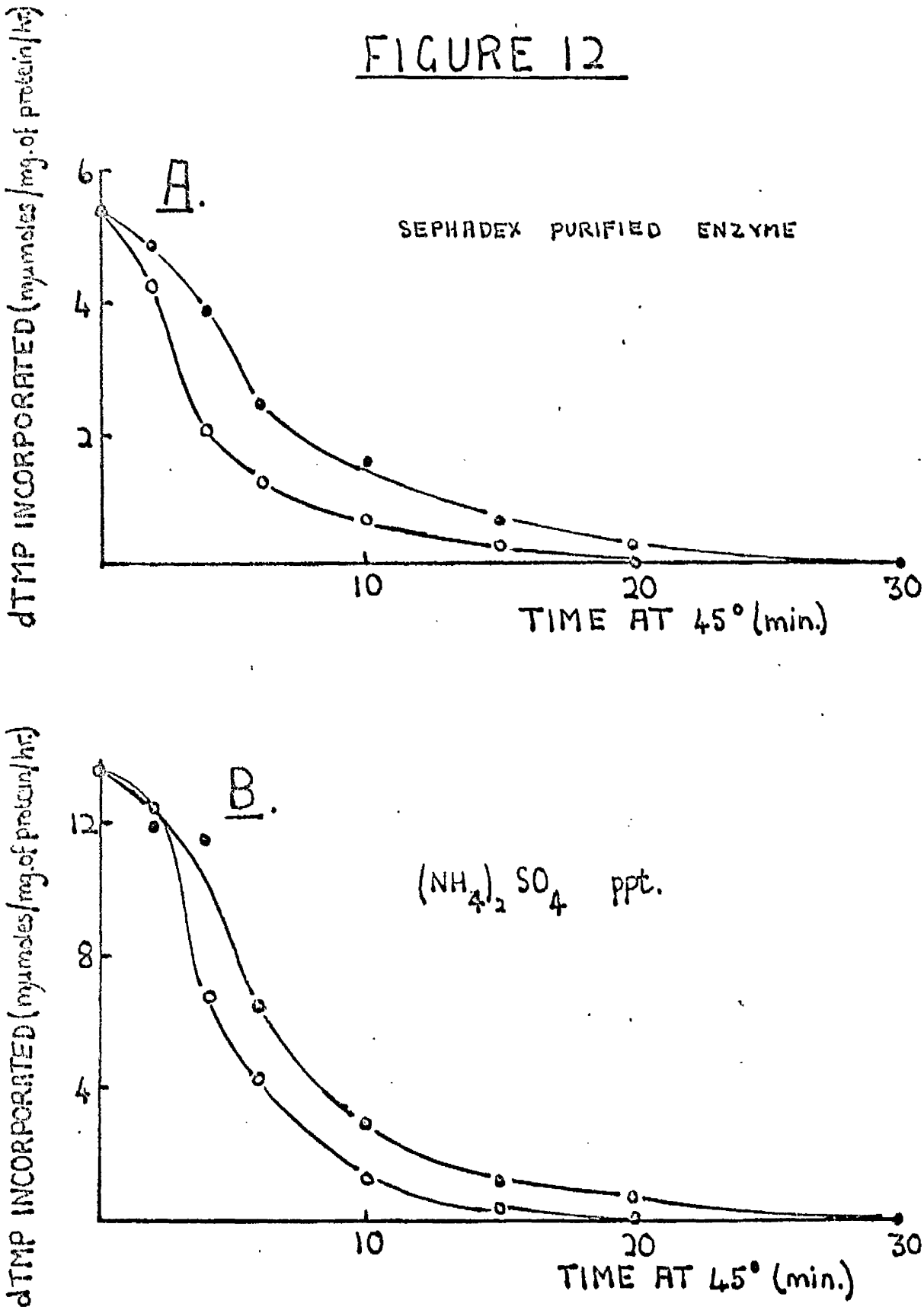
A Sephadex purified enzyme was used (134 µg. of protein/portion).

B 20-45% ammonium sulphate enzyme fraction was used (136 µg. of protein/portion).

—o— DNA polymerase in the presence of 70 µg. of heat denatured DNA.

—o— DNA polymerase in the absence of DNA.

FIGURE 12


A.
SEPHADEX PURIFIED ENZYME
dTMP INCORPORATED (mμmoles/mg. of protein/hr)
6
4
2
10
20
30
TIME AT 45° (min.)
B.
$(NH_4)_2 SO_4$ ppt.
dTMP INCORPORATED (mμmoles/mg. of protein/hr)
12
8
4
10
20
30
TIME AT 45° (min.)

II C Protection by Protein.

It was found in many instances that DNA polymerase was more labile in dilute purified solutions, suggesting that protein has a protective effect on the enzyme. A polymerase concentration curve was performed in duplicate, but in the first set of tubes BSA was added to give a constant concentration of protein at 390µg/assay. The results in Fig. 13 show the protective action of protein. The kinetic aspects of these results are dealt with more fully in III C.

D The effect of temperature of storage on DNA polymerase

0.1ml. portions of DNA polymerase were stored at various temperatures for varying lengths of time and then kept at -70° until assayed altogether. The results (Fig.14) show stimulation of DNA polymerase activity when the enzyme was stored at 4°for up to 1 week, but its activity then fell off rapidly with a half life of about 6 days. The polymerase stored at room temperature had a half life of about 4 days. The polymerase stored at -10° was also remarkably labile, with a half life of about 10 days, possibly because these samples were almost thawing while being transferred to -70°. Thus it appears that polymerase stores better at temperatures a little above freezing rather than a little below.

III DNA Polymerase kinetic studies.

A Deoxyribonucleoside triphosphates.

The majority of these studies were performed using an $(NH_4)_2SO_4$ precipitate fraction, though in later studies a Sephadex-purified enzyme was used and similar results were obtained. Initially

FIGURE 13

Effect of BSA on a DNA polymerase concentration curve

The standard assay conditions were used, though there were 8 μmoles of tris-HCl, pH 7.5/ assay. The specific activity of [α^{32}P]-dTTP was 2.6 x 10^6 counts/min./μmole. The enzyme protein concentrations were as indicated.

—⊙— BSA present in the assay giving a total protein concentration (BSA + enzyme protein) of 390 μg.

—o— No BSA present.

FIGURE 14

Effect on DNA polymerase of storage at different temperatures

0.1 ml. portions of Sephadex purified DNA polymerase containing 450 μg. of protein dissolved in Buffer 4A were stored for the indicated number of days at the indicated temperatures. They were then removed to -70° and stored till day 18 when all the samples were assayed for DNA polymerase activity by the standard procedure.

—⊙— Samples stored at -15°

—o— Samples stored at 4°

—Δ— Samples stored at 20°

FIGURE 13

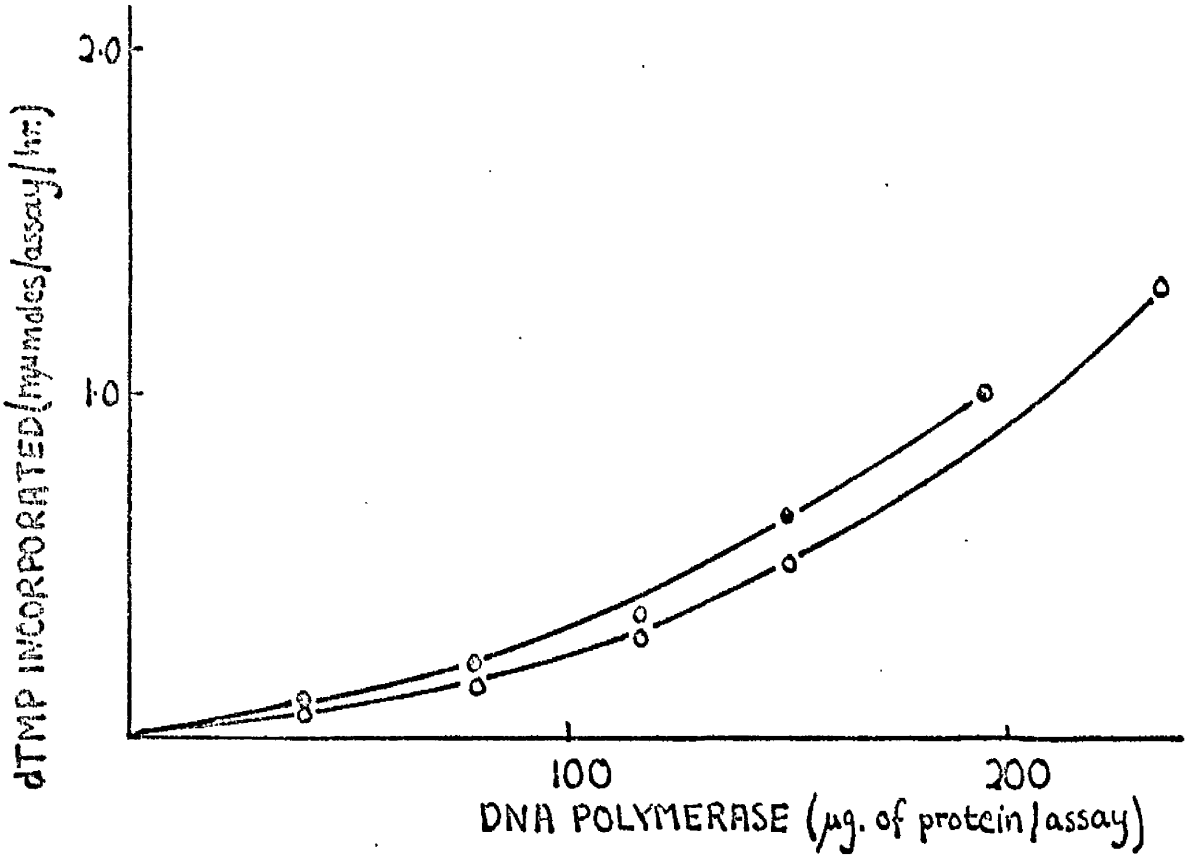
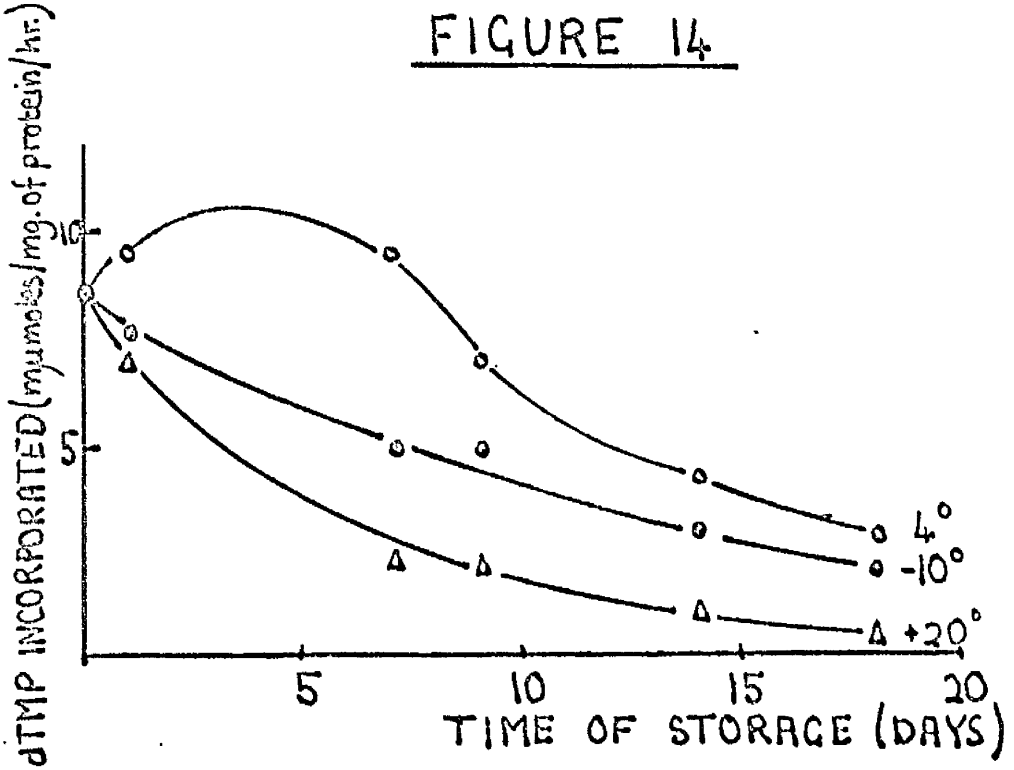


FIGURE 14



the experiments were performed by varying the concentration of one unlabelled triphosphate, while measuring the reaction by incorporation of label in one of the three remaining triphosphates, each present at 50 nmoles/assay. The radioactive triphosphates were $[\alpha^{32}P]$ -dTTP, used for studying dGTP, dCTP, dATP, and $[^{3}H]$ - dATP for dTTP incorporation. Incubations were normally for 45min. at 37° since the reaction was not linear to 60min. in the absence of one triphosphate. Anomalous results were obtained by this procedure and the experiments were later repeated using an $[\alpha^{32}P]$ - label in the triphosphate under investigation, and the expected classic results were then obtained. The differences in results of these different methods of investigation are shown in Fig. 15. dTTP concentration curves were performed with the labelled triphosphate as (i)$[^{3}H]$ -dATP, (ii)$[\alpha^{32}P]$ - dTTP. When dATP incorporation was measured the standard substrate-velocity graph, did not pass through the origin (Fig. 15A) and consequently the Lineweaver-Burk plot (Lineweaver & Burk, 1934) was not linear (Fig. 15B). In the absence of dTTP there was incorporation of $[\alpha^{3}H]$ - dATP at about 20% of the level when all four triphosphates were present at 50 nmoles/assay. It was found that a plot of $1/v$ against $1/\sqrt{S}$ was a straight line (Fig. 15C) and this was also true for each of the other three triphosphates, which was suggestive of allosteric interactions between triphosphate binding sites. The possibility of interactions between complementary triphosphates was investigated by varying the concentrations of dCTP and dGTP, (though the concentration of dCTP always equalled that of dGTP) and measuring the reaction by $[\alpha^{32}P]$ - dTTP incorporation. The plot of $1/v$ against $1/\sqrt{S}$ was still found to be a straight line.

FIGURE 15

Kinetics of dTTP incorporation into DNA

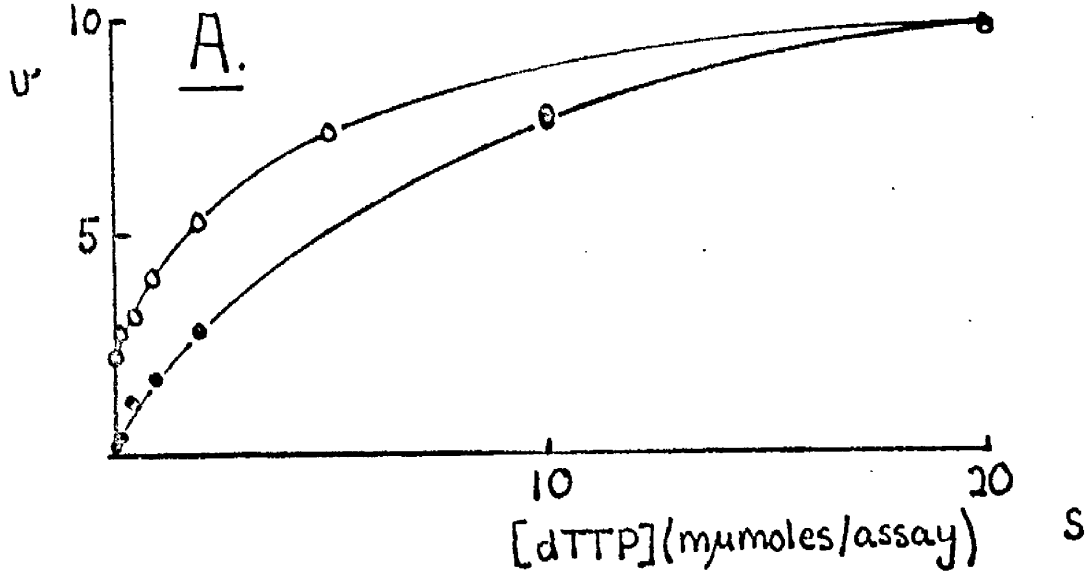
The standard assay was used with the concentrations of dTTP varying as shown. The enzyme used was 172 µg. of a 20-45% ammonium sulphate fraction and incubations were for 45 min.

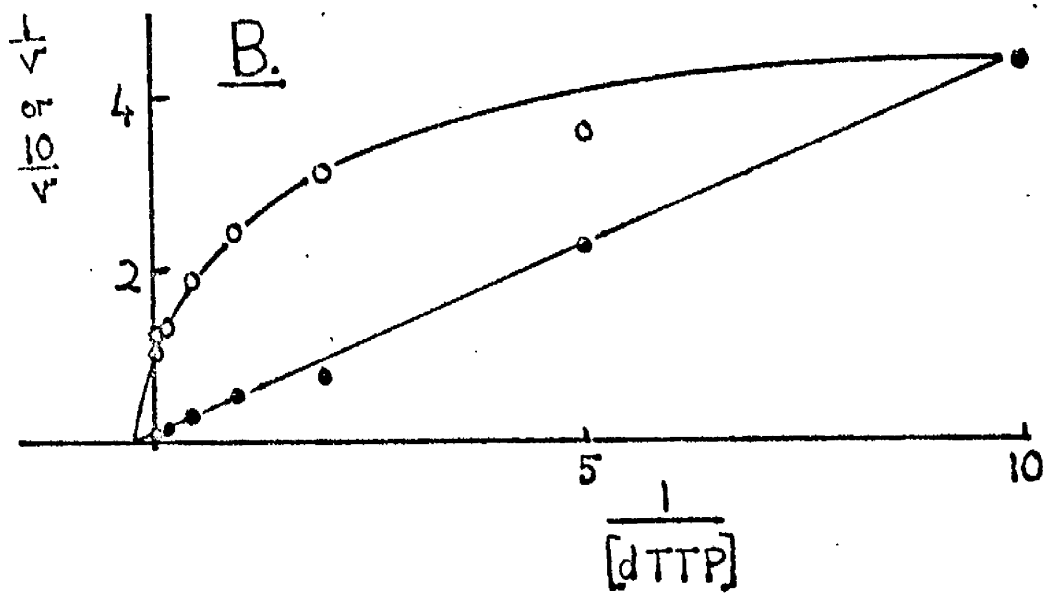
v, the velocity of the enzyme reaction is expressed in mµmoles of the radioactive deoxynucleotide incorporated into an acid insoluble product/mg. of protein/45 min.

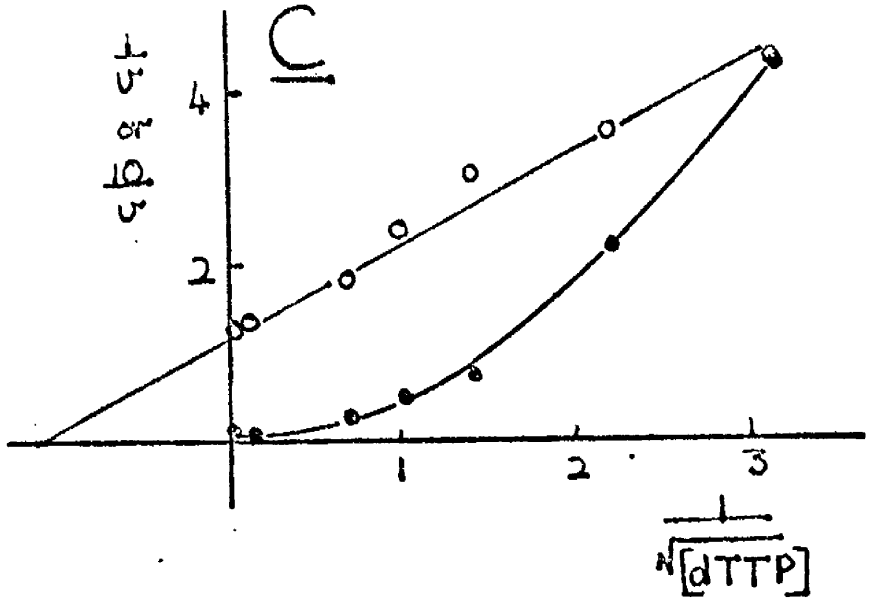
s, the substrate concentration is measured in mµmoles of dTTP/assay.

—o— Reaction measured by [^{3}H]-dATP incorporation, specific activity = 10.4 x 10^{6} disintegrations/min./µmole.

—⊙— Reaction measured by [α^{32}P]-dTTP incorporation specific activity = 3.5 x 10^{6} counts/min./µmole.


A.
v
10
5
10
20
[dTTP](mμmoles/assay)
S


B.
1/v or 10/v
4
2
5
10
1/[dTTP]


C.
1/v or 10/v
4
2
1
2
3
1/√[dTTP]

Using an $[\alpha^{32}P]$-label in one triphosphate and maintaining the others at 50 mμmoles/assay gave the classical substrate concentration curves for all four triphosphates, and the Lineweaver-Burk plots of the results are shown in Fig.16. From the intercepts, the approximate value of K_m for each triphosphate was calculated as shown.

Besides doing concentration curves of each of the triphosphates alone, concentration curves of two, three and all four triphosphates together at equal concentrations were also performed. The reaction was measured by $[\alpha^{32}P]$ - dTTP which was always one of the triphosphates whose concentration was varying. The remaining triphosphates, if any, were at a constant concentration of 50 mμmoles/assay. The standard velocity-substrate graph (Fig.17A) and the Lineweaver-Burk plot (Fig. 17B) gave the expected results in all cases excepting when all four triphosphates were varying. A sigmoid concentration curve was obtained and the Lineweaver-Burk plot curved upwards. As is shown by the K_m values the higher the concentration of the other three triphosphates in the assay the lower are the K_m values of dTTP i.e. the greater the affinity of dTTP for the enzyme.

The results of the variation of concentration of all four triphosphates were replotted in the form of a Hill plot in Fig. 18 which was a straight line of slope 3.0 suggesting that there are three allosterically connected substrate binding sites (Reinhard, 1968b).

FIGURE 16

Kinetics of Incorporation of deoxyribonucleoside triphosphates into DNA: Lineweaver-Burk plots

The standard assay was used. The concentration of one radioactive triphosphate was varied, while maintaining the other three at 50 mμmoles/assay. The enzyme used for A, B, and C was a Sephadex purified fraction (500 μg./assay) and for D a 20-45% ammonium sulphate fraction (172 μg./assay). Incubations were for 45 min.

v, the velocity of reaction, is expressed in mμmoles of the radioactive deoxyribonucleotide incorporated/mg. of protein/45 min.

s, the substrate concentration, is expressed in mμmoles of the radioactive deoxyribonucleoside triphosphate/assay.

Specific Activity of [α^{32}P]-dATP was 2.5 x 10^6 counts/min./μmole

Specific Activity of [α^{32}P]-dGTP was 2.1 x 10^6 counts/min./μmole

Specific Activity of [α^{32}P]-dCTP was 3.1 x 10^6 counts/min./μmole

Specific Activity of [α^{32}P]-dTTP was 3.5 x 10^6 counts/min./μmole

FIGURE 16

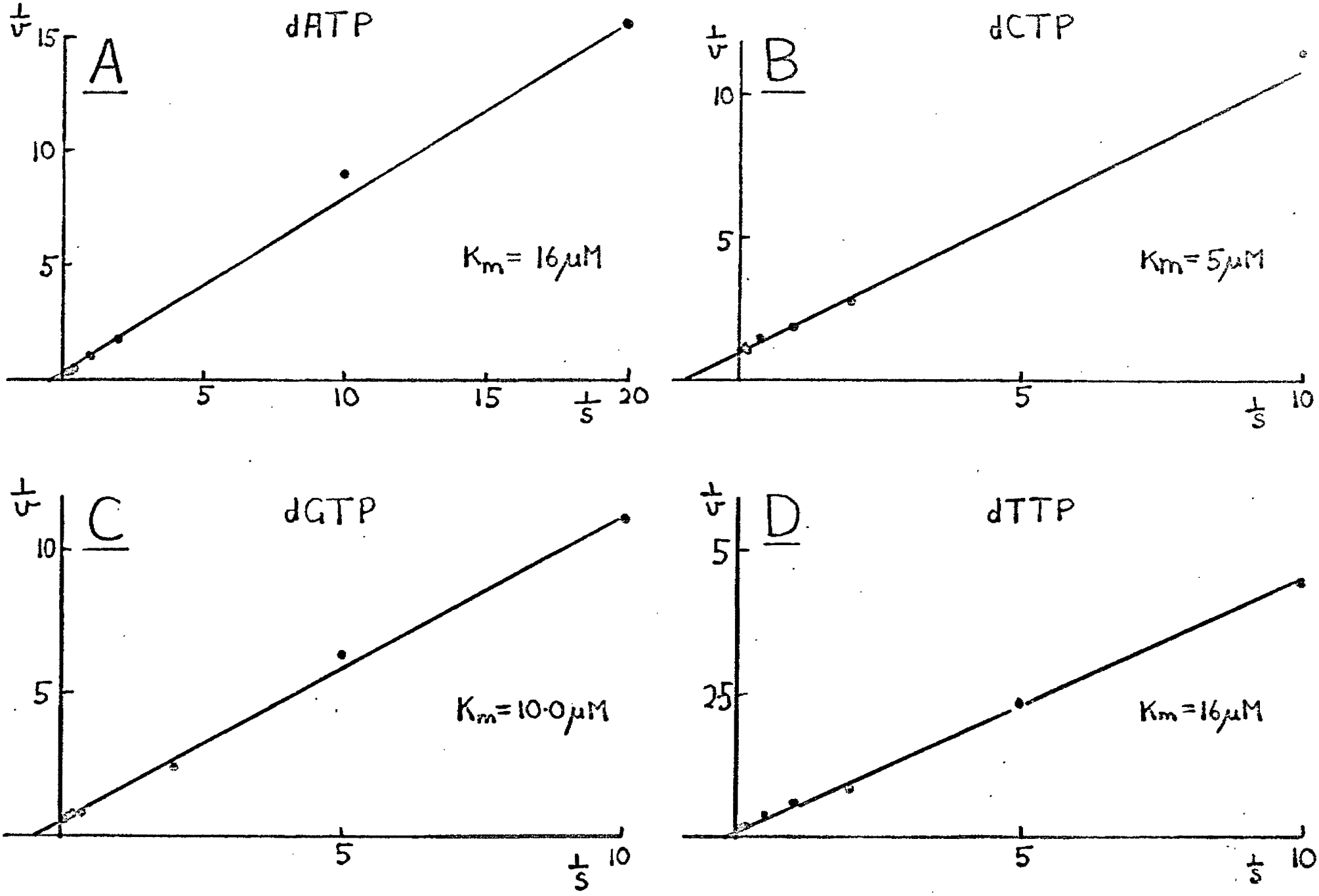

A
dATP
$\frac{1}{v}$
15
10
5
K_m = 16 μM
5
10
15
20
$\frac{1}{S}$
B
dCTP
$\frac{1}{v}$
10
5
K_m = 5 μM
5
10
$\frac{1}{S}$
C
dGTP
$\frac{1}{v}$
10
5
K_m = 10·0 μM
5
10
$\frac{1}{S}$
D
dTTP
$\frac{1}{v}$
5
25
K_m = 16 μM
5
10
$\frac{1}{S}$

FIGURE 17

Effect of the other deoxyribonucleoside triphosphates on the Kinetics of the incorporation of dTTP

The standard assay was performed with the exception of the deoxyribonucleoside triphosphate concentrations. There was 172 µg. of 20-45% ammonium sulphate fraction of enzyme per assay. Incubations were for 45 min.

The specific activity of $[\alpha^{32}P]$-dTTP was 3.6×10^6 counts/min./µmole

v, the enzyme velocity is expressed in mµmoles of dTMP incorporated/mg. of protein/45 min.

s, the substrate concentration is expressed in mµmoles/assay of each triphosphate whose concentration is varying (and equal to mµmoles of dTTP/assay).

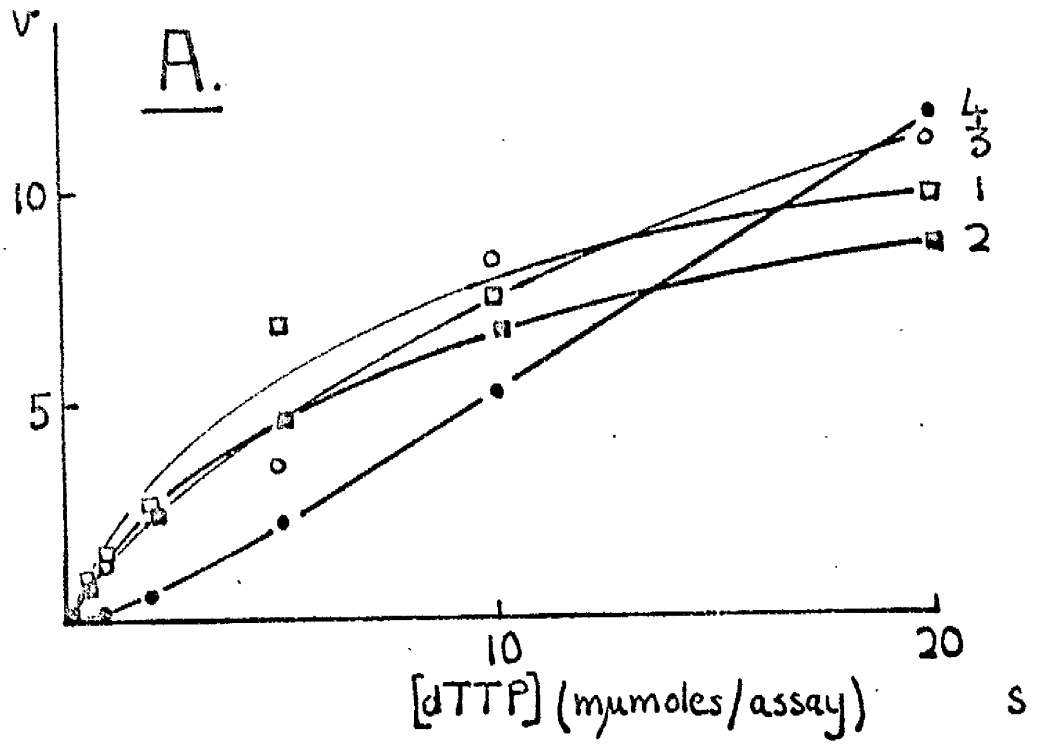
—□— dTTP alone

—⊟— dTTP and dATP

—○— dTTP, dATP and dCTP

—●— dTTP, dATP, dCTP and dGTP.

FIGURE 17


A.
V
10
5
10
20
[dTTP] (mμmoles/assay)
S
4
3
1
2

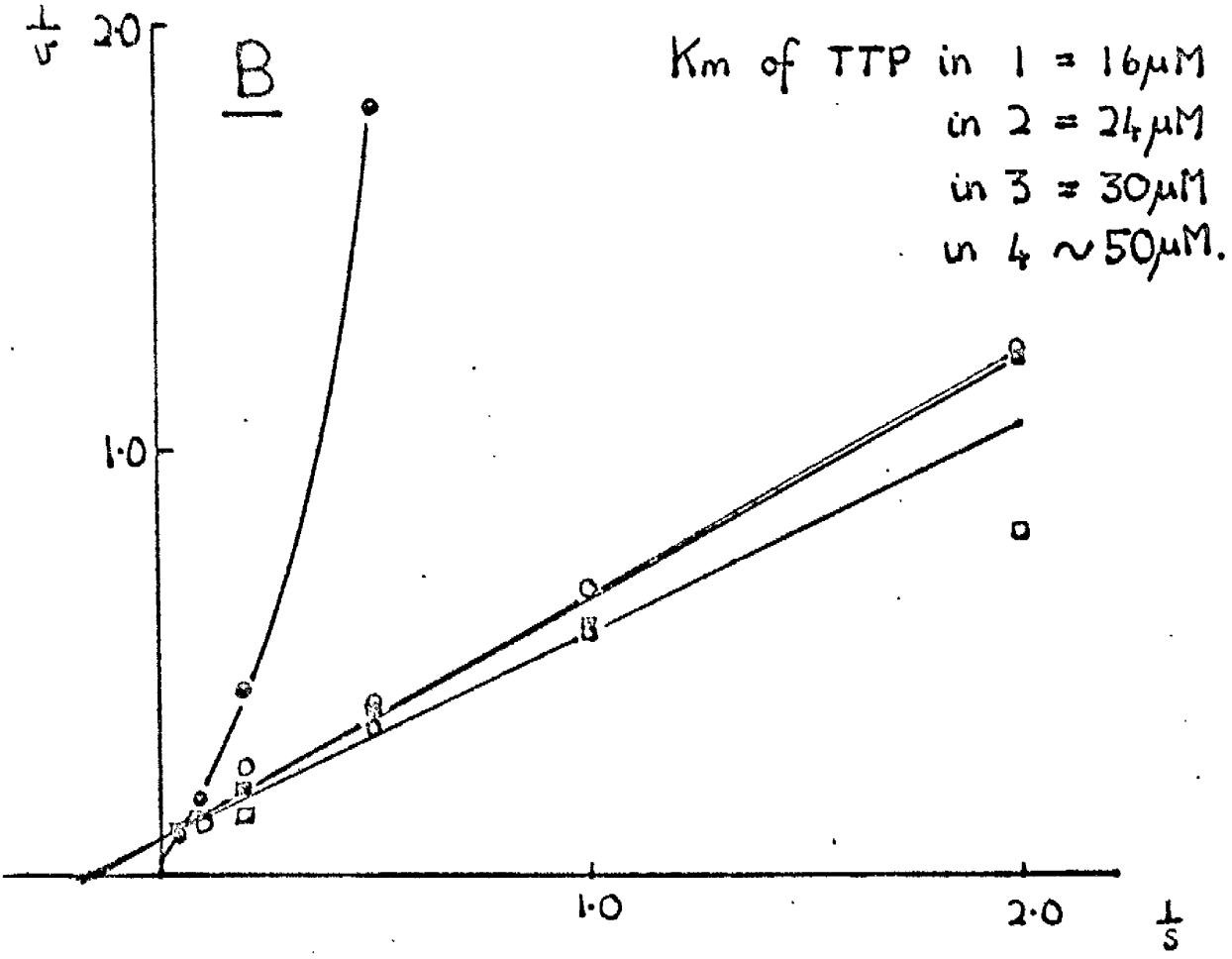

B
1/V
2·0
1·0
1·0
2·0
1/S
Km of TTP in 1 = 16μM
in 2 = 24μM
in 3 = 30μM
in 4 ~ 50μM.

FIGURE 18

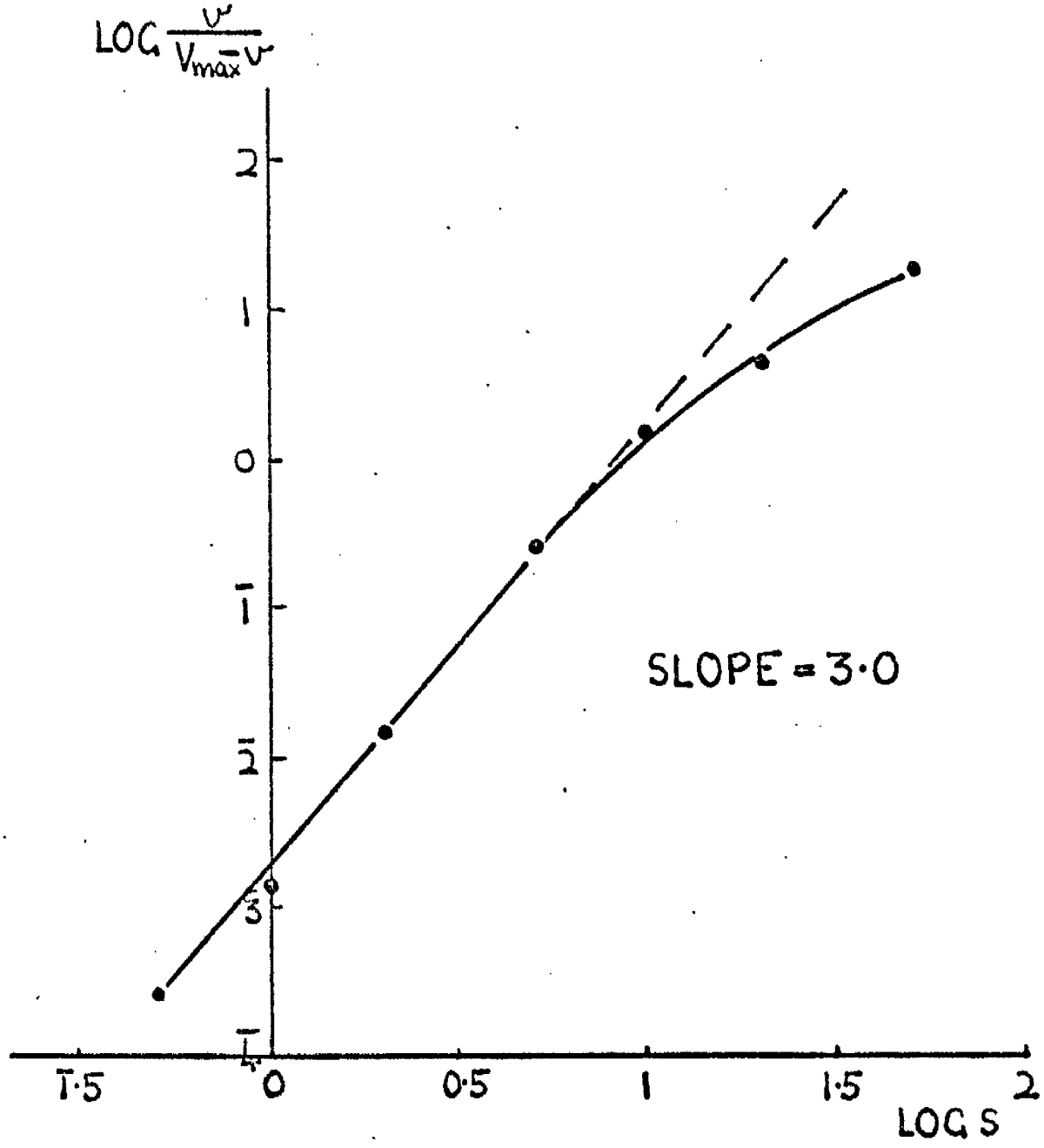
Hill Plot of the concentration curve of all four deoxyribonucleoside triphosphates together

The results of the concentration curve of all 4 triphosphates together (see Fig. 17) are replotted as Log $\frac{v}{V_{max}-v}$ against Log s. V_{max} was determined from the Lineweaver-Burk plot in Fig. 17B.

v, the reaction velocity is expressed as mμmoles of dTMP incorporated/mg. of protein/45 min.

s, the substrate concentration is expressed as mμmoles/assay of each of the four triphosphates present at equal concentrations.

FIGURE 18



III B DNA concentration curve

Plots of reaction velocity (v) against DNA substrate concentration (s), of $^1/v$ against $^1/s$, and Hill plots are shown in Fig. 19. Two enzyme preparations were used, a Sephadex purified fraction and an $(NH_4)_2SO_4$ precipitate fraction. Both fractions give similar optimum DNA concentrations of 40-80µg. DNA/assay, are inhibited at higher DNA concentrations (see Fig.29), and have sigmoid-shaped concentration curves. The Lineweaver-Burk plots are non-linear, the Sephadex purified enzyme showing this anomaly more markedly than the less purified enzyme. The Hill plots have gradients of 2.0 and 1.3 for the Sephadex and ammonium sulphate enzyme fractions respectively.

C Protein concentration curve

Protein concentration curves with and without BSA are shown in Fig.13. The non-linearity of the curve was found in all the Sephadex purified preparations. The reaction velocity, though non-linear, generally increased with protein concentration to at least 500µg. of protein/assay.

The implication of the finding that the specific activity of Sephadex purified DNA polymerase varies with the enzyme concentration is that results expressed in nucleotide incorporated/mg. protein are non-comparable, unless the actual protein concentration is also stated.

D Time curve of DNA polymerase

Under the standard assay conditions the time curve is linear to at least 60min., though at low protein concentrations the period of linearity is reduced. Fig. 20 shows that with the Sephadex purified enzyme the reaction is linear to 30 min. with 57µg. protein,

FIGURE 19

Denatured DNA kinetics with different DNA polymerase fractions

The standard DNA polymerase assays were carried out using various concentrations of denatured ascites-DNA as primer as indicated. The specific activity of [α^{32}P]-dTTP was 5.6 x 10^6 counts/min./umole. Incubations were for 1 hr. at 37°.

A & B are substrate concentration curves; C & D are the Lineweaver-Burk plots; E & F are the Hill plots, the V_{max} values having been calcuated from C.& D.

v, the reaction velocity is expressed as mμmoles of dTMP incorporated/mg. of protein/hr.

s, the substrate velocity is expressed as μg. DNA/assay

——o—— in A, C & E, 115 μg. of Sephadex purified DNA polmerase/assay

——o—— in B, D & F, 110 μg. of 20-45% ammonium sulphate fraction of DNA polymerase/assay

FIGURE 17

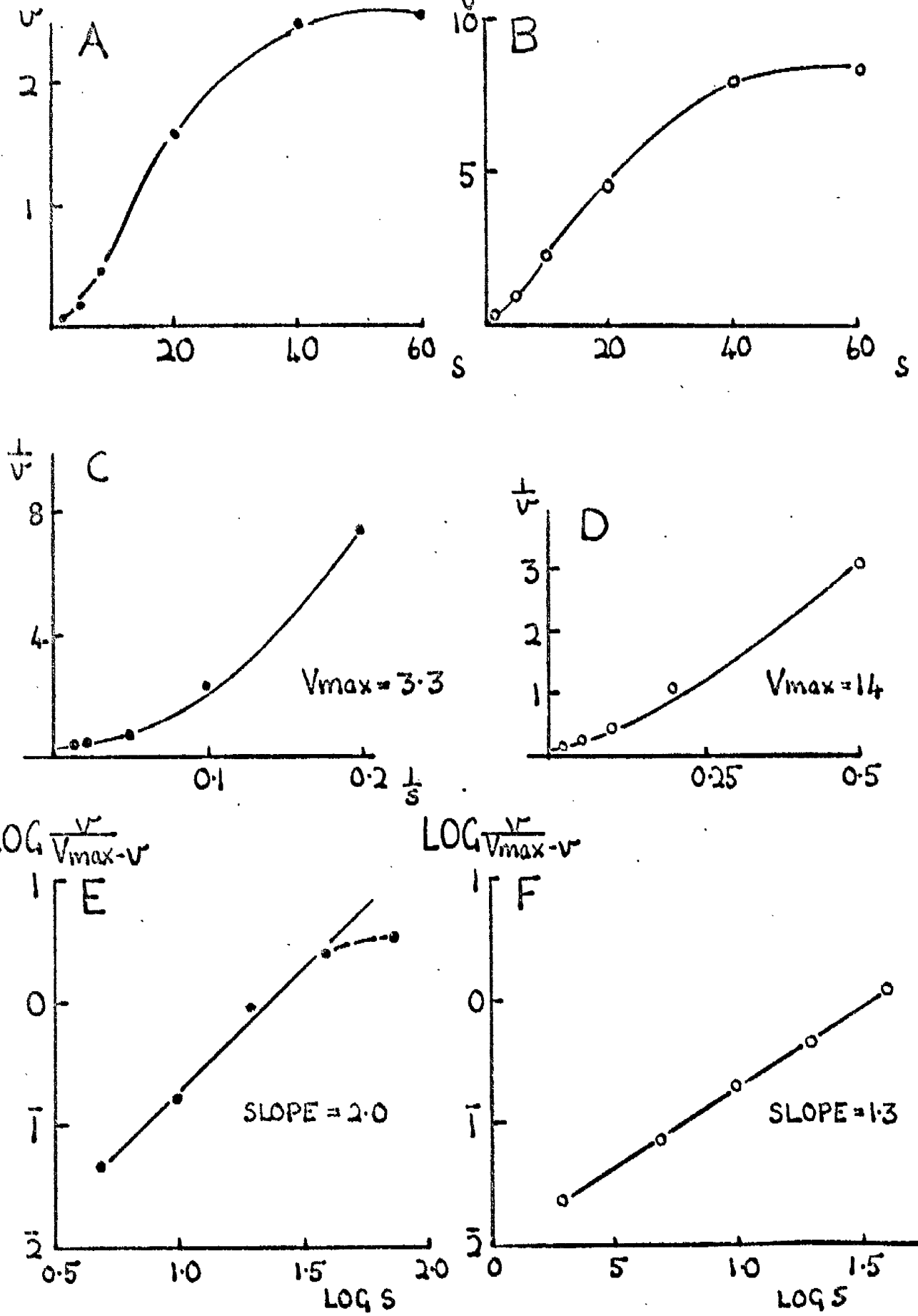

A
v
2
1
20
40
60
s
B
v
10
5
20
40
60
s
C
1/v
8
4
Vmax = 3·3
0·1
0·2
1/s
D
1/v
3
2
1
Vmax = 14
0·25
0·5
LOG v/(Vmax-v)
E
1
0
1̄
2̄
SLOPE = 2·0
0·5
1·0
1·5
2·0
LOG S
F
1
0
1̄
2̄
SLOPE = 1·3
0
5
1·0
1·5
LOG S

FIGURE 20

Time curves at different concentrations of DNA polymerase

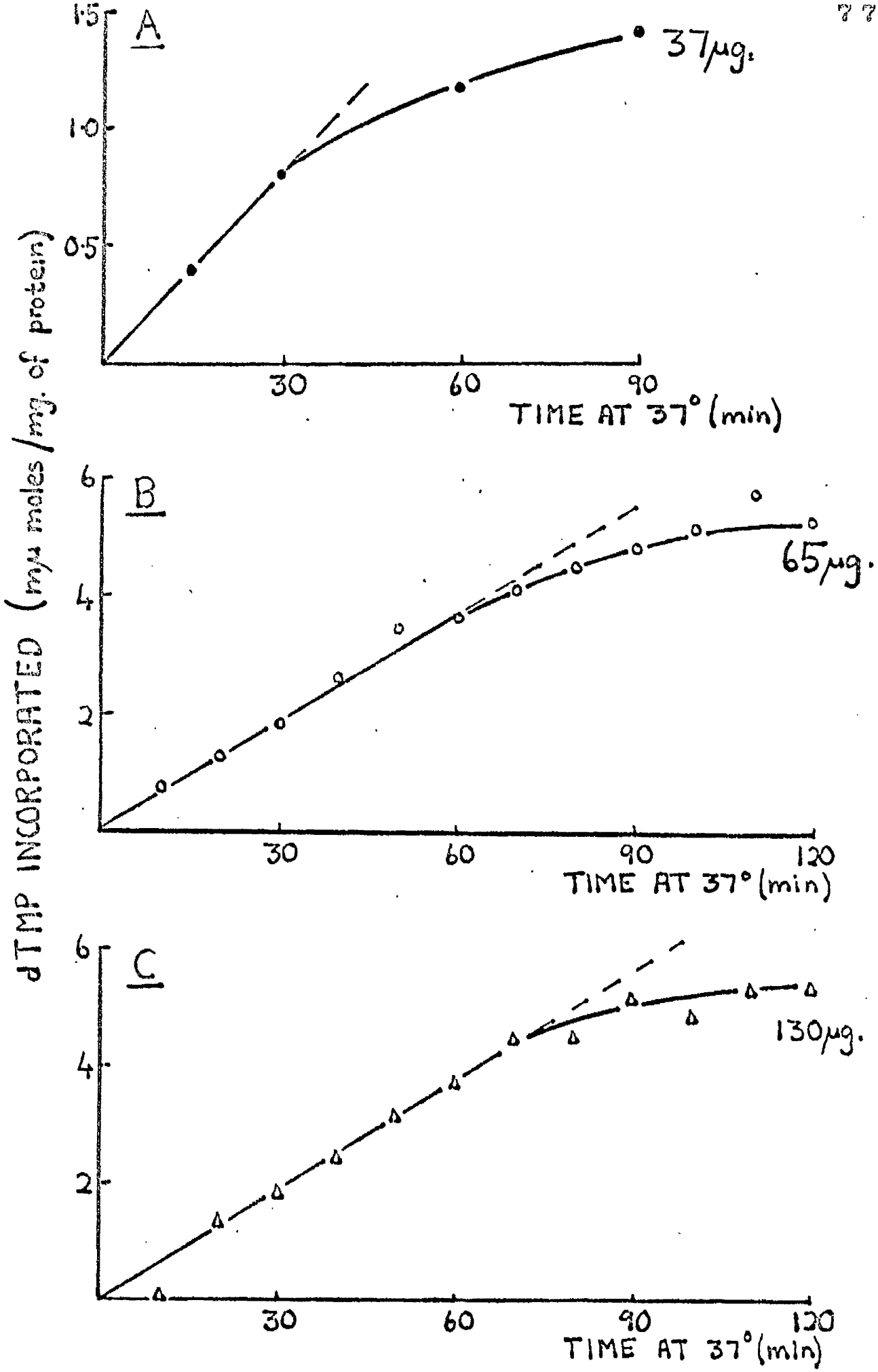
The standard assay was performed but it was scaled up 3-fold for B & C. 0.05 ml. portions were withdrawn at the indicated times of incubation at 37°. The specific activity of [α^{32}P]-dTTP was 4.3×10^6 counts/min./μmole in A, and 1.9×10^6 counts/min./μmole in B & C. The enzyme used was a Sephadex purified fraction

A 37 μg. of enzyme fraction protein/assay

B 65 μg. of enzyme fraction protein/equivalent standard assay

C 130 μg. of enzyme fraction protein/equivalent standard assay

FIGURE 20


A
37µg.
1·5
1·0
0·5
30
60
90
TIME AT 37° (min)
B
65µg.
6
4
2
30
60
90
120
TIME AT 37° (min)
C
130µg.
6
4
2
30
60
90
120
TIME AT 37° (min)
dTMP INCORPORATED (mµ moles / mg. of protein)

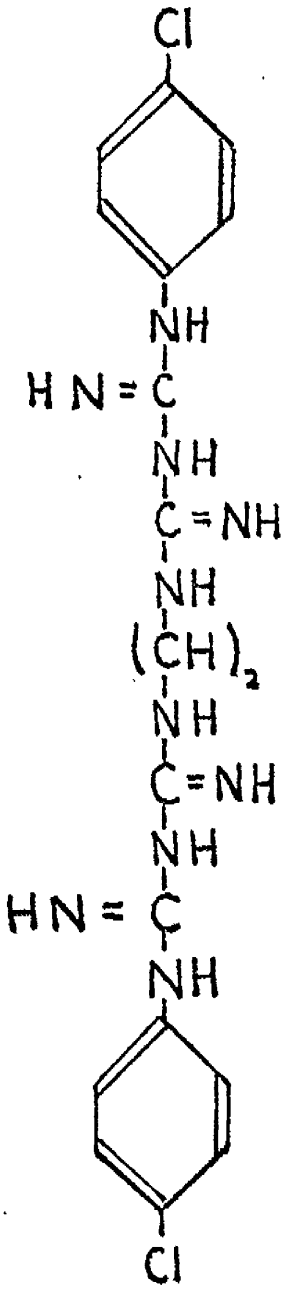
to 60min. with 65µg. protein, and to 70 min. with 130µg. protein. This shortened period of linearity of reaction with time with low protein concentration does not invalidate the protein concentration results of the previous section since sigmoid protein concentration curves were also found for 15min. reactions. At low DNA concentrations the time curve is linear to 60 min.

Over longer periods of incubation there is net synthesis up to 4hr., there being only a doubling of incorporation at 4hr. over that at 1hr. However this can be increased by adding more enzyme and triphosphates, and maintaining the other reaction components at their correct concentrations without adding more DNA. By adding these at half hourly intervals there was net synthesis to at least 5hr. and a trebling of the incorporation at 1hr. Under these conditions up to 30% replication of the primer was achieved.

E Effect of hibitane on DNA polymerase

On one occasion hibitane, bis-(p-chlorophenyldiguanido)-hexane digluconate, (Fig. 21) which was used as a bacteriostatic agent on the Sephadex G-150 column between runs, was incompletely removed causing great inactivation of the following batch of DNA polymerase. Inhibition studies were then performed, and Fig. 22 shows a hibitane concentration curve. Hibitane at 5µg/assay inhibited the reaction 12%. and at 100µg/assay about 98%. The hibitane probably reacted by complexing with the small molecules in the polymerase reaction. Solutions of hibitane at 20µg/ml. gave white precipitates when added to equal volumes of 0.8M-tris-HCl,pH75, to 1.0M-KCl, to 0.02M-EDTA, and to 0.5M-$MgSO_4$.

FIGURE 21



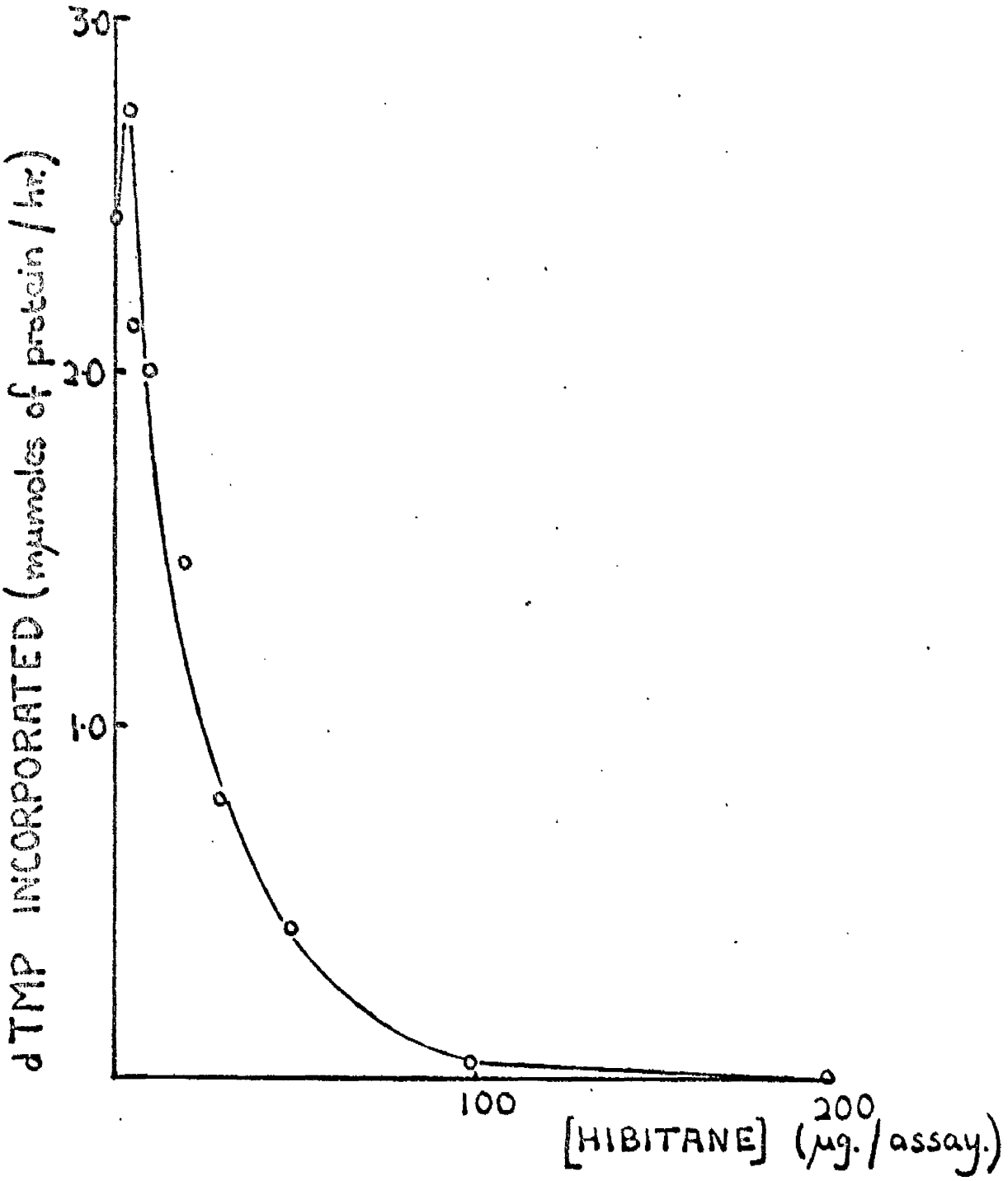
HIBITANE

FIGURE 22

Inhibition of DNA polymerase by hibitane

Varying amounts of hibitane were added to each standard assay. Incubations were at 37° for 1 hr. The specific activity of [α^{32}P]-dTTP was 2.73 x 10^6 counts/min./μmole. There was 195 μg. of Sephadex purified DNA polymerase/assay.

FIGURE 22


30
20
10
dTMP INCORPORATED (mμmols of protein / hr)
100
200
[HIBITANE] (μg./assay.)

IV Sedimentation of DNA polymerase, DNase and DNA on sucrose density gradients.

These experiments were performed with the purpose of investigating the possibility of specificity in binding of DNA polymerase to DNA.

The results refer to the sedimentation of a 20-45% ammonium sulphate precipitate of DNA polymerase and/or mammalian DNA in 5-25% sucrose gradients (Methods Dii a).

IV A The effect of the composition of the sucrose solvent buffer on sedimentation of polymerase and DNA

(i) Sucrose dissolved in distilled water.

The results are shown in Fig. 23. When polymerase is sedimented alone it forms a sharp peak in the top third of the gradient (Fig.23A). Native ascites-DNA when spun alone remains even higher up the tube (Fig. 23B). But when polymerase and DNA are sedimented together, (Fig.23C), both their sedimentation patterns are greatly altered. The DNA forms two main peaks, the smaller coincident with the peak formed by DNA when spun alone, suggesting that it is unbound. The main peak is further down. The polymerase forms two peaks, the upper one being coincident with the polymerase when spun alone, and the lower peak is coincident with the main peak of the DNA suggesting possible binding. Fig. 24 shows the results of the same runs performed replacing native DNA with denatured. Denatured DNA alone barely sediments down the tube, Fig. 24B, but in the presence of polymerase it forms two main peaks (Fig. 24C). The polymerase spun in the presence of denatured DNA sediments as one peak, but a little further than in the absence of DNA.

FIGURE 23

Sedimentation of DNA polymerase, native DNA and DNA polymerase + DNA, in sucrose (dissolved in water) density gradients

0.4 ml. of enzyme and/or DNA were layered on top of 5-25% sucrose gradients which were then spun at 50,000 rev./min. in the Spinco Model L ultracentrifuge in the SW50 rotor for 3 hr. (Methods II D ii a). The gradients were harvested after passage through the Spectronic 505 spectrophotometer where the E_{260} was recorded and approximately 30 fractions (0.17 ml.) were collected. The normal polymerase assay components (including denatured DNA) were added and the standard assay performed. The 0.4 ml. samples applied to the gradient contained:

A 0.7 mg. of a pH 5 ppt. fraction of DNA polymerase

B 0.3 mg. of native ascites-DNA

C 0.7 mg. of a 20-45% $(NH_4)_2SO_4$ ppt. of polymerase and 0.3 mg. of native ascites-DNA

The specific acitvity of $[\alpha^{32}P]$-dTTP was 8.7×10^6 counts/min./μmole in A, and 9.5×10^6 counts/min./μmole in C.

—— ●—— DNA polymerase (counts/min. incorporated/hr.)

------- Native DNA (E_{260})

FIGURE 23

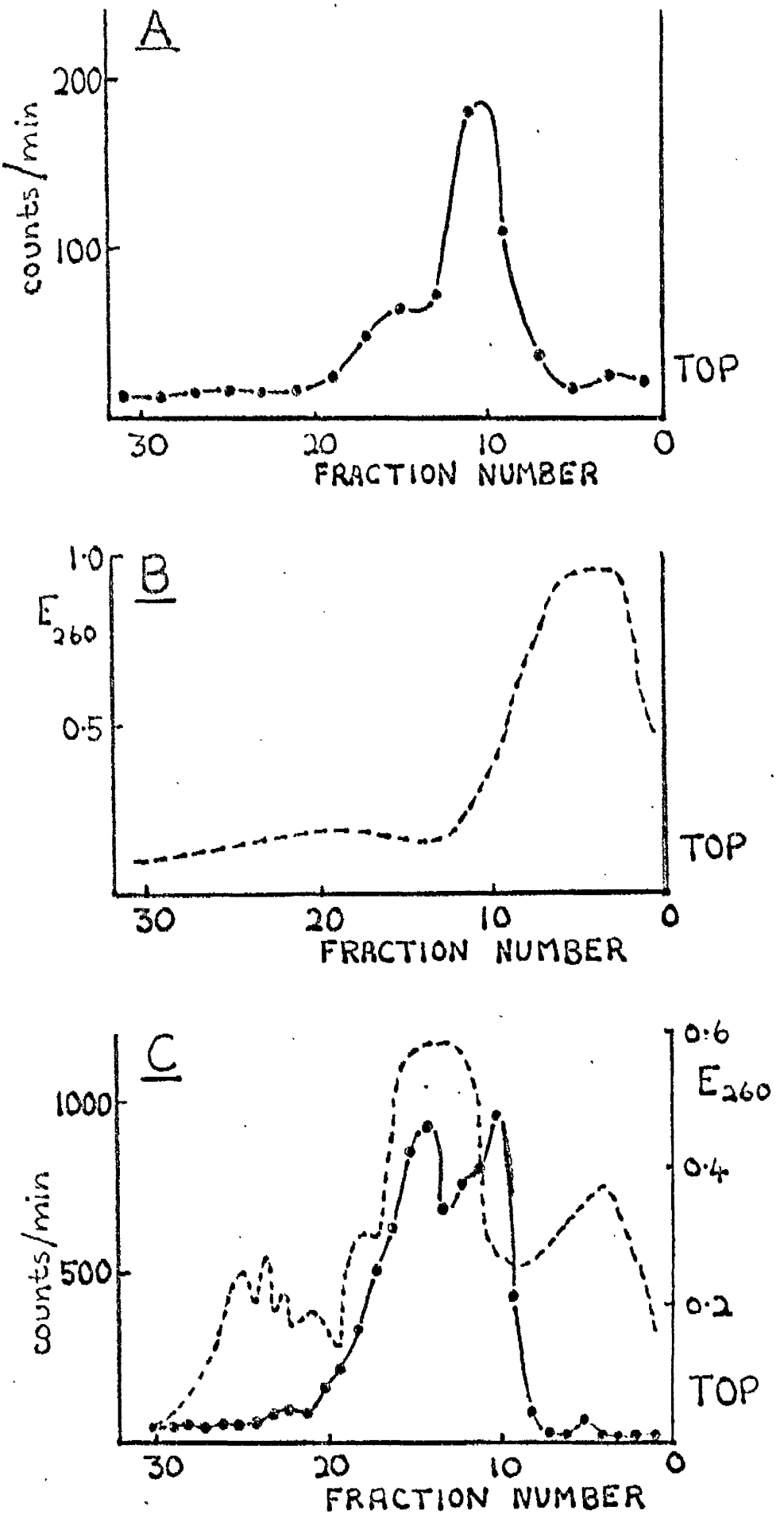

A
200
100
counts/min
TOP
30
20
10
0
FRACTION NUMBER
B
1·0
0·5
E_{260}
TOP
30
20
10
0
FRACTION NUMBER
C
1000
500
counts/min
0·6
0·4
0·2
E_{260}
TOP
30
20
10
0
FRACTION NUMBER

FIGURE 24

Sedimentation of DNA polymerase, denatured DNA, DNA polymerase + denatured DNA in sucrose (dissolved in water) density gradients

The method used was the same as that in Fig. 23, except that centrifugation was at 43,000 rev./min. for 3 hr.

The 0.4 ml. samples applied to the three gradients contained

A 0.7 mg. of 20-45% $(NH_4)_2SO_4$ ppt. fraction of DNA polymerase

B 0.3 mg. of denatured ascites-DNA

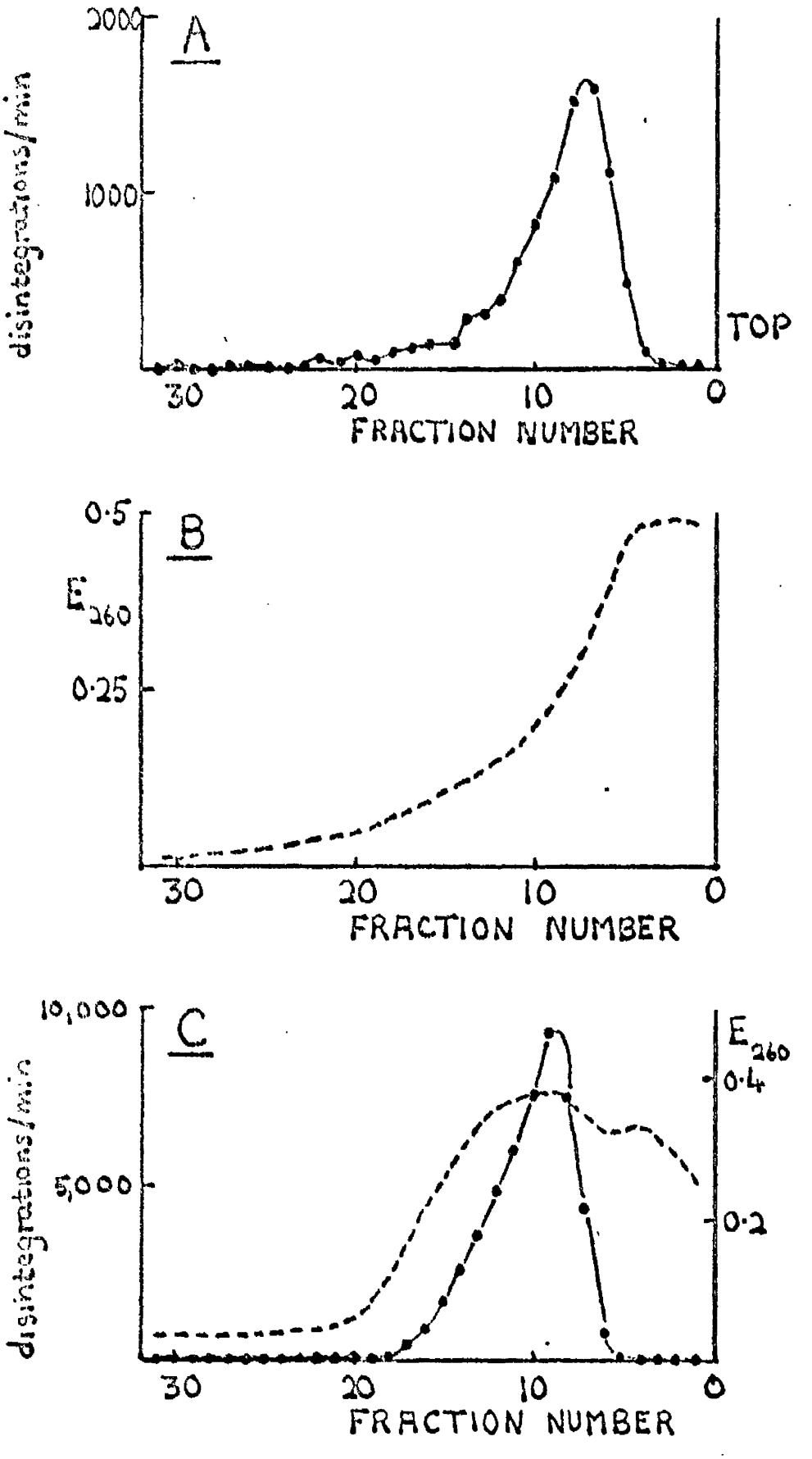
C 0.7 mg. of DNA polymerase + 0.3 mg. denatured DNA

The DNA polymerase in each fraction was measured by incorporation of $[^3H]$-dATP of specific activity 40.2 x 10^6 disintegrations/min./μmole and the radioactivity determined on the Nuclear-Chicago Liquid Scintillation spectrometer.

——⊙—— DNA polymerase (disintegrations/min./assay)

– – – – – – – DNA (E_{260})

FIGURE 24.


A
2000
1000
disintegrations/min
TOP
30
20
10
0
FRACTION NUMBER
B
0·5
E_{260}
0·25
30
20
10
0
FRACTION NUMBER
C
10,000
5000
disintegrations/min
E_{260}
0·4
0·2
30
20
10
0
FRACTION NUMBER

IV A (ii) Sucrose dissolved in various buffered solutions.

Native DNA spun alone in sucrose dissolved in 0.02M-tris-HCl, pH7.5 in anything from Buffers 1-5 gives the pattern shown in Fig.25A. In the presence of polymerase the DNA pattern is changed to that in Fig. 25B. The polymerase patterns in the presence of native DNA in sucrose solvents ranging from water to Buffer 5 are shown in Fig.26. When sedimented in sucrose dissolved in Buffers 2 and 3 there is an apparent loss of polymerase activity in the gradient which can be recovered in a gelatinous, sedimented pellet, though quantitative recovery is technically difficult to ascertain. The small peak that does occur, however, is coincident with the lower peak of polymerase in the presence of DNA in sucrose dissolved in water. This possibly corresponds to a polymerase-DNA complex. The polymerase peak in Buffers 4 and 5 is apparently unbound. The apparent loss of DNA when spun in the presence of polymerase in buffered sucrose is explicable by its sedimentation to the bottom.

The percentage recoveries of DNA polymerase from the gradients in the presence of DNA, in the various sucrose solvents are shown in Table 4. The results are expressed as a percentage of polymerase activity recovered in the sucrose-water solution. If the actual denaturation of polymerase is the same in each case, these results express the percentage of polymerase which has not sedimented to the pellet.

FIGURE 25

The effect of DNA polymerase on the sedimentation of DNA in sucrose (dissolved in 0.02 M-tris-HCl, pH 7.5, 5 m M-2-mercaptoethanol) density gradients

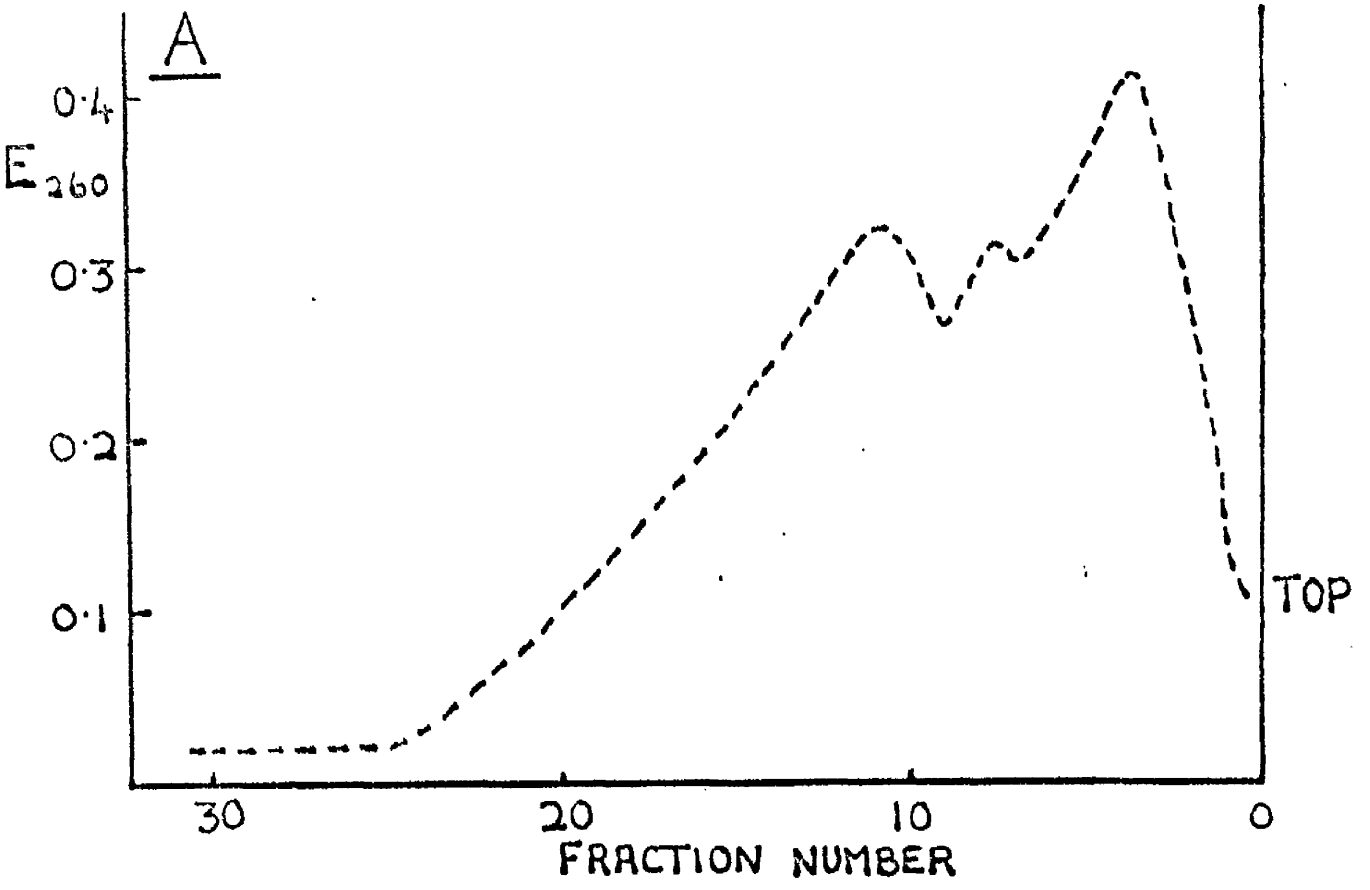
Two sucrose density gradients were performed as in Fig. 23 with sucrose dissolved in Buffer 2. The 0.4 ml. samples applied to the gradient contained:

A 0.3 mg. of native ascites-DNA

B 0.3 mg. of native ascites-DNA + 0.7 mg. of DNA polymerase fraction (ammonium sulphate fraction)

Copies of the E_{260} traces from the Spectronic 505 spectrophotometer are shown.

FIGURE 25


A
0·4
E_{260}
0·3
0·2
0·1
TOP
30
20
10
0
FRACTION NUMBER

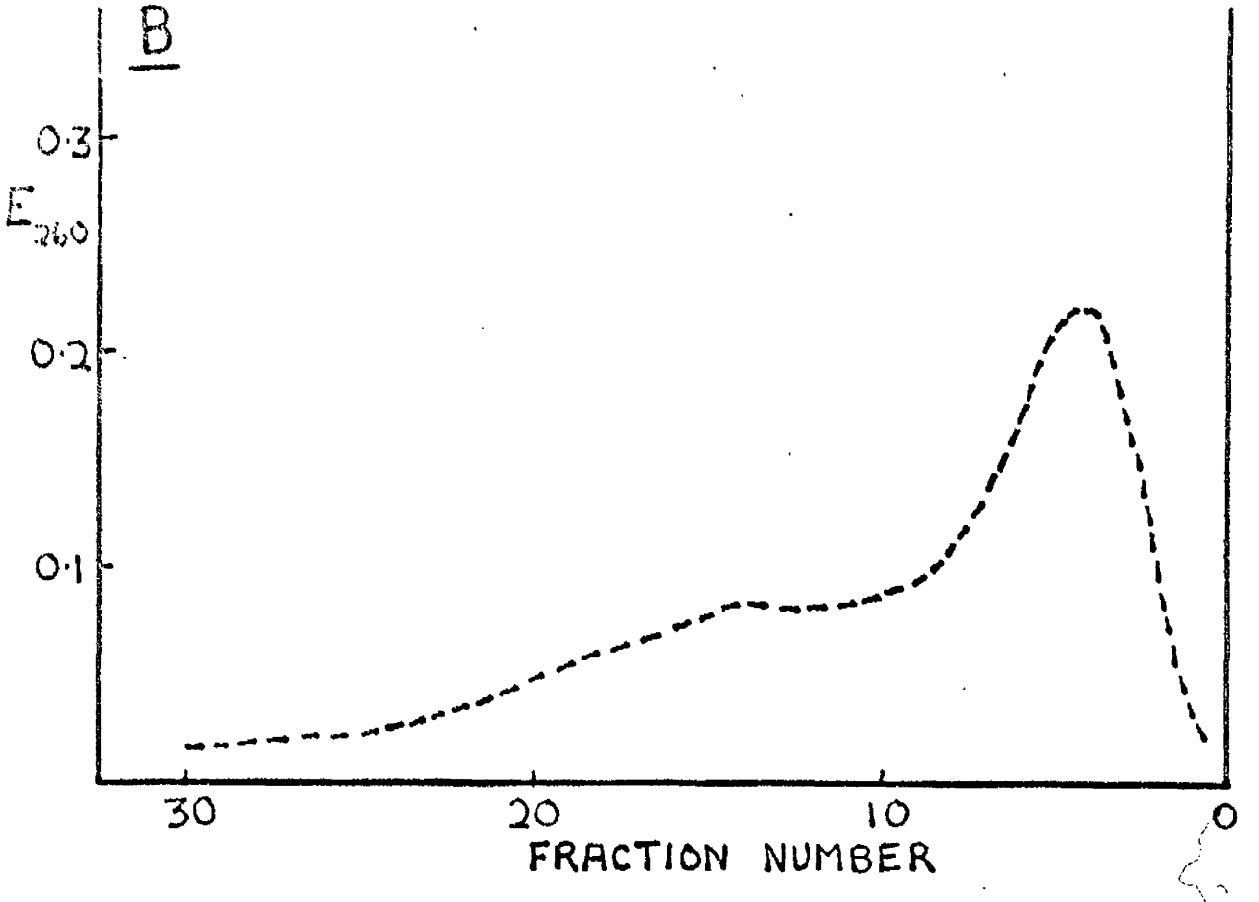

B
0·3
E_{260}
0·2
0·1
30
20
10
0
FRACTION NUMBER

FIGURE 26

The effect of differences in the buffer composition of the sucrose solvent on the sedimentation of DNA polymerase in the presence of native DNA

0.4 ml. samples, containing 0.7 mg. of ammonium sulphate fraction of DNA polymerase and 0.3 mg. of native ascites-DNA, were layered onto each of 5 sucrose gradients, the sucrose for which was dissolved in water, and Buffers 2-5 as indicated. All fractions were assayed for DNA polymerase activity under the same conditions and denatured DNA was always added as primer. The [α^{32}P]-dTTP differed in specific activity and was 9.5×10^6 counts/min./µmole for water and Buffer 4A, 9.0×10^6 counts/min./µmole for Buffers 2 and 3, and 3.3×10^6 counts/min./umole for Buffer 5.

FIGURE 26

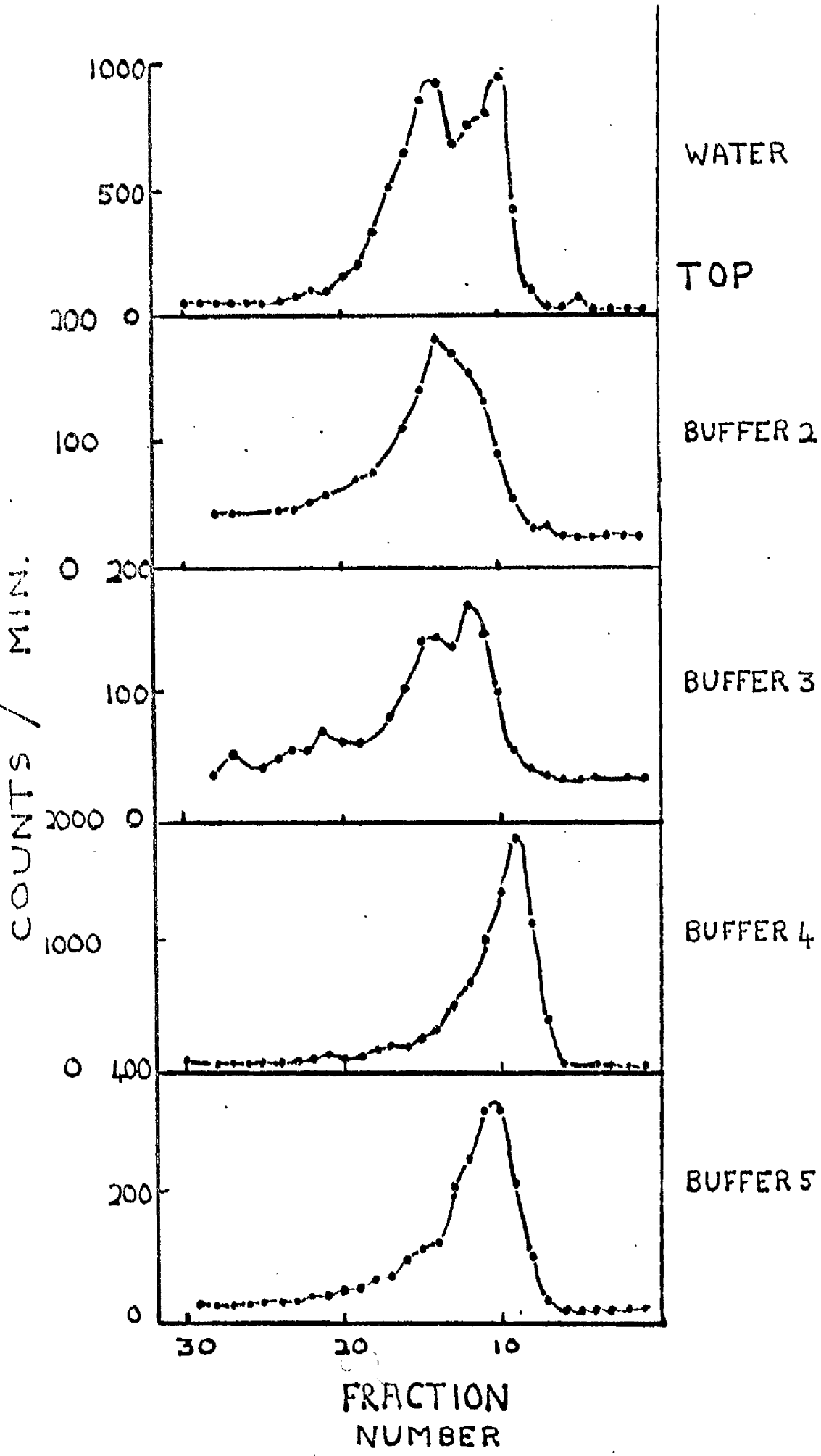

1000
500
200
0
WATER
TOP
100
0
200
BUFFER 2
100
0
200
BUFFER 3
2000
1000
0
400
BUFFER 4
200
0
BUFFER 5
COUNTS / MIN.
30
20
10
FRACTION NUMBER

TABLE 4

Percentage DNA Polymerase remaining in sucrose density gradients in the presence of native DNA, in different sucrose solvents

Sucrose solvent	% DNA Polymerase activity (water = 100)
Water	100
Buffer 1	29
Buffer 2	28
Buffer 3	43
Buffer 4A	68
Buffer 5	75

5-25% sucrose gradients dissolved in water or buffer as indicated were used for sedimentation of native DNA with DNA polymerase as described in Fig. 23. After harvesting the gradients the fractions were assayed for DNA polymerase under the standard conditions and the total polymerase units/gradient were expressed as a percent of those recovered when water was the solvent.

IV B Sedimentation of DNase

The DNA polymerase pH5 precipitate fraction was used for these DNase studies. DNase when sedimented alone in a sucrose-water gradient gave a single peak (Fig.27A), and in the presence of native DNA gave two peaks (Fig.27B), the smaller of which was coincident with the previously mentioned DNase peak. The larger was apparently bound to DNA.

C Sedimentation coeffients of polymerase and DNase.

By the method of Martin and Ames (1961) the sedimentation coeffients of DNA polymerase and DNase were found by comparison with that of a known protein, haemoglobin, by sedimentation in a sucrose density gradient (Methods Diia).

The sedimentation coeffient of haemoglobin was calculated directly from centrifugation on the Spinco Model E Analytical Ultracentrifuge (Methods Di a) and is shown together with the results of the determination of $S_{20,w}$ of polymerase and DNase in Table 5. The results are the mean of three determinations.

V Investigation of the primer for DNA polymerase.

A Unprimed reaction

A polymerase assay incubation was performed with a Sephadex purified enzyme preparation in the absence of DNA primer. 190µg. of polymerase fraction protein were added in 0.05ml. to the tube at hourly intervals, and 190µg. of BSA added to a control tube. The results in Fig. 28 show there to be a very low level of incorporation - in fact of the order of 0.1% of that in the presence of 60µg. of denatured DNA primer. There was a lag phase for the first hour in which there was no significant difference between the control

FIGURE 27

Sedimentation of DNase in the presence or absence of native DNA in sucrose (dissolved in water) density gradients

The DNase preparation was a pH 5 ppt. of DNA polymerase. The gradients were merely a repeat of those performed for DNA polymerase (Figs. 23A and C). The gradients were harvested into 18 fractions and the DNase assay components, including 10 µg. of denatured Esch. coli [^{32}P]-DNA added.

A 1.4 mg. of pH 5 ppt. protein was applied to the gradient.

B 0.7 mg. of pH 5 ppt. protein + 0.3 mg. of native DNA

FIGURE 27

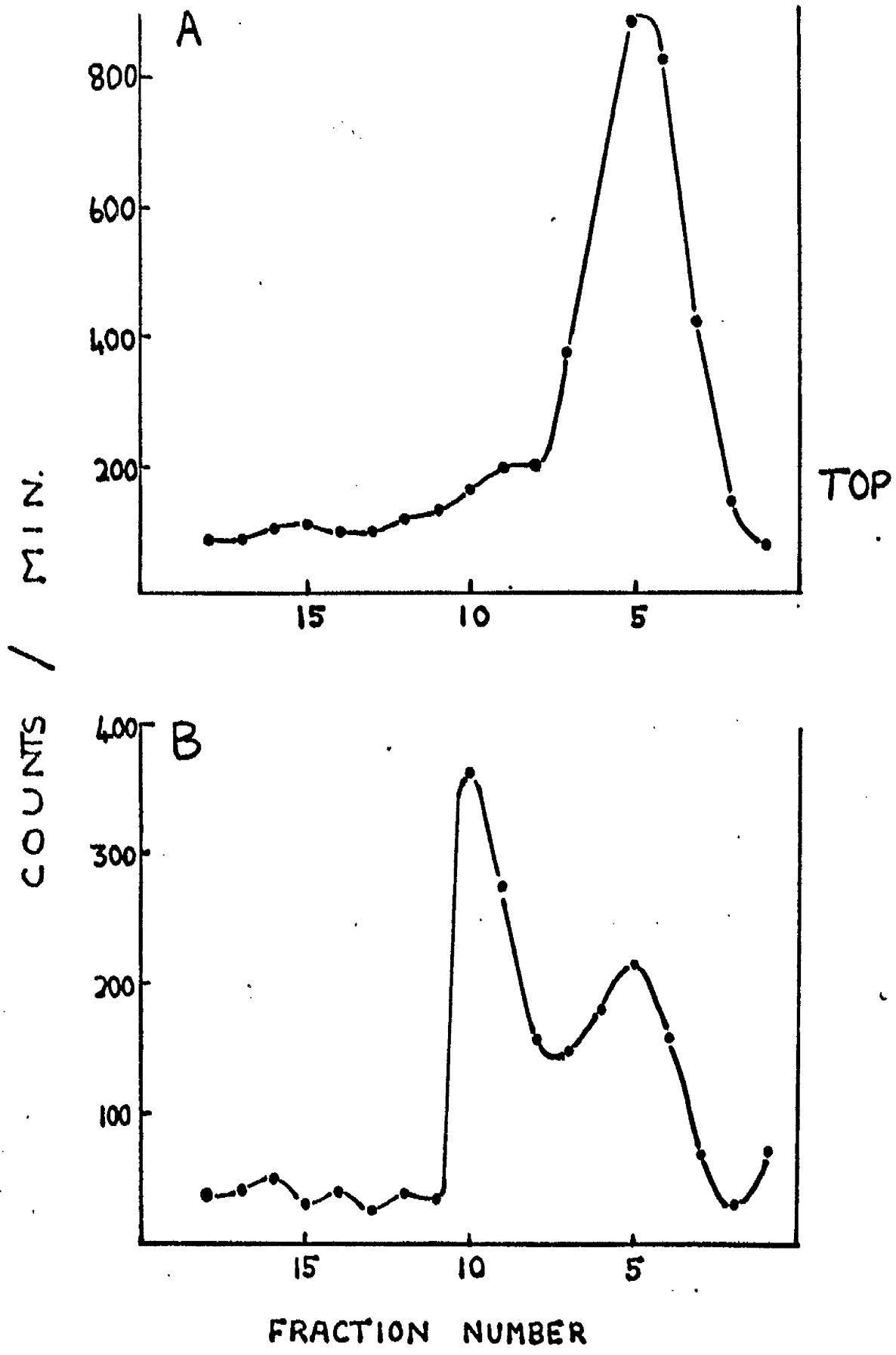

A
800
600
400
200
MIN.
TOP
15
10
5
B
400
300
200
100
COUNTS / MIN.
15
10
5
FRACTION NUMBER

TABLE 5

Sedimentation coefficients and molecular weights of DNA polymerase and DNase

	$S_{20,w}$	Molecular weight (approx.)
Haemoglobin (standard)	3.97	64,500
DNA polymerase	7.1	154,000
DNase	4.3	73,000

FIGURE 28

DNA polymerase activity in the absence of primer

Two polymerase assays, scaled up 2-fold, with no DNA primer and 35 mμmoles of [α^{32}P]-dTTP (13.3 x 10^5 counts/min./μmole) were set up. The test assay contained 370 μg. of Sephadex purified enzyme, and the control 310 μg. of BSA. 0.05 ml. samples were withdrawn at the times indicated and 155 μg. of enzyme added to the test and 155 μg. of BSA added to the control, dissolved in 0.05 ml. of Buffer 4A, at hourly intervals as indicated by the arrows. The results are corrected for the dilutions.

—Δ— Test

—▲— Control

FIGURE 29

Comparison between priming abilities of native and denatured DNA

DNA concentration curves were performed with native and denatured DNA which had been further purified (Methods II A i). The enzyme was a Sephadex purified fraction (357 μg./assay). The specific activity of [α^{32}P]-dTTP was 5.1 x 10^6 counts/min./μmole.

—⊙— Denatured DNA

—○— Native DNA

FIGURE 28

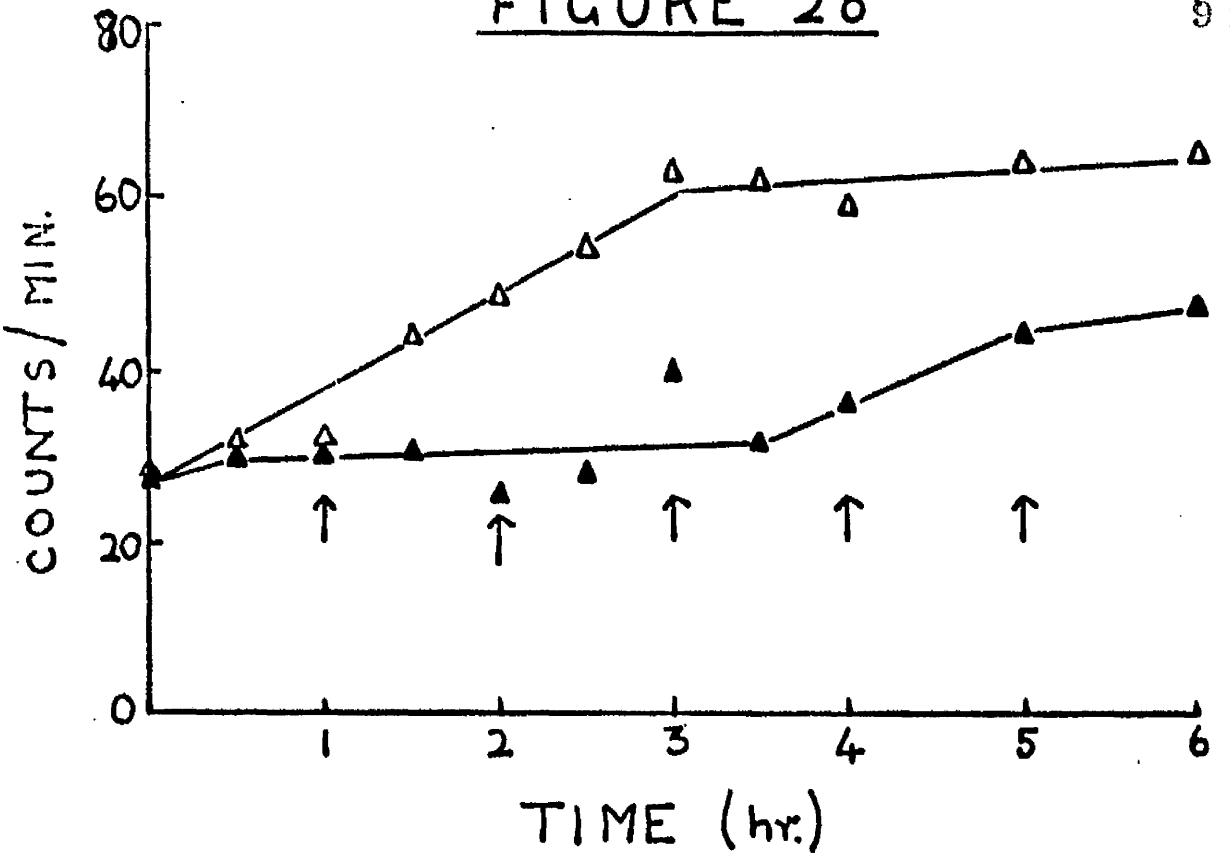
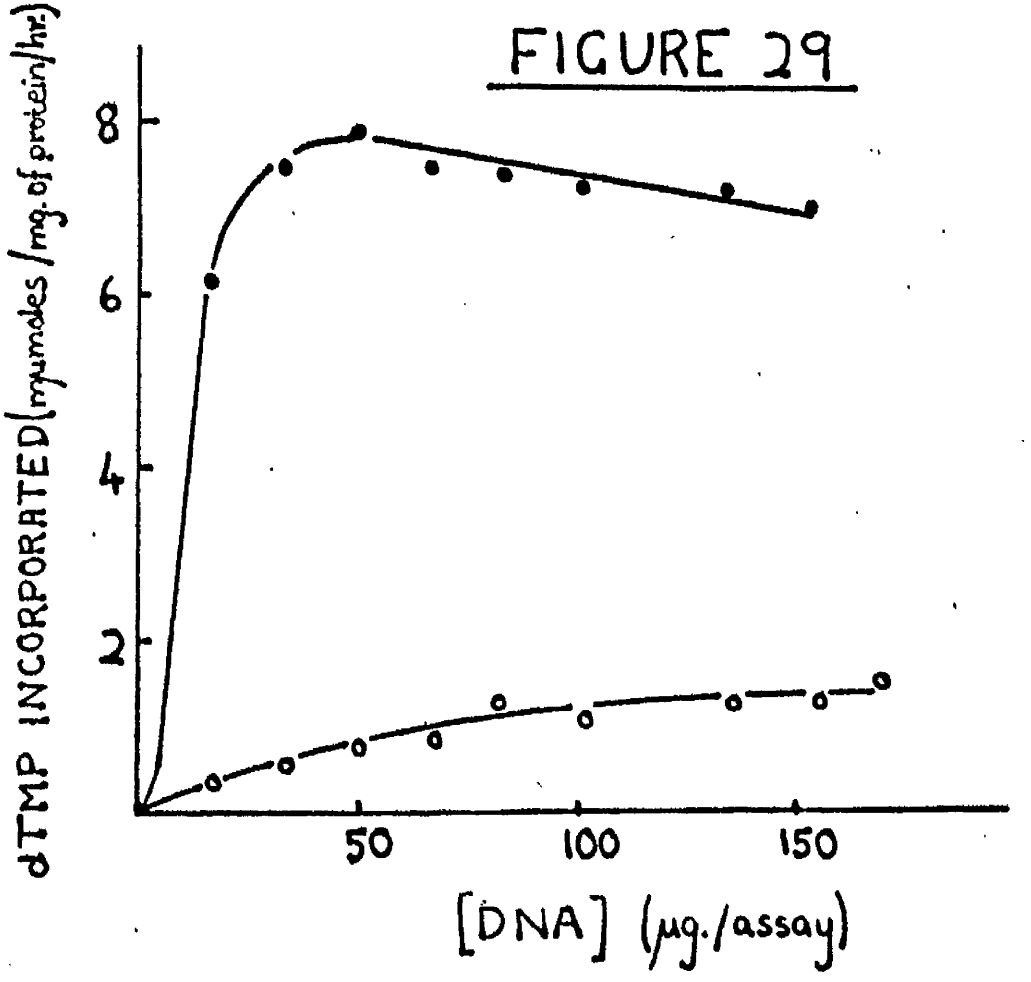


FIGURE 29



and test samples. Unprimed incorporation was maximal at 3-4hr.

V B Comparison of priming by native and denatured DNA.

Fig. 29 shows the difference between the native and denatured DNA substrate concentration curves. The DNA used was further purified as described in Methods IIA i). Denatured DNA has an optimum concentration at about 50µg./assay and then falls off slightly (the usual sigmoid-shape of the curve is not evident when points at lower DNA concentrations are omitted). Native DNA however in the range used, is much less effective and does not reach an optimum concentration. At 50µg./assay native DNA primes at ~10%, and at 150µg./assay ~20% of denatured DNA.

C Effect of Time of denaturation, and DNA concentration during denaturation on its priming ability.

0.2ml. portions of DNA were heated separately at 100° for various precise times, then rapidly cooled in ice, and centrifuged to spin down the condensation on the sides of the tube. The other polymerase assay components and enzyme were added and the priming ability of the DNA assayed. Fig. 30 shows time of denaturation plotted against enzyme velocity at three different DNA concentrations. They are all alike in their optimum time of 0.5-1.5 min. at 100°, though they differ after longer at 100°. In Fig. 30A with DNA at 0.1 mg./ml. the priming ability falls to about 60% of the optimum after 10min. In Fig. 30B with DNA at 1.0 mg./ml. it falls to about 80%, and in Fig. 30C with DNA at 1.5 mg./ml. it falls to about 90%.

FIGURE 30

Effect of concentration of DNA while undergoing heat denaturation, and of the time at 100° of DNA, on its priming ability

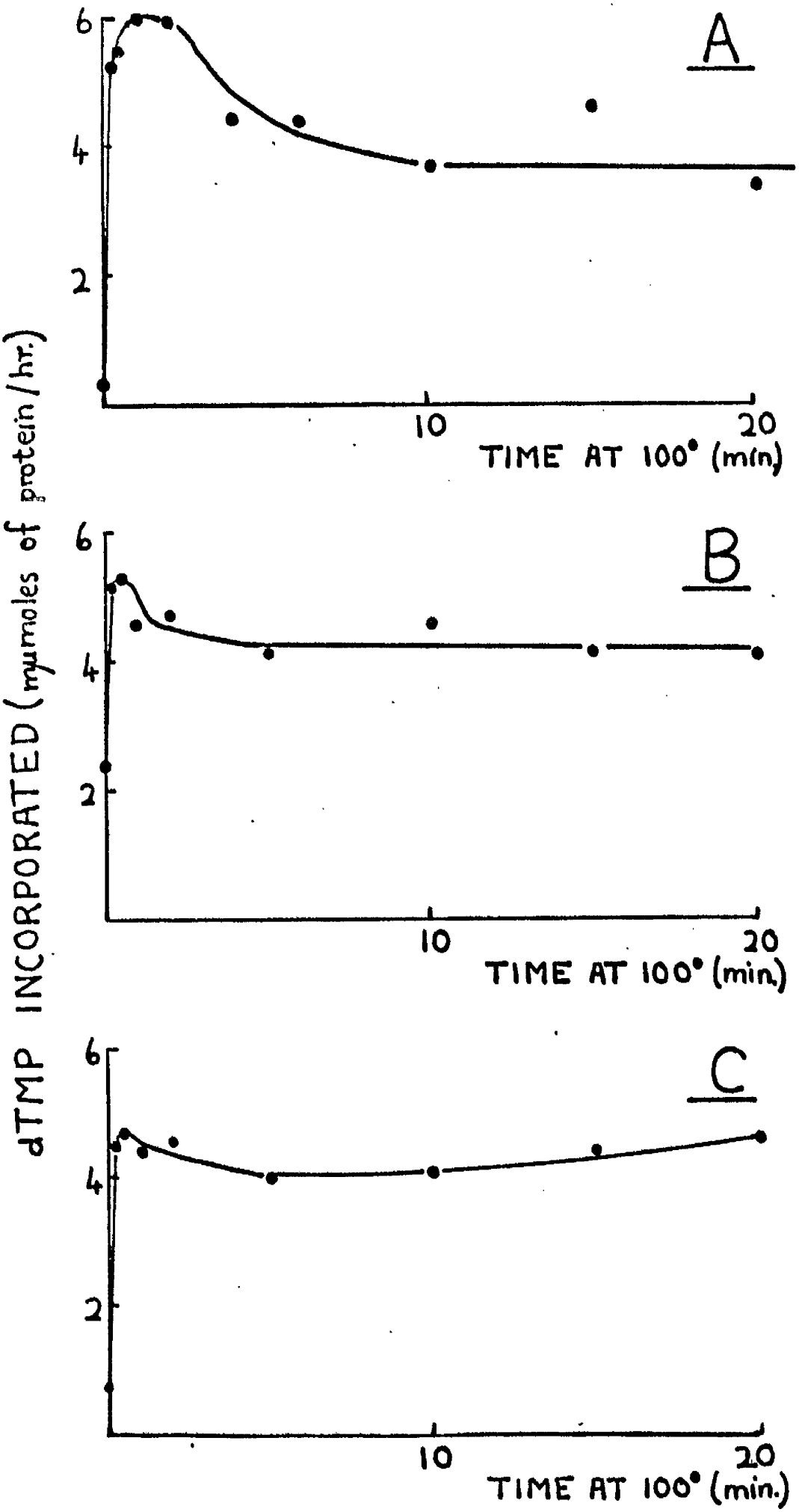
0.2 ml. portions of native ascites-DNA were heated in small centrifuge tubes at 100° for various times (as indicated) and were stoppered after the first 20 sec. to minimise evaporation. They were then plunged into an ice/water bath, and centrifuged to spin down any condensed steam on their inside walls. DNA polymerase (0.145 mg./assay) and the other assay components were added to the appropriate volume of DNA and incubations were performed for 1 hr.

A DNA present at 0.1 mg./ml. while denaturing, and the assays performed at 20 µg. of DNA/assay

B DNA was present at 1 mg./ml. while denaturing, and at 100 µg./assay

C DNA was present at 1.5 mg./ml. while denaturing and at 60 µg./assay

FIGURE 30


A
B
C
6
4
2
10
20
TIME AT 100° (min.)
dTMP INCORPORATED (mμmoles of protein / hr.)

VI Investigation of the product of DNA polymerase action.

A CsCl gradients

A DNA polymerase reaction was performed under conditions to promote maximum replication of the DNA and the reaction mixture was applied directly with added native and denatured DNA on top of a CsCl solution and subjected to centrifugation at 35,000g for 40hr. as described in Methods D iib. The primer and marker DNAs were salmon testes since this was more homogeneous and of higher molecular weight than the ascites DNA (see Discussion II). Fig. 31 shows the results in terms of E_{258} and counts/min. in the fractions. 30% of E_{258} is due to the primer and product DNA and the remainder to the added marker DNAs. There is only slight separation of the native and denatured DNA markers, the denatured sedimenting further down the tube. The radioactivity forms two peaks - one small one corresponding to denatured DNA and a longer broader peak probably corresponding to small heterogeneous acid precipitable oligonucleotides. The polymerase product before and after centrifugation was also analysed by DEAE-cellulose chromatography, as described in the following sections.

B Chromatography on sheets of DEAE cellulose.

This technique is described in Methods F i). Initially these results were complicated by the presence of glycerol in the polymerase reaction medium. Glycerol retarded the movement of non-incorporated deoxyribonucleoside triphosphate so that its R_F value was reduced from 0.84 to approx. 0.40 in the presence of 25% glycerol. A spot of glycerol applied on top of a sample already on the chromatogram had a similar effect.

FIGURE 31

CsCl density gradient centrifugation of the product of DNA polymerase

A standard DNA polymerase reaction mixture (with only 20 µg. of heat denatured salmon testes DNA) was incubated for 5 hr. at 37°. 0.2 ml. reaction mixture + 0.01 ml. each of native and denatured salmon testes DNA (20 µg. of each) were layered on top of 5 ml. of CsCl soln. (13.5 g of CsCl in 10 ml. of 0.08 M-tris-HCl, pH 7.5). This was subjected to centrifugation at 35,000 g for 40 hr. (Methods D ii b). The radioactivity and E_{258} of alternate fractions were determined.

——o—— E_{258}

– – – – – – – counts/min.

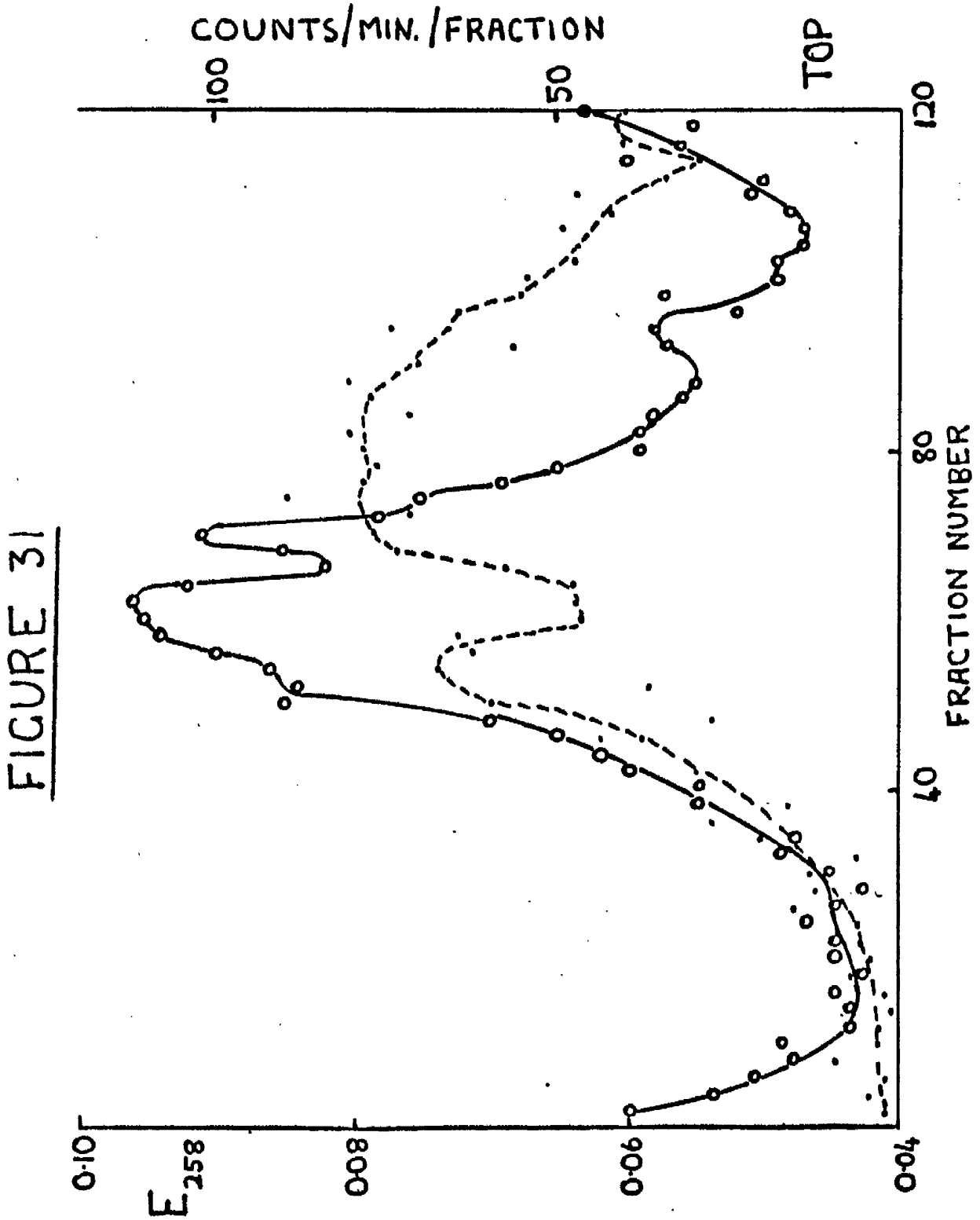

COUNTS/MIN./FRACTION
100
50
TOP
120
80
40
FRACTION NUMBER
0·10
0·08
0·06
0·04
E_{258}

FIGURE 31

The results of chromatography of the product of the DNA polymerase reaction after different incubation times run on DEAE-cellulose in 0.75M-NH_4HCO_3, pH8.6 are shown in Fig. 32. The zero time control shows only one peak of radioactivity - presumably of $[\alpha^{32}P]$ - dTTP although the R_F value is approximately 0.79-0.81, compared with the literature value of 0.67-0.70 (Furlong, 1967). However after incubation at 37° a peak appeared at the origin with R_F value 0.01-0.02 equivalent to high molecular weight DNA. The area of the peak was found to be directly proportional to the acid-insoluble radioactivity at the different incubation times. After incubation for 2hr. another broad peak was found at R_F = 0.30, suggestive of oligodeoxyribonucleotides. This was not apparent for incubations of 1hr. or less but was twice observed after 2hr. incubations.

There was no detectable triphosphatase activity under polymerase reaction conditions since the R_F value of the triphosphate peak remained unchanged (R_F = 0.80 at zerotime, R_F = 0.81 after 60min. at 37°).

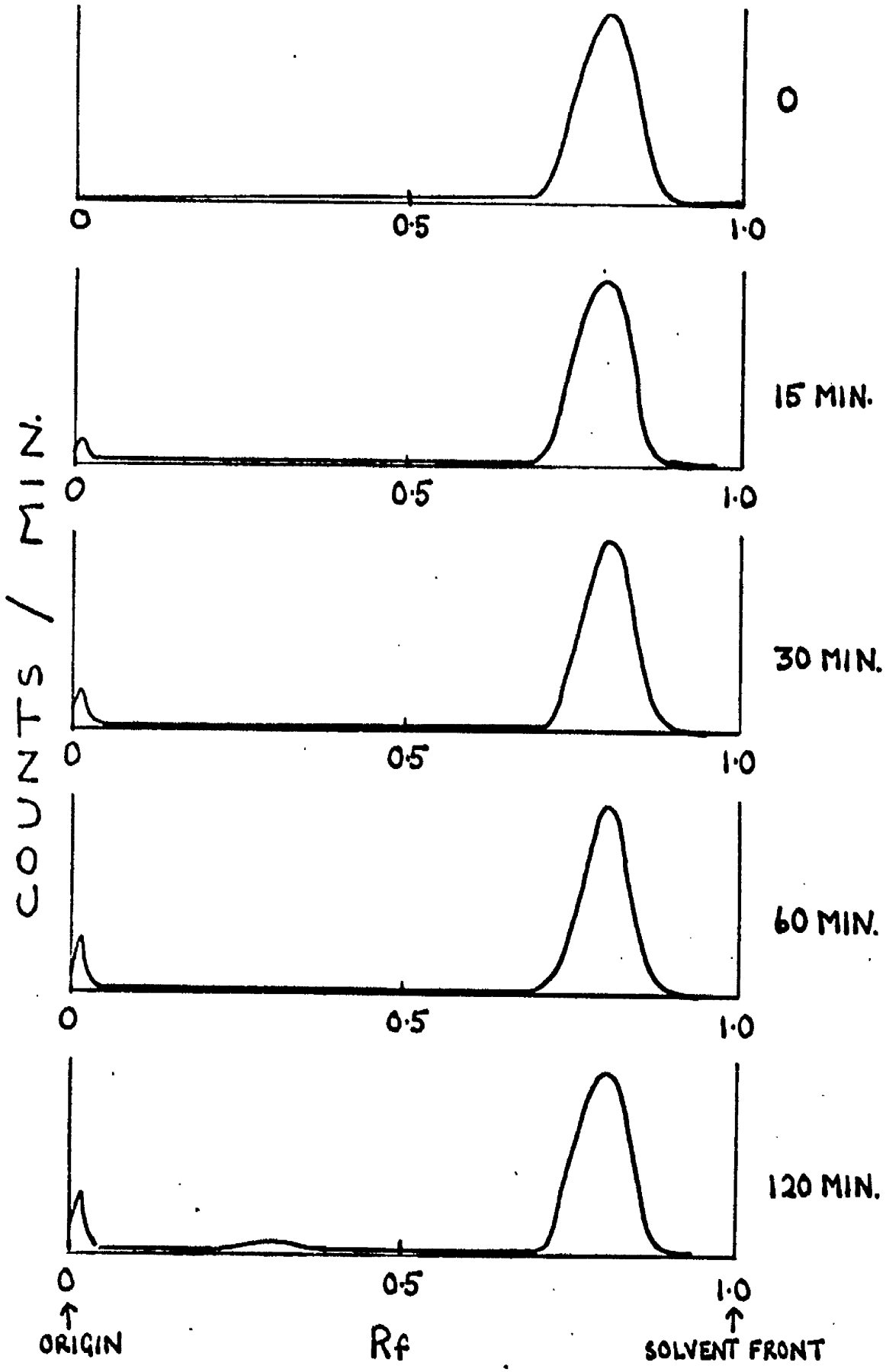
DNase activity of this Sephadex purified enzyme was not detectable by this method. The product of the standard DNase reaction in which $[^{32}P]$ - DNA from Esch. coli was incubated under polymerase conditions (in the absence of triphosphates) gave only one peak at the origin (R_F = 0.03) after zero and 2hr. incubation at 37°.

FIGURE 32

Chromatography of the product of DNA polymerase on sheets of DEAE cellulose

A standard polymerase reaction scaled up two-fold was incubated at 37° and 0.05 ml. samples withdrawn at the times indicated. They were applied to DEAE-cellulose paper and subjected to descending chromatography in 0.75 M ammonium bicarbonate, pH 8.6. Copies of the ^{32}P-scans from the Nuclear-Chicago Actigraph III are shown plotted against the R_F values.

FIGURE 32


0
0
0·5
1·0
15 MIN.
0
0·5
1·0
30 MIN.
0
0·5
1·0
60 MIN.
0
0·5
1·0
120 MIN.
0
0·5
1·0
COUNTS / MIN.
ORIGIN
Rf
SOLVENT FRONT

The product of the DNA polymerase reaction (after 5hr. incubation), which was submitted to CsCl density gradient centrifugation (Results VI A), was chromatographed on DEAE cellulose. There was a broad double peak at the origin (R_f values 0.025 and 0.080) as well as the triphosphate peak. By comparison of the areas of the triphosphate and origin peaks there was found to be approximately 30% replication of the DNA primer. After CsCl density gradient centrifugation (Fig. 31) fractions 20, 40, 60, 100, 120 were analysed on DEAE cellulose and all gave triphosphate peaks of the same area. In addition there was a broad peak at the origin in fractions 60, 80, 100, 120. The largest peak was in fraction 80, and those of 60 and 120 were barely significant. This corresponds to the acid precipitable radioactivity in Fig. 31.

VI C Polyethylene glycol-dextran two phase separation of the product.

The variation of log-(partition coefficient) of native and denatured DNAs at various concentrations of phase system solution [13.3% dextran (W/w) - 10% polyethylene glycol (W/v)] is shown in Fig. 33. 1ml. portions of well-dialysed native or heat-denatured ascites DNA were thoroughly mixed with varying amounts of phase solution and the phases separated as described in Methods F(iii). The DNA concentrations were measured by their E_{258} in the Unicam SP500 spectrophotometer, and their Kd values (the ratio of the concentrations in the upper layer over the lower layer) calculated. There was no separation of layers when < 0.5ml. phase soln. was added to 1ml. DNA soln. In all volumes of phase solution assayed the native DNA partitioned into the top layer and the

FIGURE 33

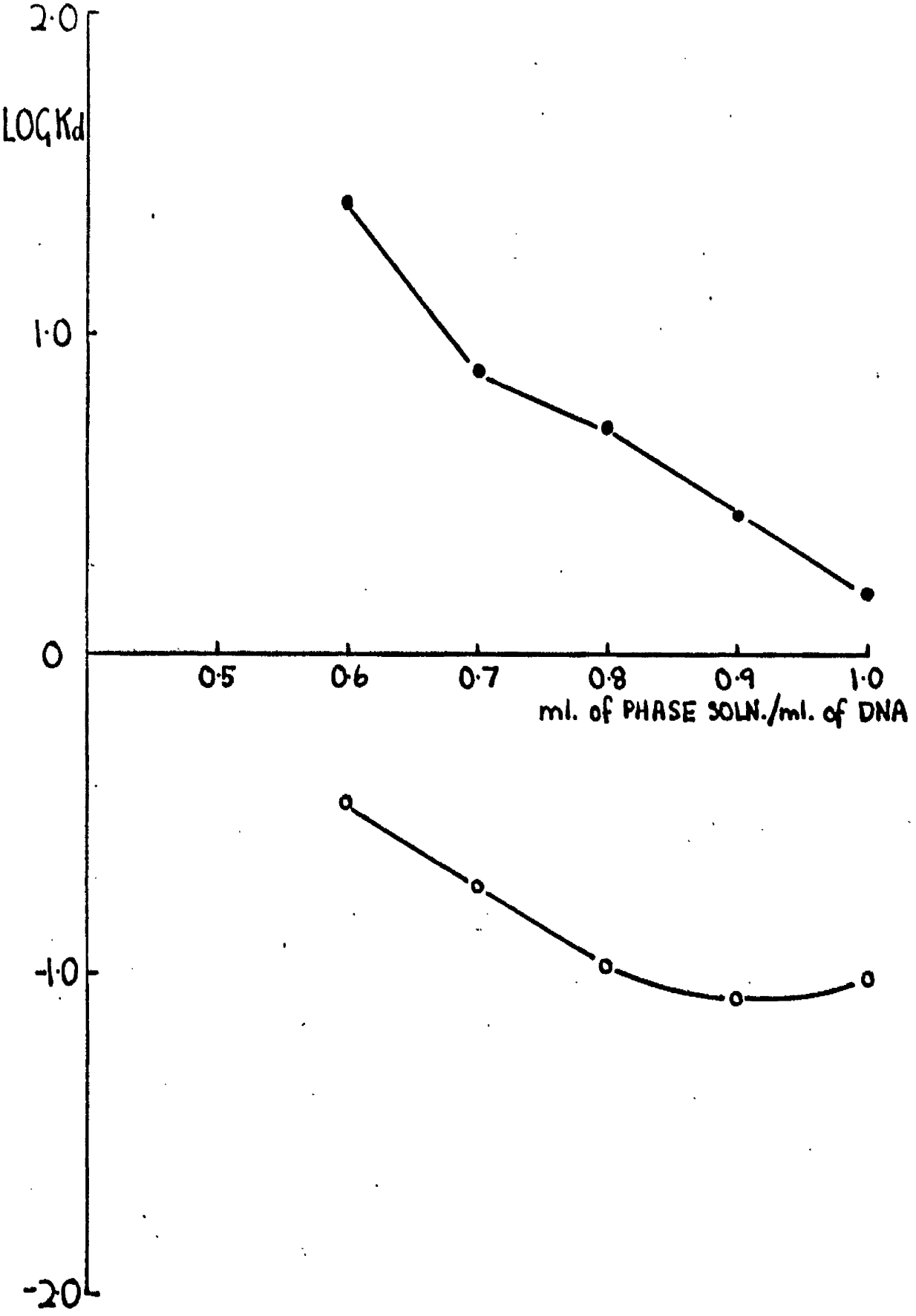
Fractionation of native and denatured DNA in polyethylene glycol-dextran two phase systems

Ascites-DNA at 0.1 mg./ml. was dialysed against 0.01 M-phosphate buffer, pH 6.8 overnight. 1 ml. portions of native DNA, heat-denatured DNA (10 min. at 100°) and 0.01 M phosphate buffer, pH 6.8 were added to various volumes of phase soln. [13.3% dextran ($^{W}/_{W}$) - 10% polyethylene glycol ($^{W}/_{W}$)] as indicated. After thorough shaking and centrifugation, all at 1°, the E_{258} of diluted portions of the layers was measured and the partition coefficients calculated after subtraction of the appropriate blanks.

—○— Native DNA

—○— Denatured DNA

FIGURE 33



denatured into the lower layer.

Partitioning of the product of DNA polymerase was similarly performed. A standard polymerase reaction, scaled up 5-fold, was set up and 0.25ml. samples were withdrawn at various times and diluted by adding 1ml. of 0.01M-sodium phosphate buffer pH6.8. These solutions were subjected to dialysis at 4°for 40hr. against four changes of 0.01M-phosphate buffer, 1mM-sodium pyrophosphate being present in the first dialysis. 0.9ml. of phase solution was added to 1ml. portions of each of the dialysed solutions. The DNA concentration of the subsequently formed layers were measured by the micro-Burton method (Methods Fv), and the ^{32}P in each layer determined directly on the Nuclear Chicago gas-flow counter. There was a native and a denatured DNA control which contained the same polymerase constituents as, and were dialysed together with, the test samples, although they were never incubated at 37°. The levels of radioactivity in these controls were 10-20% of the test samples (about 99.5% of nonincorporated [$\alpha^{32}P$] - dTTP was removed by dialysis).

The results are shown in Fig. 34. The denatured DNA primer initially partitioned into the lower layer but even after 1 hour at 37°it partitioned into the upper layer. The results calculated from ^{32}P in each layer are similar to but more accurate than those calculated from the microBurton method since the sensitivity of this method is lower, especially at extreme values of Kd. The native and denatured controls are of the same order as those in Fig. 33 in the presence of 0.9ml. of phase soln./ml. of DNA.

FIGURE 34

Fractionation of the product of DNA polymerase in polyethylene glycol-dextran two phase system

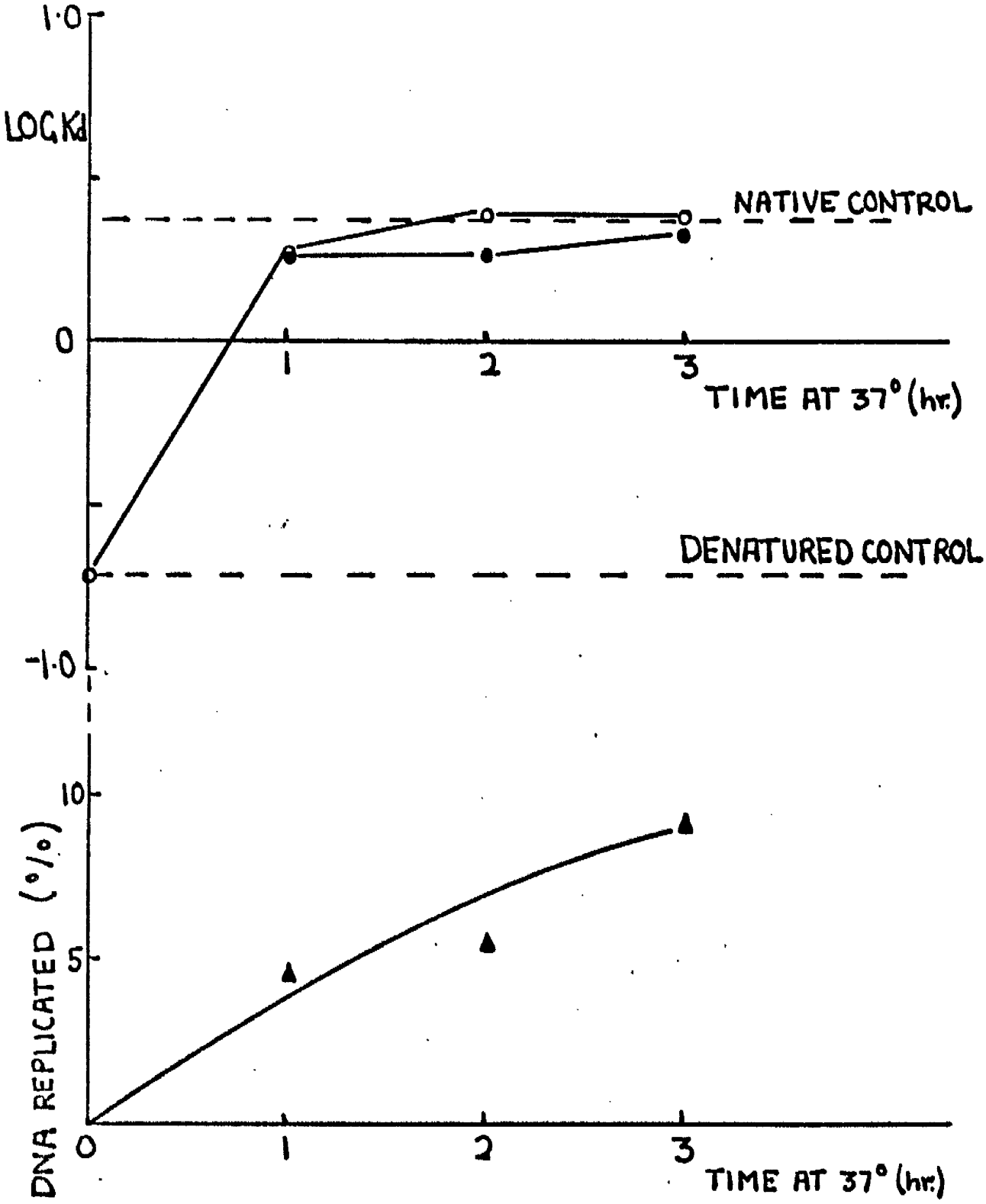
Samples of a standard DNA polymerase reaction with 20 µg. of denatured ascites-DNA primer/0.25 ml. were diluted, dialysed and fractionated in the two phase system as described in the text. Denatured DNA (zero time), and native DNA controls were performed and their partition coefficients are indicated.

——○—— DNA concn. measured by micro Burton method

——o—— DNA product concn. measured by ^{32}P estimation

——△—— % DNA replicated (determined by ^{32}P incorporation)

FIGURE 34.


1·0
LOG Kd
0
NATIVE CONTROL
1
2
3
TIME AT 37° (hr.)
DENATURED CONTROL
-1·0
10
5
DNA REPLICATED (%)
0
1
2
3
TIME AT 37° (hr.)

DISCUSSION

I Purification of DNA polymerase

The two great problems encountered during purification of DNA polymerase from Landschütz ascites-tumour cells were the removal of interfering activities and the prevention of loss of enzyme activity. In neither case was the problem solved with complete success, and success at solving one could only be achieved at the expense of the other. A balance was therefore sought, in which there was maximum removal of interfering activities with the minimum loss of enzyme.

I A Removal of Interfering Activities

Fractionation of DNA polymerase on Sephadex G-150 afforded almost total separation of DNase and polymerase. Ninety-nine per cent of the DNase was removed from the polymerase peak by this one step (Fig. 6). However, the remaining DNase was significant and accounted for a 4-8% breakdown of [^{32}P]-DNA to acid soluble material. This was equivalent to approximately 1-2 mμmoles of nucleotide rendered acid-soluble/hr./mg. of protein. The lower figures were for polymerase preparations which had undergone pH 5 precipitation and ammonium sulphate fractionation before gel filtration whereas the higher figures were for initial extracts applied directly onto the column.

The polymerase batches which were pooled and resubmitted to gel filtration contained virtually no DNase after recycling, since $< 0.5\%$ of the ^{32}P-DNA was rendered acid-soluble in 2 hours as determined by chromatography on DEAE-cellulose paper. This is

equivalent to < 0.15 mμmoles of nucleotide released/hr./mg. of protein. However, the polymerase activity of the recycled fractions was reduced by about 50%.

By the same criterion of chromatography on DEAE paper the polymerase was endonuclease free. This method is, however, relatively insensitive since oligonucleotides of chain length greater than 30 remain at the origin (Furlong, 1966). The more sensitive test which was not performed is the assay with circular DNA since a single nick converts the molecule to the linear form which has very different sedimentation properties from the circular form.

On the synthesis side using low concentrations of denatured DNA as primer, up to 30% net replication of the primer was achieved after 5 hours incubation. The specific activity of the pooled polymerase fractions was about 6-10 mμmoles of dTMP incorporated/hr./mg. of protein, corresponding to 24-40 mμmoles nucleotide incorporated/hr./mg. of protein.

The actual degradation by DNase during the polymerase reaction cannot be calculated accurately by extrapolation of the results of DNase assays and polymerase assays, since the conditions are somewhat different. The DNase assays are performed with 10 μg. of denatured ^{32}P-DNA from Esch. coli and about 50-200 μg. of enzyme protein in the absence of triphosphates. The polymerase assays were performed with 50 μg. of denatured ascites DNA and up to 500 μg. of protein in the presence of the triphosphates. It

is very possible that exonuclease activity is inhibited under replicating conditions; the T4 phage exonuclease, which is associated with the polymerase, is greatly inhibited by triphosphates (Goulian, Lucas & Kornberg, 1968). Also the DNase may not act on the product if it is double stranded since ascites DNase is generally much more active on denatured rather than native DNA. On the other hand if the DNase is an exonuclease acting at the 3'-OH terminals it will preferentially degrade the product.

It seems unlikely that the remaining exonuclease activity is associated with the polymerase molecule since recycling of the enzyme permits further removal of DNase. The ratio of optimal activities of polymerase to DNase is 1.8 for T4 polymerase and 2.8 for Esch. coli DNA polymerase (Richardson, Schildkraut, Aposhian & Kornberg, 1964). Although the corresponding ratio for the optimal activities of the ascites enzyme(s) cannot be given it is extremely unlikely that, even if repeated recycling of the enzyme did not remove all exonuclease, such a low ratio of optimal activities of the two enzymes would be found.

The enzyme appeared to possess no triphosphatase activity judging by the unaltered R_p value of the dTTP peak after chromatography on DEAE paper. There was no apparent broadening or splitting of the peak after up to 5 hours under polymerase reaction conditions. After acid treatment of the polymerase reaction medium there were two significant peaks demonstrating that degradation products were detectable. According to Furlong (1967) there are significant differences of about 10% between the R_p

values for the deoxyribonucleoside mono- and triphosphates, and presumably the R_F value of the diphosphate, although not quoted, is intermediate. It is conceivable, however, that the triphosphates other than dTTP may be enzymically or thermally hydrolysed. The purine triphosphates obtained commercially tended to contain more di- and monophosphates than the pyrimidine triphosphates indicating that they are more labile. The soluble extract, pH 5 precipitate and 20-45% ammonium sulphate fraction were found to contain negligible deoxyribonucleoside triphosphatase activity by Shepherd (1965).

I B Loss of enzyme activity

The labile nature of the enzyme was a constant problem especially during purification. Activity was lost during pH 5 precipitation though this could be minimised by speed and using concentrated protein solutions. Substantial activity was lost during Sephadex fractionation. This method of enzyme fractionation was chosen as being gentle, but Yoneda & Bollum (1965) lost activity of the terminal addition enzyme during gel filtration on Sephadex G-200 (though less on G-100).

Freezing and thawing inactivated the enzyme, and incubation at 45° resulted in complete loss of activity in 20 min., in both the Sephadex-purified enzyme and a 20-45% ammonium sulphate preparation, when dissolved in Buffer 4A. This contrasts with Shepherd & Keir's (1966) finding of complete inactivation within 2 min.,

explicable possibly by the protection afforded by the components of the buffer solution.

The enzyme can be partially stabilized by high protein concentrations, DNA or glycerol. Stabilization of labile enzymes by protein, although well known, is little understood (Putnam, 1953). Possibly the enzyme may dissociate into inactive subunits at low concentrations, as does L-glutamic dehydrogenase (Olson & Anfinsen, 1952, 1953; Kubo et al., 1958). The BSA added to the eluting buffer during Sephadex chromatography prevented some loss of polymerase activity. Gottesman & Canellakis (1966) found that BSA protected their more purified fractions of the terminal addition enzyme.

Protection of an enzyme by its substrate against denaturation is also a well known phenomenon (Bernhard, 1968a), and the stability of the enzyme which was prepared by sonication rather than homogenisation was possibly due to the presence of oligonucleotides bound to the enzyme. Inactivation during dialyses following ammonium sulphate fractionation may be due in part to the removal of these protective oligonucleotides. Of considerable interest in this connection is the recent report of purification of Micrococcus lysodiekiticus DNA polymerase on a column of DNA bound to a cellulose matrix (Litman, 1968). In this method DNA polymerase binds more strongly than the other proteins and is selectively purified as well as protected.

The studies with glycerol have demonstrated its protection of the enzyme. Rat testes DNA polymerase is stimulated 2-4 fold

by glycerol (Calvin, Kosto & Williams-Ashman, 1967). The mode of action of glycerol is uncertain but it is known to protect enzymes against denaturation; it has a very marked effect on the cold-inactivated enzyme 17-β-hydroxysteroid dehydrogenase (Jarabak, Seeds & Taladay, 1966). The storage properties of DNA polymerase (Fig. 14) show that the polymerase loses activity more quickly at 20° than at temperatures below 20° so it cannot be classified as a cold-labile enzyme. The protective effect of glycerol may be due to its effect on the structure of the water molecules around the protein surface (Jarabak et al., 1966). This would be especially important during freezing and thawing of the enzyme.

The ascites polymerase is considerably more labile than the Esch. coli enzyme, the most purified fraction of which can be stored for one year at 0° with only 20% loss of activity (Richardson, Schildkraut, Aposhian & Kornberg, 1964). This reflects a difference in structure of the enzymes. The ascites enzyme is sensitive to sulphydryl agents and therefore is likely to resemble the T4 polymerase with its many cystine residues, rather than Esch. coli polymerase with only 3 half-cystine residues and which is not inactivated by sulphydryl blockers. Breakage of some of the disulphide bonds by sulphydryl blockers would cause collapse of the secondary structure and inactivation of the enzyme, whereas the bacterial enzyme would be able to withstand more severe conditions. 2-Mercaptoethanol greatly minimises this inactivation in ascites polymerase (Keir & Shepherd, 1965).

II Sucrose density gradient studies

The results of the sucrose gradient centrifugations of DNA and DNA polymerase appear anomalous in many respects. The native DNA sedimented very slowly in the sucrose-water solutions (Fig. 23B) though more rapidly in sucrose-buffer solutions (Fig. 25A); denatured DNA sedimented slowly regardless of the inclusion of buffer (Fig. 24B). A possible explanation of this is that the ascites DNA preparation is considerably degraded during, or before, isolation. The native double helix could contain many single strand nicks so that denaturing would release low molecular weight DNA fragments of much lower sedimentation coefficients. The occurrence of slow sedimenting native DNA in the pure sucrose solutions (Fig. 23B) is probably caused by partial denaturation of the DNA, releasing low molecular weight DNA in the absence of other ionic species. The inclusion of 0.02 M-tris-HCl and 5 mM-2-mercaptoethanol was sufficient to prevent this denaturation.

The actual sizes of the native DNA species in the sucrose gradients (in the presence of buffer) have been calculated very approximately using the relationship of Burgi & Hershey (1963) for two DNA molecules of molecular weights M_1 and M_2 which sediment distances D_1 and D_2 respectively under the same conditions of centrifugation.

$$\frac{D_1}{D_2} = \left(\frac{M_1}{M_2}\right)^{0.35}$$

The standard taken for the calculation was the sedimentation of T7

phage DNA by Oishi (1968a). This communication, in Fig. 7A, shows that native T7 DNA sedimented 19/30 down the 5 ml. tube of the SW50 rotor of the Spinco Model L, while spinning at 36,000 rev./min. (105,000 g) for 4 hr. The sedimentation of ascites DNA in buffered sucrose at 50,000 rev./min. (204,000 g) for 3 hours is shown in Fig. 25A. Therefore the equivalent distance sedimented by the T7 DNA under the same conditions of sedimentation is $\frac{19}{30} \times \frac{204,000}{105,000} \times \frac{3}{4} = 0.915$. The molecular weight of T7 DNA was taken as 25×10^6 daltons (Harpt, Krasna & Zimm, 1965). These values of D_1 and M_1 and the distances sedimented by each of the three peaks of ascites DNA (Fig. 25A) were substituted into the Burgi and Hershey equation. The molecular weight of each of the three peaks was found to be 7×10^4, 6×10^5 and 2×10^6 corresponding to 200, 1800 and 6200 nucleotides in length.

Similarly by comparison with denatured T7 DNA [cf. Oishi (1968a), Fig. 7B], the bulk of the denatured ascites DNA in buffered sucrose gradients (not shown) which sediments in a very broad peak with a mean at ⅓ of the distance down the tube extending over the top ⅔ of the gradient, has a molecular weight of 5×10^5 corresponding to 1,700 nucleotides.

The existence of this very low molecular weight DNA is more likely to be caused by DNase action rather than mechanical shearing, since the latter is minimal with low molecular weight DNA. The significance of the triple peak is unknown.

In the presence of polymerase, the native and denatured DNA solutions do not sediment similarly in the sucrose-water gradients (cf.

Fig. 23C and Fig. 24C). Since any binding of proteins to DNA probably takes place on top rather than in the gradient, and since the polymerase was dissolved in Buffer 4A containing 0.15 M-KCl, the studies with native DNA are likely to reflect binding with more highly native DNA than would be present in the pure sucrose gradients in the absence of the polymerase preparation. This was confirmed by assaying the polymerase in the native and denatured DNA "complexes"; if the polymerase-DNA complex was assayed in the presence and absence of further denatured DNA primer the activity of the polymerase-denatured DNA complex was the same in both instances, but the activity of the polymerase-native DNA complex in the absence of added denatured DNA was only 4% of the level in its presence.

The native DNA binds with approximately half the polymerase in the sucrose-water gradient, and the other DNA peaks, as well as much of the main DNA peak, are probably caused by binding with other proteins in the polymerase preparation. It is difficult to ascertain whether the polymerase binds to the denatured DNA (Fig. 24) since there is only a very small increase in its sedimentation. However, since the denatured DNA is of much lower molecular weight than the native DNA a smaller increase in sedimentation rate would be expected when the polymerase binds to denatured rather than native DNA. Since there was only one polymerase peak in the presence of denatured DNA, all (or none) of the polymerase was bound to the DNA.

In connection with the occurrence of oligonucleotides present in less purified polymerase preparations (Discussion I B), evidence supporting this theory is shown in Figs. 23A and 24A; the pH 5 precipitate polymerase fraction has a shoulder which sediments at a greater rate than the remainder of the polymerase, and which is absent from the ammonium sulphate fraction and which may represent polymerase bound to oligonucleotides.

In the buffered sucrose gradients DNA-protein aggregates are formed which sediment to the bottom of the tube, explaining the apparent loss of ultraviolet absorbing material in the gradient when native DNA is sedimented with protein (cf. Figs. 25A and 25B). The polymerase is complexed in this aggregate in a somewhat unassayable form in 2 or 3-component buffered sucrose gradients. However, if the gradient contains 0.15 M KCl there appears to be no binding of the polymerase to DNA. This is in agreement with Kornberg's group who find that binding of poly d(A-T) to Esch. coli polymerase is strongly inhibited by 0.2 M potassium phosphate buffer. This may possibly explain the absence of binding of the calf thymus polymerase to DNA since in these studies the sucrose was dissolved in 0.05 M phosphate buffer (Yoneda & Bollum, 1965). Similarly Chang & Hodes (1968) could not detect binding between either of their Shope fibroma polymerases and DNA. Their sucrose gradients contained 0.1 M potassium phosphate buffer. Billen (1963) was able to reconstitute binding between DNA and Esch. coli polymerase giving a sedimentable complex, using sucrose gradients in which the sucrose was dissolved in a buffer at pH 7.4 containing

0.01 M-tris, 1 m M-succinic acid and 0.01 M-magnesium chloride. Kadoya et al., (1964) isolated from Esch. coli a deoxyribonucleoprotein complex containing polymerase using sucrose gradients in which the sucrose was dissolved in 10 m M-tris, 10 m M-magnesium chloride, pH 7.4 It is therefore possible that any failure to observe binding of polymerase to DNA is explicable by the presence of inhibiting amounts of certain ions.

Alternatively the ascites polymerase may have an especially high affinity for the ascites DNA preparation used. As discussed previously its native structure may contain many single strand nicks. Englund et al., (1968) showed that the Esch. coli polymerase only binds at nicks or ends of native DNA, though throughout the length of denatured DNA.

Originally these experiments were performed to examine specificity of binding between polymerase and DNA from the same origin, as was demonstrated for virus RNA dependent RNA polymerase for its own RNA (Haruna & Spiegelman, 1965). However since many non-related proteins, including BSA, bind to DNA, and more than half of the DNase of a pH 5 polymerase preparation was found to bind (Fig. 27) and since there was similar binding between calf thymus DNA and ascites polymerase (not shown) there was no evidence to support any specificity of binding.

The Esch. coli polymerase shows no specificity for the type of DNA, though it cannot bind to native DNA in the absence of 3'-OH groups (Englund et al., 1968).

These results raise the possibility that the binding which occurs is non-specific protein-DNA binding rather than enzyme-substrate binding, especially since there is inhibition in the presence of 0.15 M-KCl. During the *in vitro* assay the optimal activity is achieved in the presence of 0.05 M-KCl, and at 0.15 M-KCl the activity was reduced by 70% (Shepherd & Keir, 1966).

The results of determination of $S_{20,w}$ and molecular weights (Table 5) give results higher than those for other polymerases (cf. Introduction II I). The method of determination may be responsible for this since the large difference in sedimentation rates of haemoglobin and polymerase will cause a considerable error. Moreover, the measurements were performed with a pH 5 precipitate enzyme fraction which may, as previously mentioned, contain small oligonucleotides bound to the polymerase causing an increase in its sedimentation rate.

III Effect of incubation time and protein concentration on DNA polymerase reaction rate

Time curves frequently showed a slight concave curvature upwards (Fig. 8); the possible significance of this will be discussed later. Apart from this upward curvature, deviation from linearity in the polymerase reaction with time is almost certainly caused mainly by denaturation of the protein with time; the lower the protein/assay the lower the period of linearity. The addition of more enzyme and triphosphates at regular intervals increases the period of net synthesis (Results IIID), though the contribution

of the breakdown of triphosphates towards cessation of the reaction is minimal, judging by the unchanged R_F values of dTTP in chromatography on DEAE paper. The apparent slowing down of the polymerase reaction may also be caused by the action of DNase since the higher the ratio of product to primer the greater will be the ratio of product to primer degraded by DNase.

As previously mentioned (Results IIID) the deviations from linearity do not invalidate the substrate and protein concentration curves.

The non-linearity of the protein concentration curve is somewhat anomalous. These results were not obtained by Shepherd (1965) who showed linearity of activity with protein concentration up to 120 µg/assay for the crude extract pH 5 precipitate and ammonium sulphate fraction, and a subsequent tailing off. The present results show an upward curvature up to at least 500 µg/assay for all polymerase fractions (see Figs. 5, 7, 13). It is unlikely to be caused by inactivation of the enzyme at low protein concentrations since it was still apparent in the presence of BSA (Fig. 13) and of glycerol (Fig. 7).

Such upward curvatures are attributed to the presence of an activator in the enzyme preparation (Dixon & Webb, 1964). Similar results were obtained with sea urchin embryo DNA polymerase but after pretreatment of the DNA primer with DNase the protein concentration curve became linear (Loeb et al., 1967). This would suggest that the product of DNase digestion is acting as an activator. A similar result would be expected if the DNA poly-

merase contained oligonucleotides as initiators (the state of the primer and the effect of oligonucleotides will be discussed in the following section). If the presence of either oligonucleotides or of DNase in the polymerase preparation were responsible for the shape of the concentration curve it would be expected that the characteristics of the curve would be greatly altered at different stages of enzyme purification. This was not found to be the case.

Another possibility is that polymerase molecules associate to dimers at higher concentrations and that this is a more stable, or more efficient and perhaps even necessary, structure for polymerisation.

IV Kinetics of incorporation of deoxyribonucleoside triphosphates

The kinetic studies with DNA polymerase from ascites cells are subject to criticisms of non-validity since the enzyme was not a pure protein. There may be significant non-specific binding of the other proteins in the preparation to the DNA or triphosphates. In addition the triphosphate kinetics were performed with an ammonium sulphate fraction, which contains some DNase, though some of these were repeated without any apparent differences using a Sephadex fraction.

The striking point about the concentration curve of one triphosphate, while maintaining the others at their saturating concentrations of 50 mμmoles/assay, was that the level of incorporation of that particular triphosphate when present at low concentrations was much lower than that of the other three triphosphates (Fig. 15).

Indeed, in the absence of one triphosphate the incorporation of the other three was only reduced to 20% of the level in the presence of all four. This also occurs with other mammalian polymerases; the calf thymus enzyme in the absence of one triphosphate displays incorporation amounting to 10-30% of maximal incorporation, the level depending on which triphosphate is absent (Bollum, 1960a); Mantsavinos (1964) found the corresponding figure for regenerating rat liver polymerase to be 17%. This incorporation could be caused by the presence of endogenous supplies of the absent triphosphate, by synthesis of the triphosphate from endogenous supplies of the precursors, or by conversion of another triphosphate. Alternatively the product may be completely lacking in the missing base; this can be due to 1) copying a portion of the template containing little or none of the complementary base to the missing triphosphate, 2) miscopying of the template allowing another nucleotide to be incorporated in place of the missing one, 3) non-template specified synthesis. In connection with this last explanation Shepherd & Keir (1966) showed that in the presence of the full complement of triphosphates the product of ascites polymerase has the same dinucleotide frequency as the primer, and non-template specified homopolymer formation is very low. However there could be non-template specified heteropolymer formation since this has recently been demonstrated with the calf thymus cytoplasmic terminal addition enzyme (Ratcliff et al., 1968).

So when performing reaction kinetics with one labelled triphosphate at a lower concentration than the other three, the velo-

city of the total polymerase reaction i.e. total nucleotide incorporation, is not directly proportional to the incorporation of label. The Km values of the triphosphates, recorded in Fig. 16, therefore measure the concentration of that triphosphate at half the V_{max} of incorporation of that triphosphate, and not its concentration at half the V_{max} of total nucleotide incorporation. It can be seen by inspection of Fig. 15 that the former value is larger than the latter. The former value is also more likely to represent true polymerase template copying since non-template specified synthesis is suspected to influence the latter value.

It was probably because of similar difficulties that Englund et al. (1968) resorted to equilibrium dialyses to measure the equilibrium constants of the triphosphates with Esch. coli polymerase, to obtain values in the absence of polymerisation. In support of the validity of performing studies in the presence of polymerisation the following adaptation of Michaelis-Menten kinetics for the incorporation of one triphosphate in the presence of saturating amounts of the other substrates is presented.

Consider all the enzyme, E, to be bound to the template DNA, D, and an oligonucleotide, O, to which during the normal reaction nucleotides are added according to template specification (the rationale and kinetics of formation of this complex will be discussed later). This complex then exists as DEO and the incorporation of a nucleotide into the oligonucleotide can be represented in a simplified version as

$$DEO + NTP \rightleftharpoons DEON \longrightarrow DEO$$

In the last stage(s) of the reaction the enzyme covalently bonds the nucleotide to the oligonucleotide and moves along the lengthened oligonucleotide and the DNA.

For the incorporation of a specific triphosphate the DNA requires to have the complementary base to that triphosphate adjacent to the binding site of that triphosphate, e.g. for the incorporation of dTMP.

$$\overset{a}{D}EO + dTTP \rightleftharpoons \overset{a}{D}EOT \longrightarrow DEO$$

where $\overset{a}{D}EO$ represents the DNA-enzyme-oligonucleotide complex, so that an adenine moiety on the template DNA is adjacent to the active site specifying the incorporation of the thymidine nucleotide.

The total enzyme E is complexed so that

$$[E] = [\overset{a}{D}EO] + [\overset{a}{D}EOT] + [\overset{c}{D}EO] + [\overset{c}{D}EOG] + [\overset{t}{D}EO] + [\overset{t}{D}EOA] + [\overset{g}{D}EO] + [\overset{g}{D}EOC].$$

Since there is equivalence of adenine and thymine, and of cytosine and guanine in the template DNA

$$[E] = 2[\overset{a}{D}EO] + 2[\overset{a}{D}EOT] + 2[\overset{g}{D}EO] + 2[\overset{g}{D}EOC]$$

If the $\frac{G+C}{A+T}$ ratio of the template is x then

$$2[\overset{g}{D}EO] + 2[\overset{g}{D}EOC] = 2x[\overset{a}{D}EO] + 2x[\overset{a}{D}EOT]$$

$$\text{so that } \frac{E}{2(1+x)} = [\overset{a}{D}EO] + [\overset{a}{D}EOT] \text{ ------(1)}$$

(This expression does not hold if there is significant accumulation of $\overset{a}{D}EO$ at the rate-limiting step)

In the incorporation of dTTP

$$K_s = \frac{[\overset{a}{D}EO]\,[T]}{[\overset{a}{D}EOT]} \text{ ----------(2)}$$

where K_s is the equilibrium or substrate constant for dTTP, and is taken as being equal to K_m the Michaelis constant.

Let the rate of the reaction be v

$$\text{Then } v = k[{}^{a}_{DEOT}] \text{ ----------(3)}$$

where k is the velocity constant for dTTP incorporation. The maximum velocity, V_{max}, for dTTP incorporation will occur when

$$[{}^{a}_{DEOT}] = \frac{E}{2(1+x)}$$

$$V_{max} = \frac{k\,E}{2(1+x)} \text{ ----------(4)}$$

Substituting (3) and (4) into (1)

$$V_{max} = k[{}^{a}_{DEO}] + v$$

$$\text{or } k[{}^{a}_{DEO}] = V_{max} - v \text{ ----------(5)}$$

Substituting (5) and (3) into (2)

$$K_m = \frac{(V_{max} - v)\,[T]}{v} \text{ -----(6)}$$

Rearranging this gives the classic Lineweaver-Burk plot of

$$\frac{V_{max}}{v} = \frac{K_m}{[T]} - 1$$

It can be seen from Equation (4) that V_{max} is dependent on the base ratio of the template DNA, and since K_m is dependent on V_{max}, Equation (6), it is also dependent on the base ratio.

These results are valid only if the incorporation of dTTP is rate limiting, i.e. in the presence of saturating levels of the other triphosphates and of DNA. If the other reactions of polymerase are rate limiting the situation becomes very complex. The K_m of dTTP increases as the other triphosphate concentrations diminish (Fig. 17). The Hill plot of the variation in concen-

tration of all four triphosphates maintaining them at equal concentrations has a gradient of 3.0 (Fig. 18). The significance of this is not readily explicable. The Hill plot has been put to use by Changeux (1963) for analysis of allosteric interactions in threonine deaminase where he states that the slope of the Hill plot is a measure of the interaction coefficient and is less than or equal to the number of allosteric binding sites. The equation has also been used for analysis of isocitrate dehydrogenase (Atkinson, Hathaway & Smith, 1965) where it was emphasized that the slope corresponds to the number of sites only when the interactions between the sites are strong. Therefore, if the kinetics of a polymerising enzyme can be compared with those of a more standard type of reaction, the significance of the slope is that the enzyme contains at least three different allosteric sites for triphosphates; obviously four sites is a more attractive number.

However there would appear not to be a specific site for each triphosphate since the presence of only one triphosphate at high saturating concentrations activates the sites almost as fully as having three triphosphates at saturating concentrations (cf. the linearity of the Lineweaver-Burk plots, Fig. 17B).

Speyer (1966) has produced genetic evidence suggesting allosteric site(s) in T4 polymerase but Englund *et al.* (1968) find only one triphosphate site in *Esch. coli* polymerase. However mammalian and T4 polymerases have many features in common, and differences from *Esch. coli* polymerase, so it is possible that the number of active sites may be another difference.

Another possible interpretation of the Hill plot is that the slope measures another type of interaction. Under template specified conditions, the incorporation of one triphosphate is dependent on three other reactions i.e. the incorporation of the other three triphosphates. In the presence of even one of these triphosphates at saturating levels while the others are at low concentrations, the overall synthesis does not reflect template specification and so the incorporation of one triphosphate is self limiting. If this explanation were true synthesis directed by a homopolymer template would obey first order kinetics with respect to the complementary base in the presence of low levels of the other three triphosphates. If the theory of three allosteric sites were true multiple order kinetics would still be observed.

Similar results have not been obtained with other polymerase systems. Beyersmann & Schramm (1968) calculated the K_m value for Esch. coli polymerase, presumably by altering the concentration of all four triphosphates and maintaining them at concentrations equal to each other, and obtained a linear Lineweaver-Burk plot. Chang & Hodes (1968) also showed linear kinetics under the same conditions using Shope fibroma polymerase.

This suggests that the apparent interactions obtained with the ascites enzyme may be explicable by lack of sufficient enzyme purification. The triphosphates may bind non-specifically to the other proteins in the polymerase preparation. This particular result from which the Hill plot was drawn was obtained using an ammonium sulphate fraction which contains substantial nuclease

which may function preferably in the absence of polymerisation.

The actual K_m values obtained (Fig. 16), ranging from 5-16 μM, are of the same order though slightly lower than those reported in the literature:- Beyersmann & Schramm using Esch. coli polymerase obtained a K_m value of 2×10^{-5} mole of nucleotide/litre (20 μM); Chang & Hodes (1968) using Shope fibroma polymerase obtained the values of 46 μM for native DNA and 160 μM for denatured DNA, using the same method of determination; Bäglund et al. (1968) using the technique of equilibrium dialysis obtained values for each triphosphate ranging from 12-147 μM for Esch. coli polymerase.

V The role of DNA in the ascites DNA polymerase reaction

The enzyme requires DNA primer for reaction and the slight incorporation of radioactivity in the absence of added DNA is probably due to small quantities of oligonucleotides in the polymerase reaction media. It would appear that the best primer is not completely denatured DNA as judged by the variation in the time of denaturation curves (Fig. 30). The optimum time of denaturation varies with the concentration of DNA while undergoing denaturation, and the increased priming ability after short times of denaturation is more apparent with the more concentrated DNA solutions. This is probably because at low DNA concentrations denaturation of DNA will occur more readily so that the intermediate optimal primer will only occur momentarily.

What is the structure of this intermediate? The original ascites DNA as already discussed is very heterogeneous and generally of low molecular weight. When DNA denatures the two strands

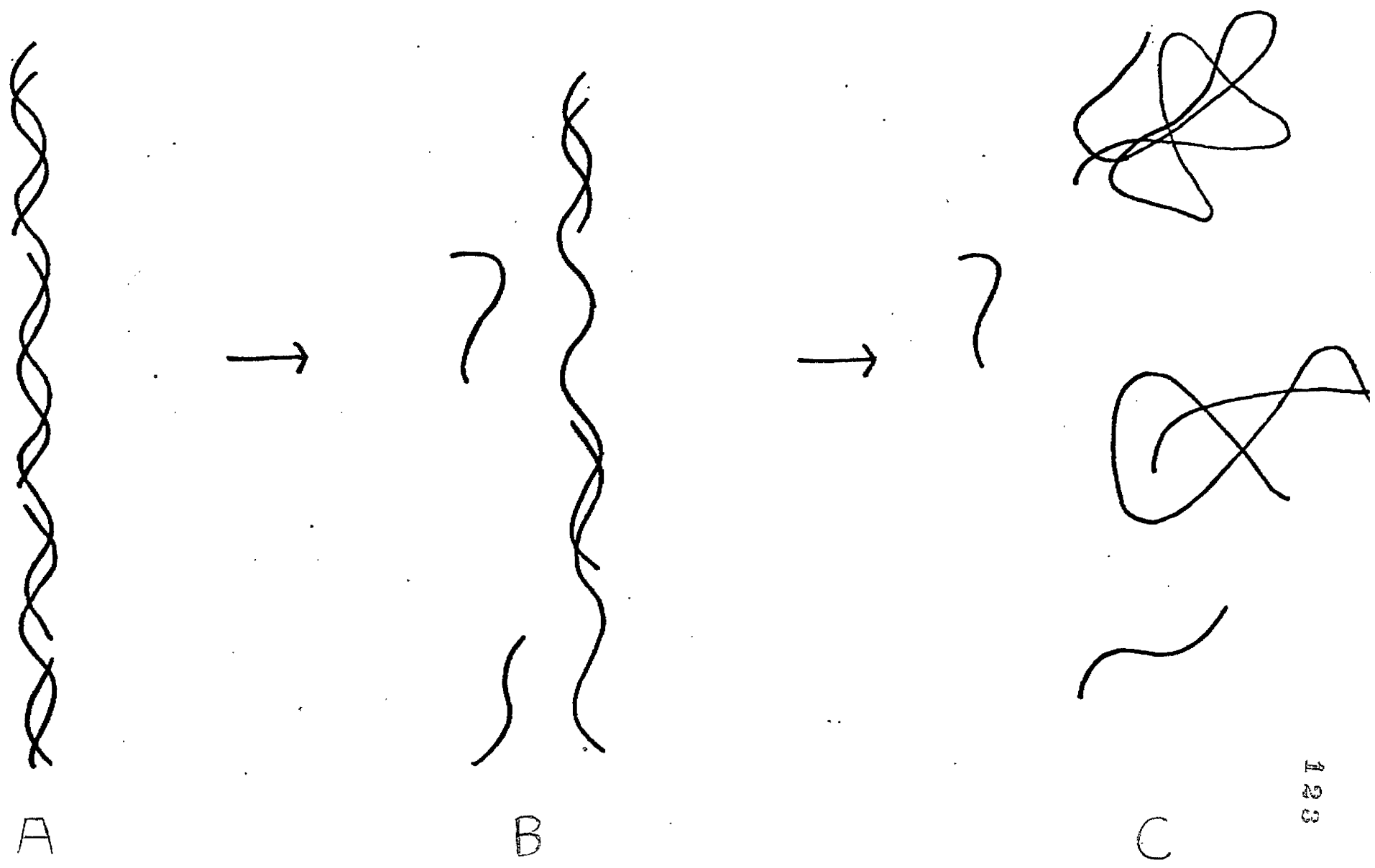
separate and the base stacking is disrupted (see Introduction I B). However, if the DNA is composed of native DNA containing single strand nicks partial denaturation will remove short oligonucleotides from the structure before separating strands with long stretches of complementarity. In completely denatured DNA the base stacking is disrupted and the strands assume a random coil formation. These stages of denaturation are depicted in Fig. 35 and the best primer (Fig. 35B) is one in which the base stacking is maintained and there are long stretches of single stranded DNA. This primer is similar to priming by DNA which has been subjected to exonuclease III digestion.

However, on the other occasions the DNA was denatured by heating to 100° for 10 min. and there could be assumed to be little or no double stranded regions. Having postulated that the ideal primer is partially double stranded DNA whereon the polymerase can perform a "repair" reaction the completely denatured primer will have to rearrange itself to a suitable primer. This is supported by evidence for two DNA binding sites; the denatured DNA concentration curve is sigmoid, the Lineweaver-Burk plot is curved, and the Hill plot is linear with a slope of 2.0 suggesting two allosteric binding sites for DNA. Keir (1965) originally postulated two binding sites on the basis of kinetic studies of the inhibition of polymerase by Actinomycin D.

One DNA molecule will act as an initiator and will bind to the enzyme at its 3'-OH terminal. The other DNA strand which

FIGURE 35

DENATURATION OF DNA



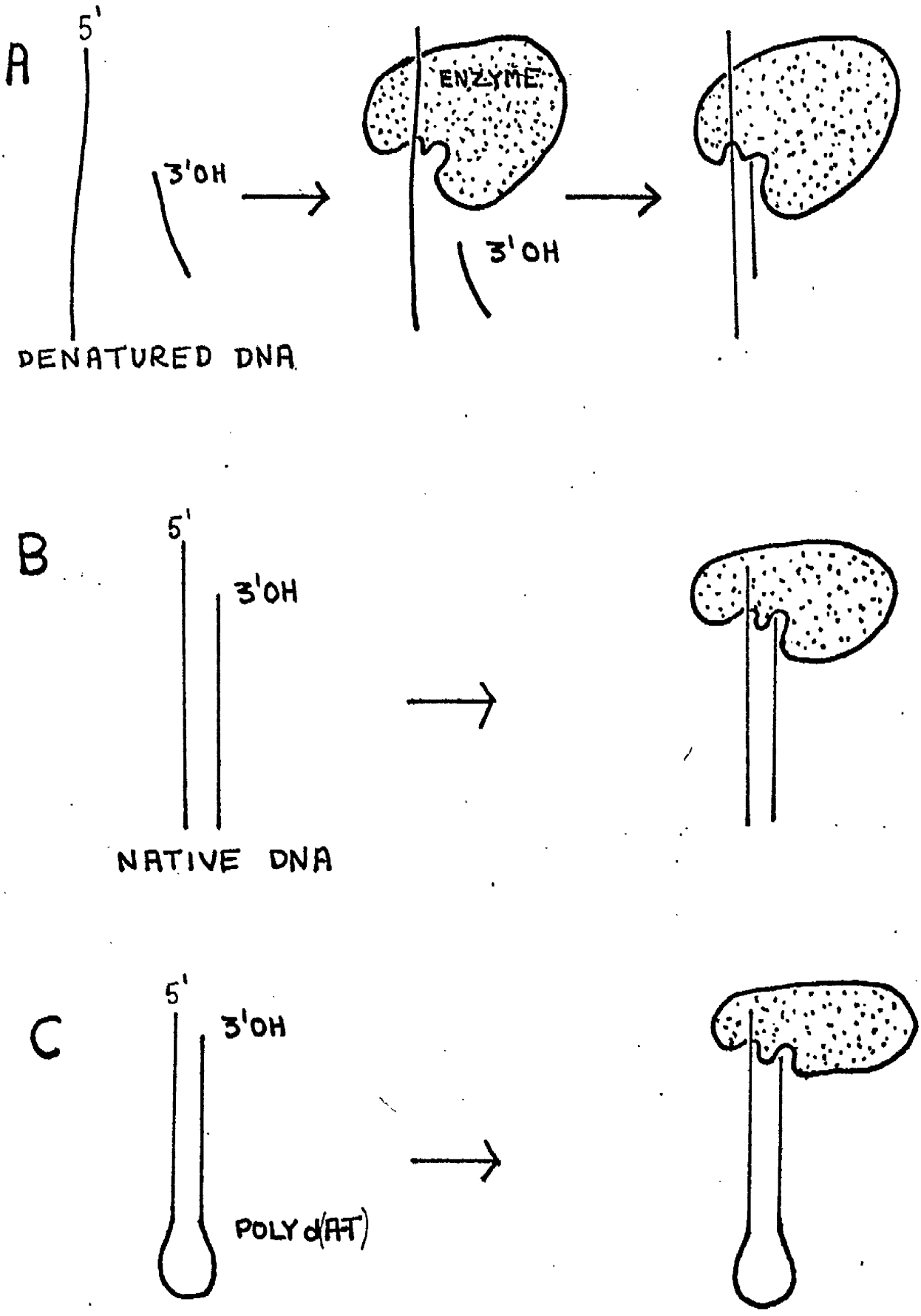
has some regions of complementarity to the initiator strand will bind to the polymerase at some point in the middle of its length adjacent to the 3'-OH terminal of the initiator (Fig. 36A).

Oligonucleotides already bound to the DNA or to the polymerase should therefore increase the reaction rate and make the kinetics first order. Oligonucleotides were shown to stimulate polymerase by Keir (1962) and more recent studies by Goulian (1968) and by Steuart et al. (1968b) confirm this.

In the absence of oligonucleotides a denatured DNA molecule can make itself a suitable primer by doubling back on itself forming a loop. This mechanism has been demonstrated for T4 and T5 phage induced polymerases (Goulian et al., 1968; Steuart et al., 1968b) and for the calf thymus enzyme (Bollum 1964; Bollum, 1968). This explains why poly d(A-T) is such a good primer since it can easily either double back on itself or two separate strands could easily anneal with overlapping ends.

A lag phase before commencement of the reaction in the absence of initiator oligonucleotides occurs with the calf thymus enzyme, which is not apparent in the presence of initiator oligonucleotides and reflects the time required for the DNA to double back along itself (Bollum, 1968). A slight lag phase or concave upwards time curve has been observed for ascites polymerase (Fig. 8) though this was not apparent if the reaction was commenced by adding enzyme, previously heated to 37°, to the other reaction constituents previously heated to 37°(Fig. 20). The total lag

FIGURE 36



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phase is unlikely to be caused by the reaction medium taking a long time to reach 37° (normally the reaction was commenced by transferring the assay tubes from ice to 37°). Heating the DNA to 37° prior to the commencement of the reaction by the addition of polymerase would allow partial association of the DNA and hence reduce or remove the lag phase.

The native DNA concentration curve though not quite linear continues to show increase in reaction rate with increase in DNA concentration to at least 170 µg. (Fig. 29). This suggests that the reaction is merely the repairing of single stranded ends in the native DNA and that "effective template" concentration curves of native and denatured DNA might be similar. Second order kinetics are not observed with native DNA since one double stranded DNA is bound to the enzyme so that each of the two binding sites is occupied by one strand (Fig. 36B).

Also the apparent discrepancy between the Esch. coli DNA polymerase having one binding site for DNA (Englund, et al., 1968) compared with two for ascites DNA is resolved since the DNA used to measure the binding with Esch. coli polymerase was poly d(A-T) in a hairpin conformation. The polymerase could then bind the one DNA molecule at two sites, one site per DNA strand (Fig. 36C).

VI The reaction product

The CsCl density gradient centrifugation of the product revealed it to contain much low molecular weight oligonucleotide since most of the radioactivity did not sediment very far down the

tube. Centrifugation was terminated before equilibrium so that separation on the basis of size as well as buoyant density was achieved. The radioactivity was acid-precipitatable since the method of assay demanded this. DEAE cellulose chromatography of the product before being applied on top of the CsCl solution confirmed the presence of oligonucleotides and chromatography of the radioactivity in some of the fractions after centrifugation confirmed that it was due to oligonucleotides. Furlong (1966) showed that oligonucleotides of length greater than 30 nucleotides remained at the origin. A broad double peak near the origin was formed when the product before centrifugation was subjected to chromatography on DEAE cellulose. About half the product was at the origin and about half had moved slightly and therefore was less than 30 nucleotides in length. In a previous CsCl centrifugation of the product, the product was dialysed extensively for 40 hours to remove non-incorporated radioactive dTTP. It was found that very little radioactivity remained after dialysis suggesting that the dialysis tubing was permeable to the low molecular weight product.

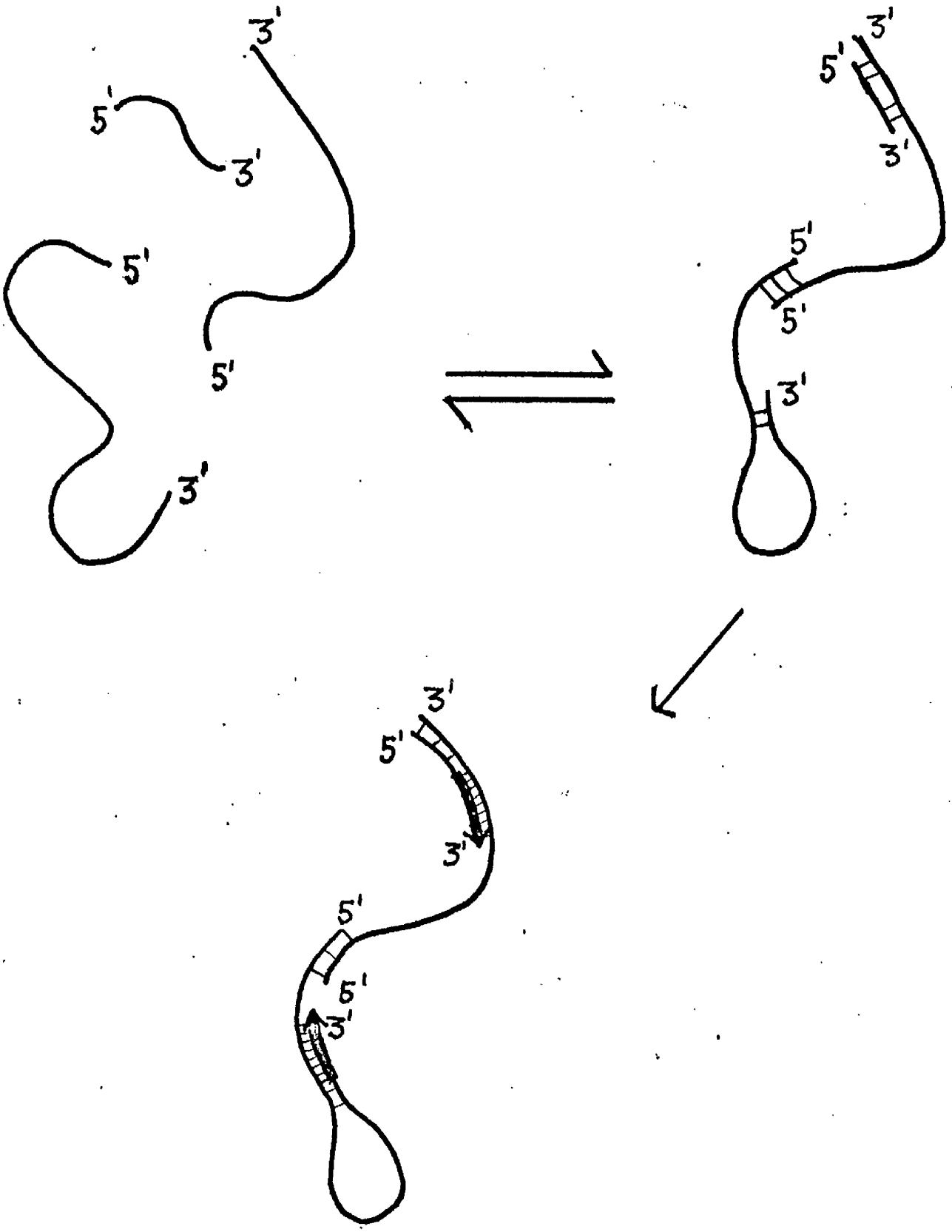
In these experiments the enzyme used had only been subjected to chromatography on Sephadex G-150 once and so contained significant DNase. Since long periods of five hours incubation were used substantial degradation of DNA probably occurred. Most of the product appeared to be separable from the bulk of the primer. These small molecular weight products might also be caused by syn-

thesis on small oligonucleotides in spite of the primer being salmon testes DNA in place of the degraded ascites DNA. Since the product was of low molecular weight under these conditions, it was difficult to determine whether it was native or denatured.

Therefore further studies were performed using polymerase which had been recycled through Sephadex, and the products after 1, 2 and 3 hours were investigated using the polyethylene glycol, dextran two phase system (Results VIC). Using this technique the product was shown to be double-stranded in spite of there being only 10% replication. This may be due to the denatured DNA molecules partially annealing forming suitable primers. This annealing may be very unstable initially so that the DNA separates into the lower layer in the control, but after a little DNA synthesis most of the primer and product DNA will behave as double stranded (Fig. 37 - the thick lines represent the newly synthesised product). Bollum (1968) found that all the primer DNA was complexed into a non-single-stranded form at only 50% replication using calf thymus polymerase. He postulated a triple-stranded form containing one product molecule and two primer molecules. Such a complex may also exist in the ascites system explaining why all the primer behaves as double-stranded DNA after only 10% replication.

Therefore, although it is now established that the product of the ascites polymerase reaction is double-stranded, further studies to ascertain its length and whether it is covalently bound to the primer require a well characterised primer, such as poly

FIGURE 37



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d A of homogeneous length, and an enzyme which is of high specific activity and 100% free of DNase.

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