Studies on the enzymic synthesis of DNA in soluble systems derived from mammalian cells

by

James Bell Shepherd. B.Sc.

The main feature of this work was the characterisation of synthesis of DNA by cell-free preparations derived from the Landschutz ascites carcinoma of the mouse. DNA synthesis was measured by the incorporation of (^{32}P)-dTMP from (α-^{32}P)-dTTP into polydeoxyribonucleotide material in the presence of dATP, dGTP, dCTP and DNA-primer. This enzymic reaction was termed replicative DNA synthesis. The incorporation of (^{32}P)-dTMP from (α-^{32}P)-dTTP on to the ends of primer DNA molecules in the absence of the other three deoxyribonucleoside 5'-triphosphates was also investigated. This was termed the terminal addition reaction.

DNA nucleotidyltransferase (E.C. 2.7.7.7.), the enzyme that catalyses the replicative reaction was readily obtained in soluble form, from the tumour cells. It was purified 12-fold by conventional enzyme fractionation techniques and freed from deoxyribonuclease by subsequent chromatography on hydroxylapatite. The enzyme fractions studied exhibited negligible phosphatase activity under conditions optimal for DNA nucleotidyltransferase activity, and ancillary techniques have shown an absolute requirement for primer DNA, heat-denatured DNA being 5-10 times more effective than native DNA. The presence of a bivalent cation was also found to be essential for enzymic activity; Mg^{2+} ions were greatly

superior to Mn^{2+} ions in this respect, while Ca^{2+} ions did not support enzyme activity. Optimal enzyme activity was observed in the pH range, 7.2 - 7.5, and the univalent cations, K^+ and Na^+, were shown to stimulate incorporation of nucleotide to variable degrees.

The presence of EDTA at 0.4mM in the assay system markedly stimulated enzymic activity, and preparation of the enzyme in 0.001M-EDTA-0.005M-2-mercaptoethanol allowed storage for two months at 0° without loss of activity. It would appear that these substances might play some role in maintenance of the integrity of the enzyme.

Optimal activity was attained in the presence of equal amounts of all four deoxyribonucleoside 5'-triphosphates, (dATP, dGTP, dCTP and dTTP). Replacement of one, two or three of these, by the corresponding mono-or diphosphates, the fourth being (α-^{32}P)-dTTP, gave a reduction of synthesis; the residual level of incorporation is suggested to be attributable to the action of enzymes capable of catalysing trans-phosphorylation reactions.

The product of enzymic activity was shown to be high molecular weight polynucleotide material, and studies on the incorporation of (α-^{32}P)-dTTP, - dGTP and - dATP suggest that the pattern of base incorporation into product DNA depends on the base ratios of the DNA-primer. In addition, the rate of DNA synthesis in these preparations appears to be commensurate with the <u>in vivo</u> DNA synthetic rate calculated from data cited in the literature. Therefore, it would seem probable that the enzyme studied in this work is well qualified to fulfil the function

of DNA replication *in vivo*.

Concurrent studies were conducted with DNA nucleotidyltransferase preparations from calf thymus gland for purposes of comparison with the enzyme derived from the tumour cells. The thymus enzyme, when subjected to the same fractionation techniques behaved in a manner similar to the Landschutz enzyme; it also exhibited similar requirements for optimal DNA nucleotidyltransferase activity. However, a survey of the *replicative* DNA nucleotidyltransferase and *terminal* addition activities from several mammalian tissues revealed that calf thymus exhibited much more *terminal* activity relative to *replicative* activity than did the following rodent tissues, rabbit spleen, liver, thymus and appendix, and rat spleen and liver. Preparations of DNA nucleotidyltransferase from Landschutz ascites-tumour cells displayed very low levels of *terminal* addition activity.

Actinomycin D was shown to inhibit DNA nucleotidyltransferase from Landschutz ascites-tumour cells in a competitive manner and it was found that *replicative* activity was much more sensitive to this inhibition than was *terminal* addition.

The inhibition of DNA nucleotidyltransferase from Landschutz ascites-tumour cells by iodoacetamide, iodoacetate, p-hydroxymercuribenzoate and sarkomycin obeyed non-competitive kinetics. Inhibition by iodoacetamide and iodoacetate was irreversible and that by p-hydroxymercuribenzoate was reversed by glutathione or 2-mercaptoethanol, while inhibition by sarkomycin was only partially released by the same procedures. The *terminal* addition reaction catalysed by the Landschutz enzyme preparations

was also inhibited by these compounds, but to a lesser extent.

Studies on the heat activation of the enzyme by prior incubation at 45°, preliminary kinetic studies involving two types of double reciprocal plots ($1/v$ against $1/s$ and $1/v$ against $1/s^2$), and more detailed investigation of the ionic requirements of the enzyme were carried out. Collectively, these studies offer some support for the concept, formed during this work, that <u>in vivo</u> DNA nucleotidyltransferase may be a highly organised entity containing two or more independently active, easily dissociable subunits.

Abbreviations:-

DNA	Deoxyribonucleic acid
dATP	Deoxyadenosine 5'-triphosphate
dGTP	Deoxyguanosine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
dTTP	Deoxythymidine 5'-triphosphate
(^{32}P)-dTMP	Deoxythymidine 5'-monophosphate labelled with a radioactive phosphorus atom
(α-^{32}P)-dTTP	Deoxythymidine 5'-triphosphate labelled with a radioactive phosphorus atom in the position, similarly for the other deoxyribonucleoside triphosphates.
EDTA	Ethylenediamine tetraacetic acid

References:-

Biochem, J. (1963). 89, 9P; 89, 425

Biochem, J. (1965). 94, 5P.

Biochem, J. (1965). in the press.

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ABBREVIATIONS

The following abbreviations will be used in this thesis:-

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
sRNA	"Soluble" or "transfer" ribonucleic acid
UMP	Uridine 5'-monophosphate
UDP	Uridine 5'-diphosphate
UTP	Uridine 5'-triphosphate
CMP	Cytidine 5'-monophosphate
CDP	Cytidine 5'-diphosphate
CTP	Cytidine 5'-triphosphate
AMP	Adenosine 5'-monophosphate
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
GMP	Guanosine 5'-monophosphate
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'-triphosphate
IMP	Inosine 5'-monophosphate
dUMP	Deoxyuridine 5'-monophosphate
dUDP	Deoxyuridine 5'-diphosphate
dUTP	Deoxyuridine 5'-triphosphate
dCMP	Deoxycytidine 5'-monophosphate
dCDP	Deoxycytidine 5'-diphosphate
dCTP	Deoxycytidine 5'-triphosphate

dAMP	Deoxyadenosine 5'-monophosphate
dADP	Deoxyadenosine 5'-diphosphate
dATP	Deoxyadenosine 5'-triphosphate
dGMP	Deoxyguanosine 5'-monophosphate
dGDP	Deoxyguanosine 5'-diphosphate
dGTP	Deoxyguanosine 5'-triphosphate
dTMP	Deoxythymidine 5'-monophosphate
dTDP	Deoxythymidine 5'-diphosphate
dTTP	Deoxythymidine 5'-triphosphate
dBrUTP	Deoxybromouridine 5'-triphosphate
dAzGTP	8-azadeoxyguanine-5'-triphosphate
PRPP	5-phosphoribosyl-1-pyrophosphate
PRA	5-phosphoribosylamine
AICAR	4-amino-5-imidazole carboxamide ribonucleotide
FAICAR	5-formamino-4-imidazole carboxamide ribonucleotide
GAR	Glycinamide ribonucleotide
FGAR	Formylglycinamide ribonucleotide
AIR	5-aminoimidazole ribonucleotide
(^{32}P)-dTMP	Deoxythymidine 5'-monophosphate labelled with a radioactive phosphorus atom
(α-^{32}P)-dTTP	Deoxythymidine 5'-triphosphate labelled with a radioactive phosphorus atom in the α position; similarly for the other radioactively labelled deoxyribonucleoside 5'-di-and triphosphates
CEP	2-cyanoethylphosphate
(^{32}P)-CEP	2-cyanoethylphosphate labelled with a radioactive phosphorus atom

DCC	Dicyclohexylcarbodiimide
DCU	Dicyclohexylurea
tris	2-amino-2-hydroxymethylpropane-1, 3-diol
EDTA	Ethylenediamine tetraacetic acid
MAK	Methylated serum albumin coated on kieselguhr
DEAE-	Diethylaminoethyl
CM-	Carboxymethyl
GSH	Glutathione
SSC	Standard saline citrate (0.15M-NaCl-0.015M-trisodium citrate)
DNaseI	Deoxyribonuclease I (E.C.3.1.4.5)
DNaseII	Deoxyribonuclease II (E.C.3.1.4.6)
ATPase	Adenosine triphosphatase (E.C.3.6.1.4)
A-T etc.	An adenine residue linked to a thymine residue by hydrogen bonds; similarly for the other base pairs.

CONTENTS

INTRODUCTION

INTRODUCTION

I General Considerations

During the last decade biochemical research has elucidated the mechanisms by which the cell synthesises three of its major component macromolecules, DNA, RNA and protein. Although the simpler aspects of their structures were described earlier, knowledge of the mode of synthesis of these molecules can be said to have originated only in the middle 1950's (Kornberg, Lehman & Simms, 1956; Grunberg-Manago, Ortiz & Ochoa, 1956; Hoagland, 1960).

Since these early discoveries, progress in uncovering the modes of synthesis and the cellular functions of nucleic acids has continued at an ever-increasing rate. As in many fields of biological research, the first understanding of the biosynthesis of DNA was obtained from work with bacteria. In 1956, Kornberg isolated an enzyme, which catalysed the synthesis of DNA, and characterised the requirements of the synthetic reaction in vitro. Since then, the existence of the enzyme has been demonstrated in several mammalian tissues, among the earliest of which were regenerating rat liver (Bollum, 1958a; Mantsavinos & Canellakis, 1958), calf thymus gland (Bollum, 1958b) and Ehrlich ascites-tumour cells (Davidson, Smellie, Keir & McArdle, 1958; Smellie, Keir & Davidson, 1959). However, subsequent reports have included many other systems, several of which are concerned with virus-infected cells (see Introduction, section V). Thus there has emerged a

general pattern of DNA biosynthesis which encompasses mammalian, bacterial and virus-infected cells and which offers attractive hypotheses regarding the replication of the DNA molecule *in vivo*. but many problems remain concerning the precise mechanism and the control of the enzymic process. It is proposed to consider some of these aspects in the succeeding sections of this Introduction.

II Structure of the Nucleic Acids

A. Deoxyribonucleic Acid

DNA is a high molecular weight polymeric substance, which is synthesised by all living systems. Fundamentally it consists of three components, an organic base, a sugar moiety and phosphoric acid. The sugar component is β -2- deoxy D-ribose, and there are four commonly occurring bases, two purines (adenine and guanine) and two pyrimidines (thymine and cytosine). The repeating unit is a nucleotide which is made up of the 5'-phosphoric acid ester of the sugar covalently linked to the base through the carbon atom in position 1 of the furanose ring. Polymerisation occurs by the formation of 3', 5'-phosphodiester bonds between the phosphate of one nucleotide and the sugar of the next, (Fig. 1). Bases other than the four common ones have been found to occur in certain DNA molecules, usually in very small quantities. Dunn and Smith (1958) found trace amounts of 6-methylaminopurine in bacterial DNA. In the T-even series of bacteriophages cytosine is replaced by 5 -hydroxymethylcytosine (Wyatt & Cohen, 1953), often with one or

FIGURE 1

A section of the polynucleotide chain in the DNA molecule.

(From Davidson, J.N., "The Biochemistry of the Nucleic Acids," 4th Ed., Methuen & Co., Ltd., London, 1960).

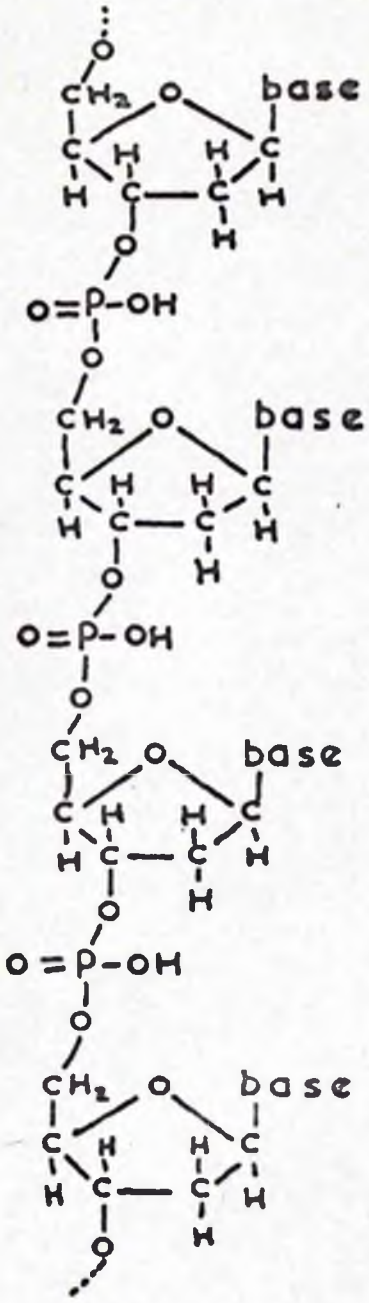


FIGURE 1.

more glucose residues attached to its hydroxymethyl group (Sinsheimer, 1954; Volkin, 1954; Lehman & Pratt, 1960). 5-methylcytosine also replaces certain cytosine residues in DNA from higher animals and plants (Wyatt, 1951; Chargaff, 1955), and it has been shown that hydroxymethyluracil and uracil respectively replace thymine in the DNAs of SP8 and PBSP2 bacteriophages (Marmur & Cordes, 1963).

Analytical evidence (Chargaff, 1955) has shown that, in almost all DNA samples investigated, the number of adenine residues equals that of the thymine residues and the number of guanine residues equals that of the cytosine residues. X-ray diffraction studies of fibres of DNA indicate that the molecule has a helical conformation and give information on the orientation of the bases within the molecule (Wilkins, Stokes & Wilson, 1953; Franklin & Gosling 1953). These observations led Watson and Crick (1953) to propose a model for DNA, in which each molecule consisted of two polynucleotide chains held together by hydrogen bonds between the bases, adenine bonding with thymine and guanine with cytosine. The hydrogen bonding accounts for the equivalence of the purine and the pyrimidine bases (Fig. 2). A space-filling molecular model of DNA (Fig.3) shows that the two chains are twisted together in a double-helical structure which has the bases towards the centre and the sugar phosphate backbones on the outside. Each turn of the double-helix accommodates ten nucleotide pairs and the component chains are arranged in an anti-parallel manner. The three-dimensional model (Fig. 3) also illustrates the two types of

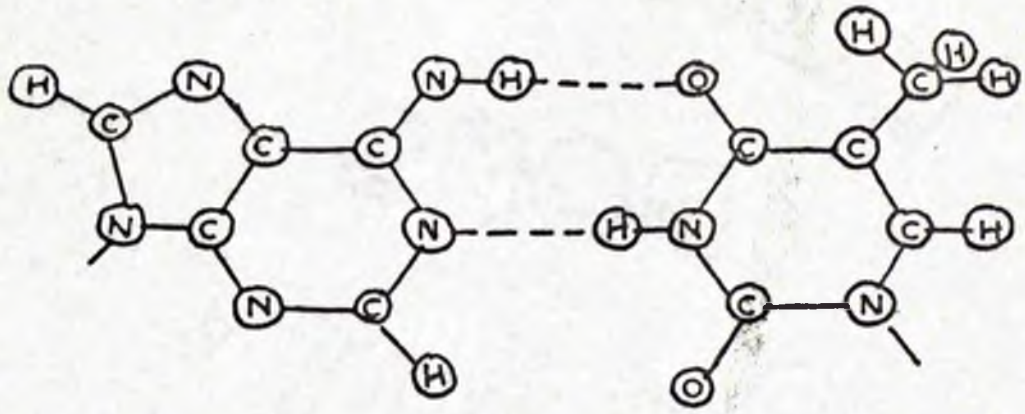
FIGURE 2

The pairing of adenine and thymine and of guanine and cytosine. Dotted lines indicate the hydrogen bonds.

(From Davidson, J. N., "The Biochemistry of the Nucleic Acids," 4th Ed., Methuen & Co., Ltd., London, 1960).

Adenine

Thymine



Guanine

Cytosine

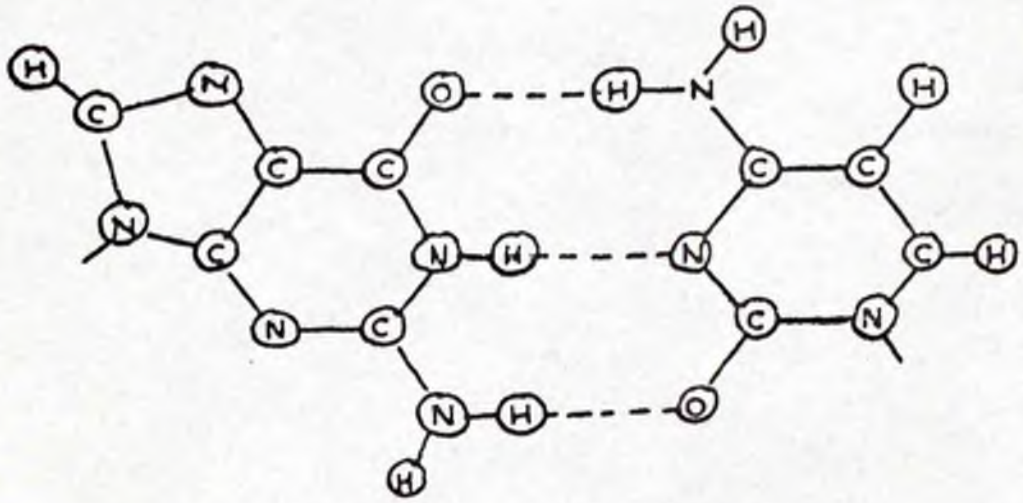


FIGURE 2

FIGURE 3

A model of the structure of DNA.

(After Feughelman, M., Langridge, R., Seeds, W.E., Stokes, A.R., Wilson, H.R., Hooper, C.W., Wilkins, M.H.F., Barclay, R.K. & Hamilton, L.D., (1955). Nature, Lond., 175, 834).

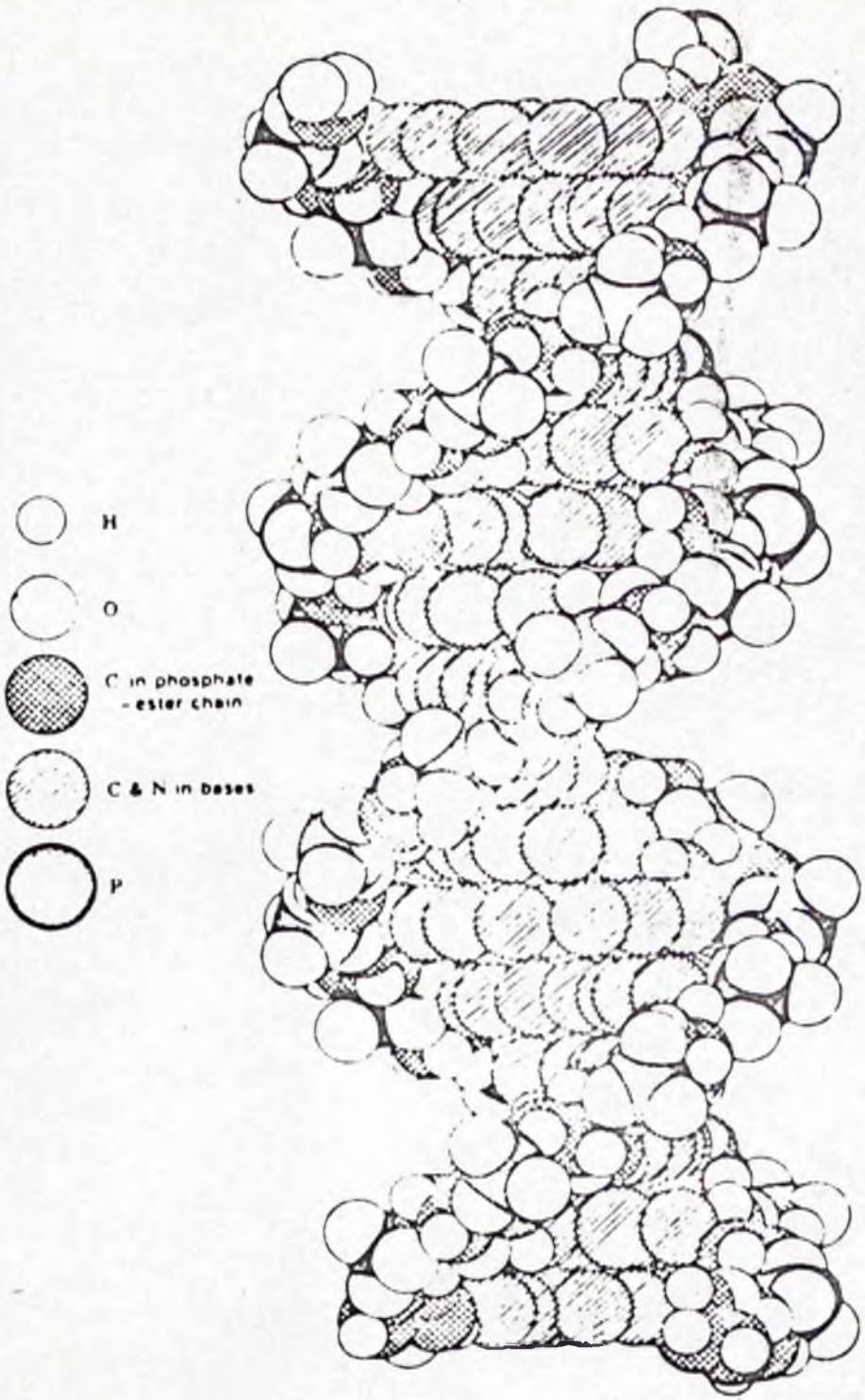


FIGURE 3.

groove arranged helically along the molecule, in such a manner that a narrow groove alternates with a deep groove. Direct evidence for the anti-parallel arrangement has been obtained from nearest neighbour frequency analysis of bases in the chains (Josse, Kaiser & Kornberg, 1961). An important consequence of this structure is that the order of bases in one chain automatically decides the order in the other.

The molecular weight of DNAs isolated from bacteria and higher organisms, by conventional methods, is of the order of 5-10 million. Molecular weights as high as 50 million have been reported for molecules obtained from the T-even bacteriophages (Levinthal, 1956; Thomas 1959), but more recently Levinthal & Davidson (1961) have suggested that the true molecular weight of the T_2 DNA particle is of the order of 100 million and it is thought that this may represent the total DNA of the bacteriophage (Rubenstein, Thomas & Hershey, 1961). Molecular weights of 1.7 million for bacteriophage ØX174 (Sinsheimer, 1959b), 50 million for herpes simplex virus (Russell & Crawford, 1963) and 33 million for bacteriophage lambda (MacHattie & Thomas, 1964), have also been reported.

Further, it appears that the DNAs of several bacteriophages are circular (Fiers & Sinsheimer, 1962; Dulbecco & Vogt, 1963; Crawford, 1964; Thomas & MacHattie, 1964).

Collapse of the helical structure of DNA can be caused by changes in temperature, pH, ionic strength and dielectric constant. This is

due to disruption of hydrogen bonds along the helix and partial or complete separation of the two component strands (Marmur, Rownd & Schildkrau 1963). This loss of double-helical structure induced by these factors is called denaturation. The collapse of the double-helix has been studied using several physical methods including spectrophotometry (Zimm & Kallenbach, 1962), sedimentation and viscosity analyses (Doty, Boedtker, Fresco, Haselkorn & Litt, 1959), optical rotation (Levedahl, 1959; Fresco, 1961) and electron microscopy (Hall & Litt, 1958; Doty, Marmur, Eigner & Schildkraut, 1960). If heat-denatured DNA is allowed to cool slowly it may reform its original double-helix; if however, the cooling is rapid the DNA may remain denatured (Doty et al. ,1960). Separation of native and denatured DNA has been attained by chromatography on MAK columns (Mandell & Hershey, 1960; Sueoka & Cheng, 1962), by countercurrent distribution in aqueous polymer systems (Albertsson, 1962) and by electrophoresis (Shack & Bynum, 1964).

One exception to the double-helical structure of DNA has so far been established. The DNA isolated from the small bacteriophage ØX174 has been shown to be single-stranded (Sinsheimer, 1959a,b).

Determination of the sequence of bases in DNA has proved a very difficult problem. The nearest neighbour frequency analysis of Josse et al., (1961) gives an estimate of the frequencies of individual dinucleotides. Chemical methods have been developed for studying the frequencies of sequences of pyrimidine nucleotides occurring

between two purine nucleotides (Burton & Peterson, 1960; Burton & Lunt, Peterson & Siebke, 1963).

Under certain in vitro conditions of enzymic synthesis by the bacterial system, a polymer composed of perfectly alternating sequences of deoxyadenylate and deoxythymidylate, poly d(A-T), is formed. Under slightly different conditions, another polymer, poly (dG) : (dC), is formed (Shhohman, Adler, Radding, Lehman & Kornberg, 1960; Radding, Josse & Kornberg, 1960). Both molecules have the properties of high molecular weight double-helices. The poly d(A-T) is a double helical molecule composed of two anti-parallel strands, each of which contains deoxyadenylate and deoxythymidylate, apparently in perfectly alternating sequence. On the other hand the double-helical poly (dG) : (dC) is made up of one strand, which is a homopolymer of deoxyguanylate and one strand, which is a homopolymer of deoxycytidylate and contains no heterogeneous covalent linkages. A polymer similar to the poly d(A-T) copolymer, but containing about 2% d(G,C), has been detected as a minor component of the DNA of various tissues of the marine crab (Cheng & Sueoka, 1964; Smith, 1964).

B. Ribonucleic Acid

RNA is a polymer similar to DNA, but differing in several important respects. The monomer unit consists of a base, a sugar and phosphoric acid linked as in DNA (Fig. 1). In this case the sugar is β -D- ribose and the common bases are adenine, guanine, cytosine and uracil. Like the bases of DNA those of RNA occasionally number among

themselves trace amounts of modified bases. sRNA, for example, contains significant amounts of 5-ribofuranosyluracil (pseudouridine) (Davis & Allen, 1957; Cohn, 1960). Among other minor bases found in RNA are thymine, 5-methylcytosine, 1-methyladenine, 2-methyladenine, 6-methylaminopurine, 6-dimethylaminopurine, 1-methylguanine, 6-hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethylaminopurine (Adler, Weissman & Gutman, 1958; Dunn, Smith & Spahr, 1960; Dunn, 1959; Dunn, 1961).

RNA also differs from DNA in its secondary structure which is much less rigid and well-defined (Rich & Watson, 1954). sRNA from yeast has been shown to have regular DNA-like double-helical regions (Spencer, Fuller, Wilkins & Brown, 1962; Luborsky & Cantoni, 1962; Brown, 1963). Double-stranded RNA with the appropriate base ratios has been described in mature reovirus (Gomatos & Tamm, 1963). Further, during the replication of certain viruses, which apparently contain single-stranded RNA, the RNA passes through a double-stranded phase (Montagnier & Saunders, 1963; Baltimore, Becker & Darnell, 1964; Weissman, Borst, Burdon, Billeter & Ochoa, 1964; Langridge, Billeter, Borst, Burdon & Weissman, 1964). However, it would appear that, with these exceptions, RNA is for the most part composed of long single-stranded polynucleotide chains, and any secondary structure which may exist arises from intramolecular hydrogen bonding between adenine and uracil on the one hand, and guanine and cytosine on the other.

III Function of DNA

The role of DNA, as the molecule which determines and maintains genetic characteristics, has been well established by experiments which demonstrate its transforming ability (Avery, MacLeod & McCarty, 1944; Hershey, 1953; Hotchkiss, 1957). In order to fulfil this role the DNA molecule must perform two functions. Firstly, it must be able, through the medium of a chemical code, to direct the development of the cell according to its inheritance, and secondly, it must have the ability to reproduce itself exactly, within the cell, for the transmission of this inheritance to future generations (Kornberg, 1961).

The fulfilment of the first of these roles, through DNA-directed synthesis of RNA and of protein, is becoming more fully understood (Hurwitz & August, 1963; Lipmann, 1963). It is now well established that the chemical code resides in the order of the bases in the DNA molecule (Crick, 1963).

The structure of DNA is particularly suited for its second role, since the order of the bases in one chain automatically determines the order in the complementary chain. This has led to various theories for the mechanism of replication of DNA *in vitro*, all of which are based on the complementarity rule (Richardson *et al.*, 1963a; Baldwin & Shooter, 1963; Cavalieri & Rosenberg, 1963; Cavalieri, 1963). Within the cell the problem of DNA replication mechanisms becomes more complex because of the association of protein with DNA. Nevertheless, the work of Cairns (1963a,b) and Nagata (1963) has created much understanding of the *in vivo* replication of the bacterial chromosome. The

mammalian chromosome is even more complex, and with the techniques at present available it is almost impossible to distinguish the fine structure of the DNA during mitosis.

The constancy of the amount of DNA in, e.g., all the somatic diploid cells of a given mammalian species, and the remarkable metabolic stability of DNA are properties which are consistent with its role within the living cell. (Davidson & Leslie, 1950).

IV Biosynthesis of the Component Nucleotides of DNA

The biosynthesis of the dexyribonucleoside triphosphates which are polymerised in the DNA synthetic reaction involves three stages, which can be conveniently described in the following order A) Biosynthesis of ribonuoleotides, B) Reduction of the ribonucleotides to deoxyribonucleotides and C) Phosphorylation of the deoxyribonucleotides to the triphosphate level.

A. Biosynthesis of the Ribonucleoside 5'-monophosphates

Purine and pyrimidine bases are synthesised from their component parts in covalent attachment to a ribose 5'-monophosphate unit and the end product of the synthetic pathway in both cases is the ribonuoleoside 5'-monophosphate and not the free base.

1) Biosynthesis of the Purine Ribonucleoside 5'-monophosphates

De novo biosynthesis of purine ribonucleotides has been reviewed by Buchanan (1960). The mode of synthesis of these molecules has been elucidated by investigation in many biological systems, but the greatest contributing factor to the full characterisation of the pathway

was that all the enzymes of purine synthesis can be obtained in a soluble form and in relatively high activity from avian liver. The pathway is described in detail in Fig. 4. IMP, the first complete purine nucleotide formed in the reaction sequence, is the precursor of both purine ribonucleotides commonly found in RNA, namely AMP and GMP (Buchanan, 1960).

2) Biosynthesis of the Pyrimidine Ribonucleoside 5'-monophosphates

The mechanisms of formation of pyrimidine ribonucleotides within the cell have been investigated more extensively in bacterial systems than in mammalian systems. Fig. 5 outlines the pathway of pyrimidine nucleotide synthesis, which has been reviewed by Reichard (1959) and Crosbie (1960). The final product of the synthetic sequence is UMP, which has been shown to be a precursor of both CMP and dTMP (Crosbie, 1960). Uridine is converted to cytidine at the level of the nucleoside di - or triphosphate (Lieberman, 1956; Kammen & Hurlbert, 1959). The conversion of UMP to dTMP is described in section IV B of the Introduction.

B. Reduction of Ribonucleotides to Deoxyribonucleotides

Although enzymic reactions catalysing the condensation of glyceraldehyde 3'-phosphate to produce 2-deoxyribose 5'-phosphate have been observed in Escherichia coli (Racker, 1951; Racker, 1952), and in rat liver (Boxer & Shonk, 1958), most evidence favours the mechanism by which deoxyribonucleotides are formed by reduction of ribose at the ribonucleotide level. Kornberg (1957a) could find no evidence for the existence of a 5'-phosphodeoxyribosyl pyrophosphate derivative, which

FIGURE 4

The enzymic synthesis of inosinic acid de novo.

(After Davidson, J. N. "The Biochemistry of the Nucleic Acids", 4th Ed., Methuen & Co., Ltd., London (1960).

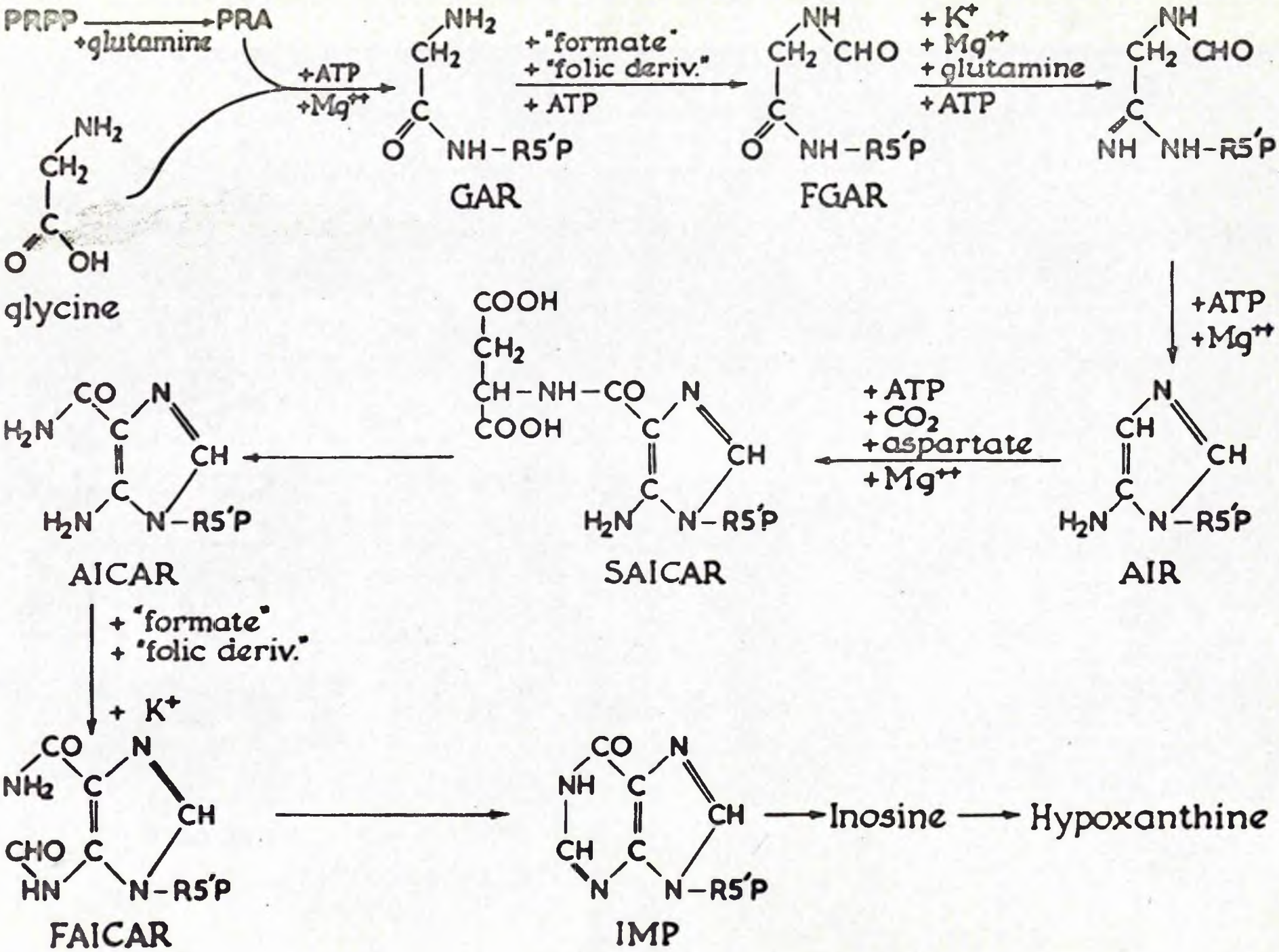

PRPP
+glutamine
PRA
+ATP
+Mg++
glycine
GAR
+"formate"
+"folic deriv."
+ ATP
FGAR
+ K+
+ Mg++
+glutamine
+ATP
+ATP
+Mg++
AIR
+ ATP
+ CO2
+aspartate
+Mg++
SAICAR
AICAR
+ "formate"
+ "folic deriv."
+ K+
FAICAR
IMP
Inosine
Hypoxanthine

FIGURE 4

FIGURE 5

The enzymic synthesis of uridylic acid *de novo*.

(After Birnie, G.D. (1959) Ph. D. Thesis, The University, Glasgow).

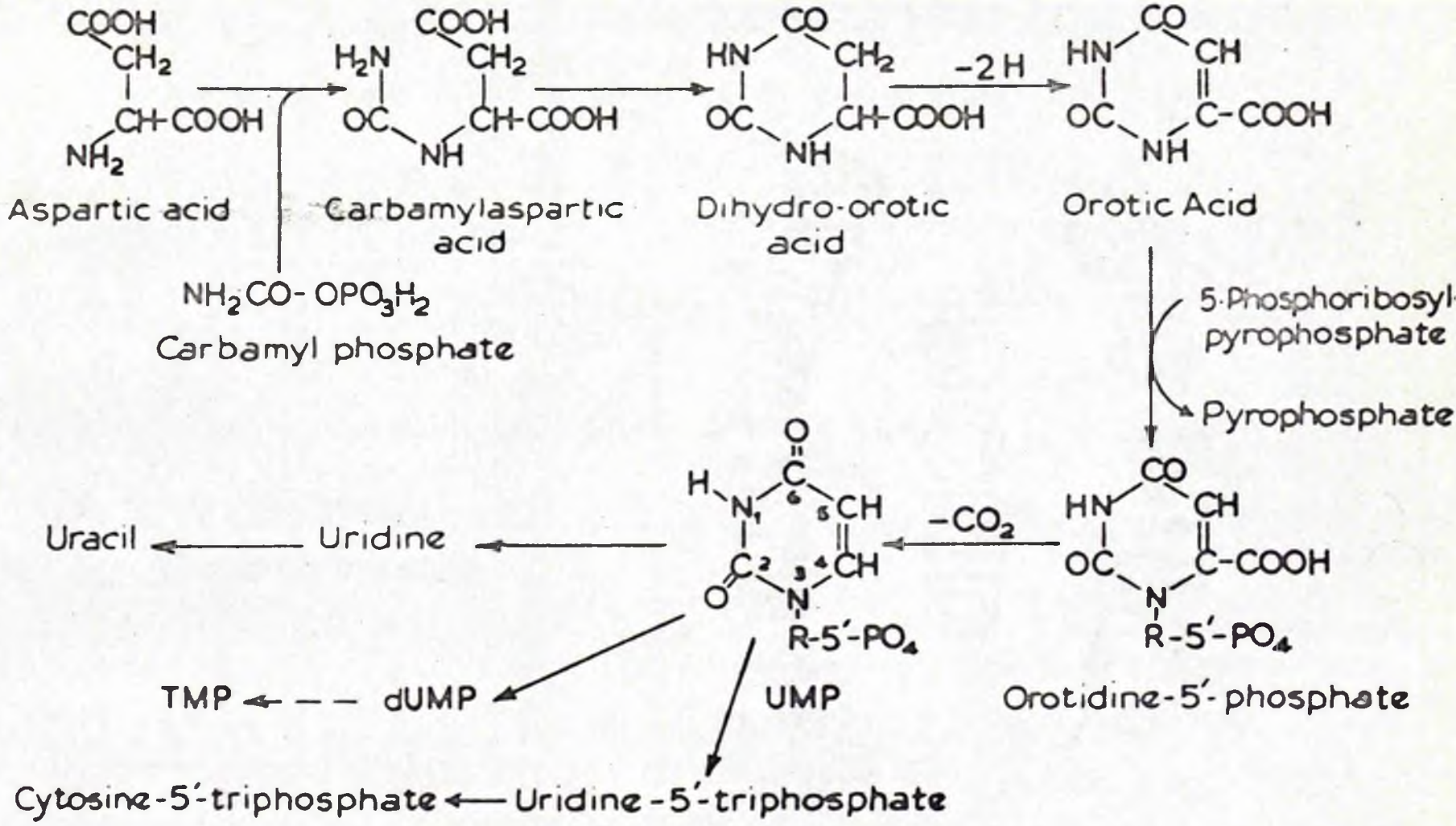

Aspartic acid
Carbamylaspartic acid
Dihydro-orotic acid
Orotic Acid
-2 H
Carbamyl phosphate
5-Phosphoribosyl-pyrophosphate
Pyrophosphate
Uracil
Uridine
-CO2
R-5'-PO4
Orotidine-5'-phosphate
TMP
dUMP
UMP
Cytosine-5'-triphosphate
Uridine-5'-triphosphate

FIGURE 5

might correspond with the role played by PRPP in purine and pyrimidine nucleotide synthesis (See Figs. 4 & 5). Besides, there is a great deal of evidence that the reduction of the ribose moiety occurs when the sugar is covalently bound to the base (Rose & Schweigert, 1953; Roll, Weinfeld & Carroll, 1956; Reichard, 1958). Enzymes, which catalyse the reduction of cytidine and uridine at the nucleoside diphosphate level have been demonstrated in E. coli B and Pseudomonas saccharophila (Reichard, Baldesten & Rutberg, 1961; Bertani, Haggmark & Reichard, 1961; Bertani, Haggmark & Reichard, 1963; Fossit & Bernstein, 1963). Apparently, the purine ribonucleotides are reduced to the corresponding deoxyribonucleotides in a similar manner at the nucleotide diphosphate level (Reichard, 1960; Larsson, 1963).

Early work indicated that UMP was also a precursor of dTMP (Reichard, 1949; Green & Cohen, 1957), and the most important problems became those of the origin of the 5 -methyl group in thymine, and the levels of nucleotide phosphorylation and reduction at which the methylation takes place. It was shown that the conversion of both dUMP and dCMP to dTMP occurs at the nucleotide level (Reichard & Estborn, 1951; Rose & Schweigert, 1953; Amos & Magasanik, 1957), and that the β-carbon of serine and the α-carbon of glycine are incorporated into the 5'-methyl group of thymine (Elwyn & Sprinson, 1950). Totter (1954) demonstrated that formate and formaldehyde could also provide the one-carbon unit, N^5, N^{10} - methylenetetrahydrofolic acid being the active intermediate in the transfer, which also involved the action of the

enzyme serine hydroxymethylase on serine (Jaenicke, 1956). A deoxyuridine derivative was shown to be the one-carbon unit acceptor in rat liver (Reichard, 1955). in E. coli (Friedkin & Kornberg, 1957), and in calf thymus (Greenberg, Nath & Humphreys, 1961; Whittaker & Blakley, 1961; Jenny & Greenberg, 1963).

Finally, the whole pathway in the formation of dTMP from UMP was elucidated by the discovery of enzymes which catalyse the reduction of CDP to dCDP (Reichard & Rutberg, 1960), and of deaminases which convert dCMP to dUMP (Scarano, 1958; Maley & Maley, 1959). Recently a phosphatase specific for dUTP has been discovered in E. coli B. The products of the cleavage are dUMP and pyrophosphate and this may be the mechanism by which dUTP is excluded from the DNA polymerisation reaction, the remaining dUMP being used for dTMP formation (Bertani et al., 1961; Reichard, 1962; Bertani et al., 1963), (see Fig. 6).

C. Phosphorylation of Deoxyribonucleotides

The initial products of the deoxyribonucleoside biosynthetic pathways are the deoxyribonucleoside monophosphates (see Figs. 4 & 5). However, as the immediate precursors of DNA synthesis in all characterised biological systems are the deoxyribonucleoside triphosphates (see Introduction, section V A3), a description of the phosphorylation of deoxyribonucleotides to the triphosphate level is required.

Enzymes have been demonstrated which catalyse the formation of deoxyribonucleoside triphosphates from deoxyribonucleoside monophosphates before incorporation into DNA in E. coli (Bessman, Lehman, Simms, & Kornberg, 1958; Lehman, Bessman, Simms & Kornberg, 1958), in ascites-

FIGURE 6

The formation of the four deoxyribonucleoside 5'-triphosphates from their ribonucleoside 5'-monophosphate precursors.

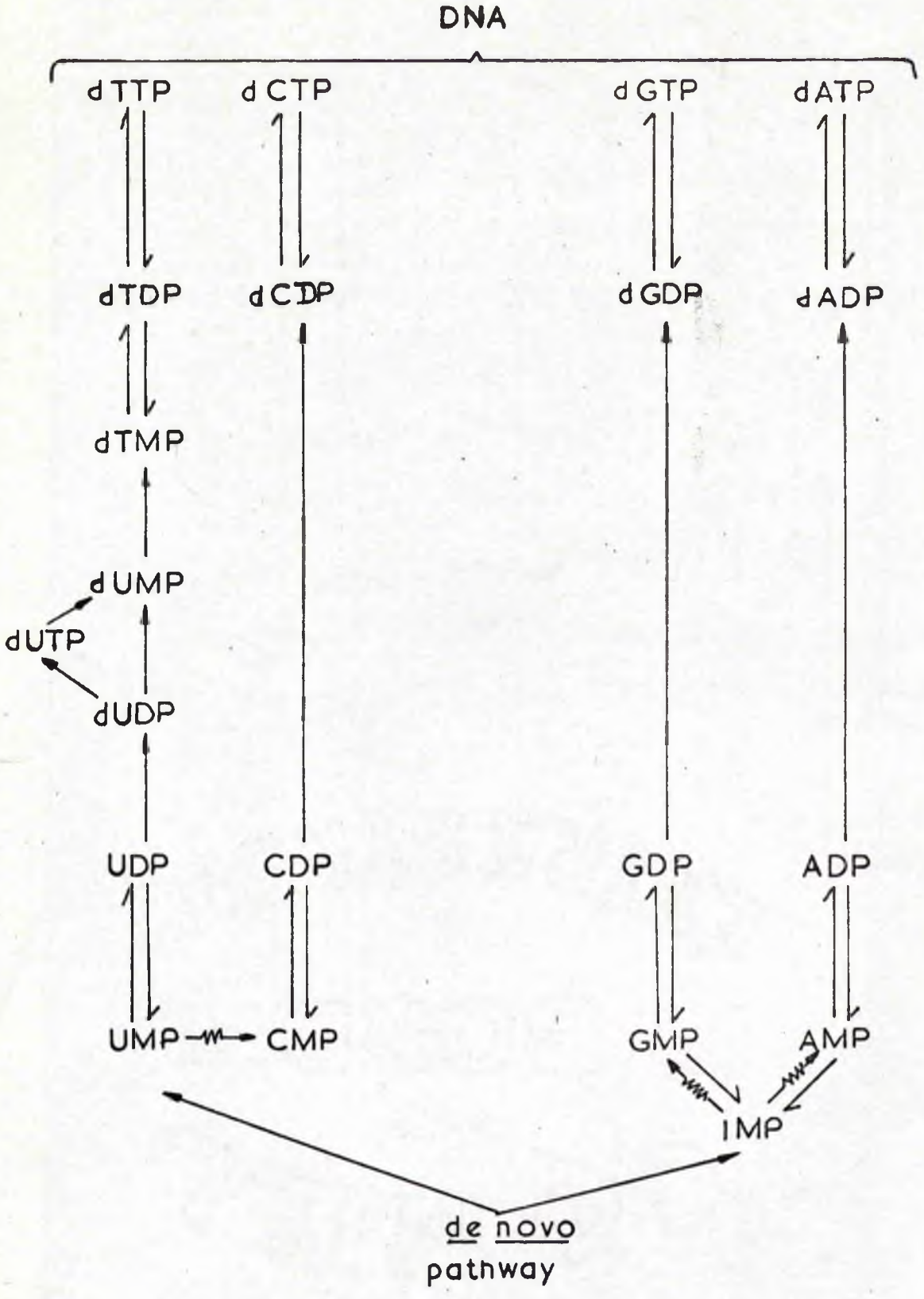

DNA
dTTP
dCTP
dGTP
dATP
dTDP
dCDP
dGDP
dADP
dTMP
dUMP
dUTP
dUDP
UDP
CDP
GDP
ADP
UMP
CMP
GMP
AMP
IMP
de novo
pathway

FIGURE 6

tumour cells (Keir & Smellie, 1959; Coddington & Bagger-Sørensen, 1963) and in rat liver (Hecht, Potter & Herbert, 1954; Canellakis & Mantsavinos, 1958; Bollum, 1958a). At present, evidence strongly suggests that there are kinase systems specific for each of the deoxyribonucleoside monophosphates (Lehman et al. 1958; Bessman, 1963), although the ability of these enzyme systems to distinguish between ribonucleotides and deoxyribonucleotides is still a matter of doubt.

Detection of deoxyribonucleoside diphosphates as intermediates in the phosphorylation sequence has proved a more difficult problem. However, in rabbit kidney cortex mitochondria and muscle, enzymes have been found which are capable of forming both dADP and dATP from dAMP (Sable, Wilber, Cohen & Kane, 1954) and the mono-, di- and triphosphates of deoxythymidine and deoxycytidine have been demonstrated in acid extracts of calf thymus tissue (Potter, Schlesinger, Buettner-Janusch & Thomson, 1957).

The role which dTTP and dTMP may play in the control of DNA biosynthesis has led to extensive investigations into the mechanisms of the phosphorylation reactions involved (Weissman, Smellie & Paul, 1960; Gray et al., 1960). As a result it is now well established that, in ascites-tumour cells, the phosphorylation of dTMP to dTTP proceeds via the intermediate stage of dTDP (Smellie, 1963; Grav & Smellie, 1964). It therefore seems probable that the formation of all four of the deoxyribonucleoside triphosphates involves the intermediate formation of

the appropriate deoxyribonucleoside diphosphate.

Deoxythymidine kinase, which catalyses the phosphorylation of deoxythymidine to dTMP has been widely reported (Weissman et al. 1960; Wanka, Vasil & Stern, 1964; Okasaki & Kornberg, 1964a, b). Fig. 6 summarises the formation, from the ribonucleoside monophosphate stage, of the four deoxyribonucleoside triphosphates commonly involved in DNA biosynthesis.

V Biosynthesis of DNA

A. General Considerations

1) History

The demonstration that DNA synthesis is a necessary prerequisite of cell division was accomplished twenty or so years ago by the use of cytological and histological techniques. There then followed a period of stagnation in the field of DNA biosyntheses, until more became known about the structure of DNA itself. The necessary information was accumulated over an interval of years and culminated in the publication of a proposed structure for DNA (see Introduction, section II). This not only provided a satisfactory molecular explanation for heredity, but also furnished enzymologists with a working hypothesis as a basis for investigation of the enzymic synthesis of DNA. Kornberg and his co-workers, using extracts of E. coli, were the first group to demonstrate the existence of a DNA-synthesising enzyme. This discovery was the culmination of several years spent in the investigation of the biosynthesis of purine and pyrimidine nucleotides and of nucleotide coenzymes, such as

flavin adenine dinucleotide and nicotinamide adenine dinucleotide, where the mechanism of the formation of the internucleotide bond helped in the understanding of polynucleotide synthesis (Kornberg, 1957a,b; Kornberg, 1959). During this time it was concluded that deoxyribonucleotides, probably deoxyribonucleoside triphosphates, were the immediate precursors of DNA. The first full paper on the subject was published in 1958 (Lehman et al., 1958), although earlier preliminary reports were available (Kornberg, Lehman & Simms, 1956; Kornberg, Lehman, Bessman & Simms, 1956; Bessman, Lehman, Simms & Kornberg, 1958a) The enzyme was termed DNA polymerase (Lehman et al. 1958), but will be referred to here as DNA nucleotidyltransferase (EC 2.7.7.7. deoxynucleoside triphosphate-DNA deoxynucleotidyltransferase).

2) Distribution of the Enzyme

DNA nucleotidyltransferase, the enzyme which catalyses the synthesis of DNA from deoxyribonucleotide precursors, has been observed in many biological systems. In bacteria it has been most extensively investigated in E. coli and Bacillus subtilis (Richardson et al., 1964a; Okazaki & Kornberg, 1964). The work with E. coli was quickly followed by reports of the enzyme in several mammalian tissues (Harford & Kornberg, 1958), among the earliest of which were regenerating rat liver (Bollum & Potter, 1957; Bollum, 1958a, b; Mantsavinos & Canellakis, 1958), calf thymus (Bollum, 1960) and Ehrlich ascites-tumour cells (Davidson, Smellie, Keir & McArdle, 1958; Smellie, Keir & Davidson, 1959; Smellie et al., 1960). More recently, the enzyme has

also been studied in cell free extracts of, or partially purified preparations from rat thymus gland, sea urchin embryos, mouse fibroblast L cells, cultured rabbit kidney cortex cells and Walker carcinoma. DNA nucleotidyltransferase has also been widely investigated in virus-infected cells. T_2 bacteriophage infection of *E. coli* has been shown to promote the synthesis of a new DNA nucleotidyltransferase different from that of the host cells (Aposhian & Kornberg, 1962). On the other hand *E. coli* cells, which have been lysogenically induced to form bacteriophage do not, on the basis of several criteria, elaborate a new enzyme (Priser & Weissbach, 1964). The DNA nucleotidyltransferase activities of cells infected by several mammalian viruses have also been investigated (see Introduction, section V B2 c).

3) The General Reaction

Kornberg and his collegues were the first group to specify the requirements for, and possible mechanism of, the reaction catalysed by DNA nucleotidyltransferase. However, all the enzymes mentioned in the previous section, in so far as they have been characterised, seem to be analogous to the *E. coli* enzyme (Kornberg, 1961). Briefly, the reaction can be described by the equation shown in Fig. 7. The course of the reaction may be followed by incorporation of radioactive label from a ^{14}C-labelled precursor deoxyribonucleoside triphosphate or an α-^{32}P deoxyribonucleoside triphosphate into an acid-insoluble product.

For net synthesis of DNA the enzyme requires all four of the

FIGURE 7

The chemical representation of DNA biosynthesis.

(After Lehman, I.R., Bessman, M.J., Simms, E.S. & Kornberg, A. (1958). J. biol. Chem., 233, 163).

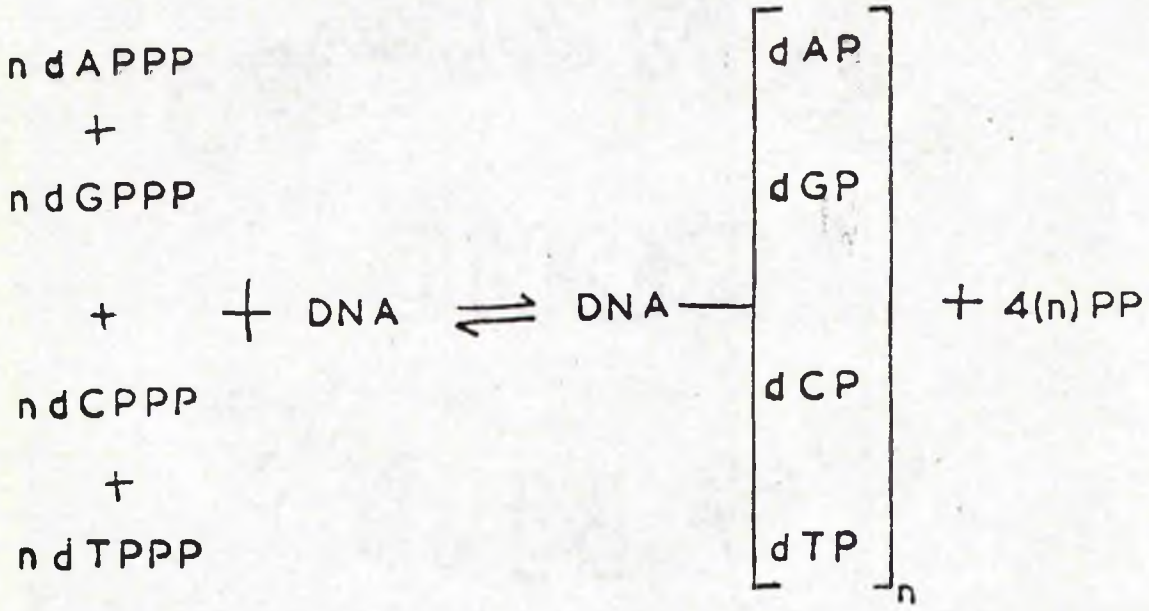


FIGURE 7

deoxyribonucleotides which form adenine-thymine and guanine-cytosine base pairs in DNA. The deoxyribonucleoside triphosphates alone are active, only the deoxy sugar compounds being incorporated into DNA. However, purified preparations of the enzyme can also catalyse the incorporation of ribonucleoside monophosphates into DNA in the presence of Mn^{2+} and primer DNA (Berg, Fancher & Chamberlin, 1963). DNA must be present and there is an absolute requirement for a bivalent cation. DNA from plant, bacterial, viral and animal sources serves equally well in the synthetic reaction, provided that its molecular weight is high. The product of the reaction is high molecular weight double-stranded DNA, and inorganic pyrophosphate is released in quantities equimolar to the deoxyribonucleotide incorporated. In the E. coli system native and denatured DNA serve equally as primer in synthesis.

If one of the four deoxyribonucleotide substrates is omitted, the magnitude of the synthetic reaction is decreased more than one hundred-fold. However, under these conditions there still remains addition of a small number of deoxyribonucleotide residues to the 3'-hydroxy-terminal positions of the DNA chains.

The standard reaction does not proceed at all in the absence of DNA primer, and the role which it plays would appear to be that of a template directing the synthesis of exact replicas of itself, rather than that of a simple primer or initiator of polymerisation. The means by which synthesis is directed is the formation of hydrogen-bonded base pairs. Evidence that the product of the reaction is a high molecular

weight, double-stranded copy of the DNA template is both varied and abundant (Shachman et al., 1958; Kornberg, 1961). Substitution in the product of analogues of the common bases, e.g., by replacing dCTP with dBrUTP or dGTP with dAzGTP in the reaction mixture, can only be accomplished if these analogues fulfil the hydrogen-bonding conditions of the natural base (Bessman et al., 1958a). The product of the enzymic reaction has equivalence of adenine to thymine and of guanine to cytosine, and the chemical composition of the product reflects that of the template DNA (Lehman et al., 1958a; Josse et al., (1961). The technique used by Josse et al., (1961) for determining the nearest neighbour frequency analysis of bases in DNA demonstrated conclusively the mechanism of replication by base pairing and antipolarity of the component strands of the product. Thus, from its in vitro properties, it would seem that DNA nucleotidyltransferase from E. coli would have the ability to replicate the DNA molecule faithfully, in vivo.

In his description of the probable mechanism of the formation of the phosphodiester bond, Kornberg (1961) draws an analogy with the formation of the dinucleotide coenzymes. He suggests that the α-phosphate of the nucleoside triphosphate is subjected to nucleophilic attack by the 3'-hydroxyl group at the growing end of the polydeoxyribonucleotide chain together with the elimination of pyrophosphate as is shown in Fig. 8. Evidence which shows that the introduction of 3'-hydroxyl groups into DNA stimulates DNA nucleotidyltransferase activity, while introduction of 3'-phosphoryl

FIGURE 8

Postulated mechanism for extending a DNA chain.

(After Kornberg, A., "Enzymatic Synthesis of DNA," John Wiley & Sons, Inc., New York - London, 1961).

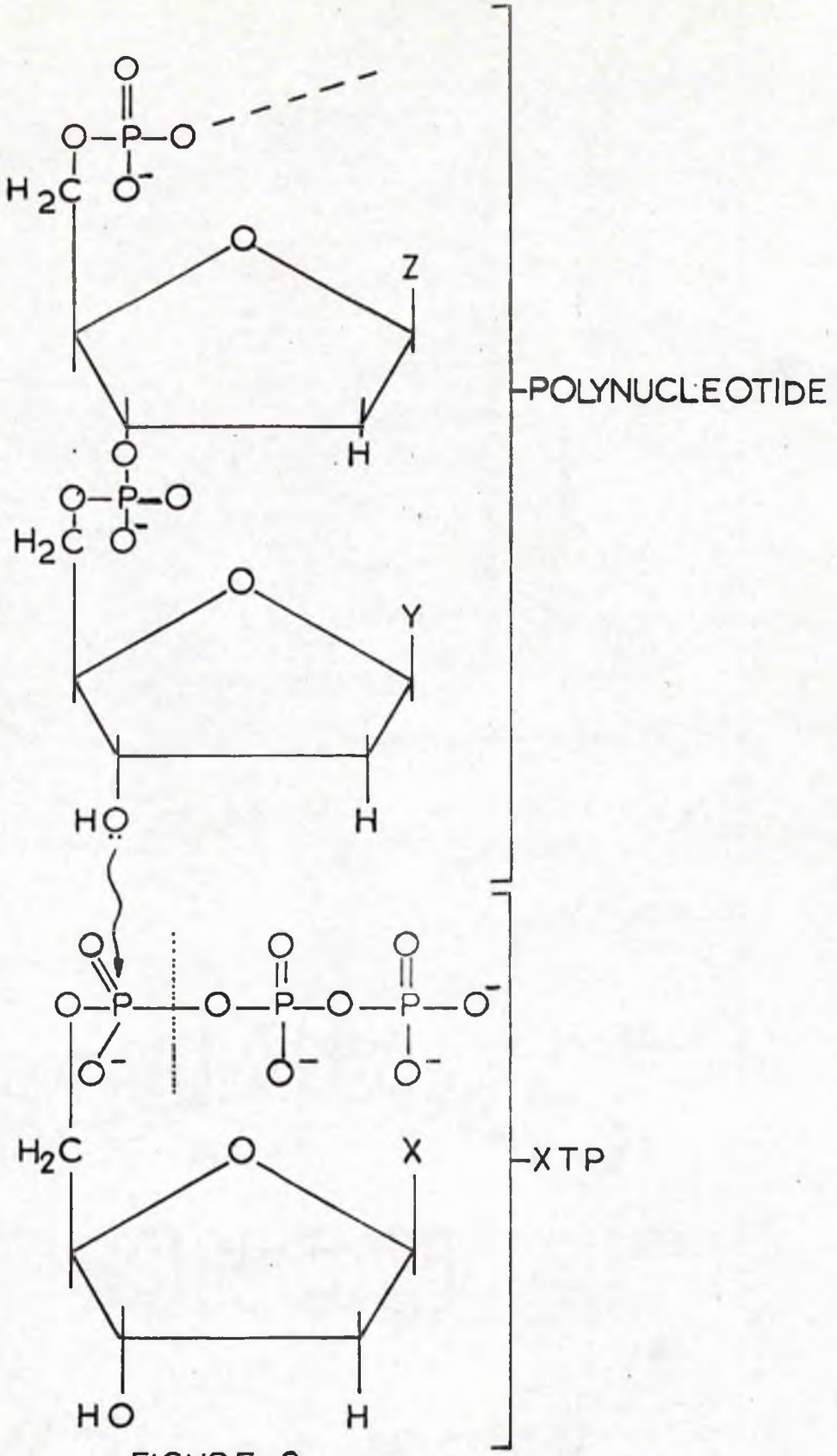


FIGURE 8

groups inhibits activity, to some degree lends support to this mechanism (Richardson et al., 1963a; Richardson, Schildkraut & Kornberg, 1963b). The presence in E. coli of a DNA phosphatase-exonuclease which cleaves the 3'-phosphoryl linkage of mono - or diesterified phosphate residues of a DNA chain, suggests also that the product 3'-hydroxyl groups may have an important role to play in the initiation of DNA synthesis (Richardson & Kornberg, 1964).

B. DNA Nucleotidyltransferases of Mammalian Tissues

1) General Considerations

Although, in time, reports of mammalian DNA nucleotidyltransferases succeeded those of the E. coli enzyme by only two years, both their early and subsequent characterisation has been much less comprehensive. The main reasons for this would seem to be the facts that the mammalian enzymes are much less stable than their bacterial counterparts, and that large amounts of logarithmic phase bacterial cells are much more readily obtainable than are comparable mammalian cells. Thus, when investigating the problem of DNA biosynthesis in mammalian cells, one tends to favour a tissue which is reasonably easily obtained, and which has appreciable DNA nucleotidyltransferase activity. It must also be emphasised that the description of the general reaction in the previous section applies in toto only to the E. coli enzyme characterised by Kornberg and his colleagues. However, almost all of the mammalian enzymes, so far investigated, appear to resemble the bacterial enzyme in most respects.

2) Distribution and Properties of DNA Nucleotidyltransferase in Mammalian Tissues

This subject can be conveniently reviewed in four sections, namely, the distribution of DNA nucleotidyltransferase in (a) normal cells (b) tumour cells (c) virus-infected cells and (d) other systems.

a) Normal Cells

Regenerating rat liver was one of the first mammalian tissues in which DNA nucleotidyltransferase activity was observed (Bollum, 1958a, b; Mantsavinos & Canellakis, 1958). Since then investigation of the properties of this enzyme has lagged behind that of other tissues. However, recent work of Mantsavinos (1964) reported a substantial purification of the regenerating rat liver enzyme, which was shown to have an absolute requirement for a bivalent cation and to be primed equally well by native and by denatured DNA. On the other hand it has also been found that denatured DNA is a somewhat better primer, than native DNA, of cell free extracts of regenerating rat liver (De Recondo, Frayssinet & Le Breton, 1964; De Recondo 1964).

The best characterised of the mammalian DNA nucleotidyltransferases is that of calf thymus gland, which has been purified over the crude preparations some fiftyfold (Bollum, 1960). The enzyme has an absolute requirement for a bivalent cation and denatured DNA primes the reaction to a much greater degree than native DNA (Bollum, 1959). It is in fact claimed that native DNA is incapable of priming the activity of calf thymus DNA nucleotidyltransferase (Bollum, 1963a).

Walwick & Main (1962) have shown that DNA nucleotidyltransferase

from rat thymus gland expresses a preference for denatured DNA and also a requirement for a univalent cation.

DNA nucleotidyltransferase has also been demonstrated in partially synchronized cultures of mouse fibroblast cells - L cells, (Gold & Helleiner, 1963), and in cultured rabbit kidney cortex cells (Lieberman, Abrams, Hunt & Ove, 1963). The L cell enzyme, which has been purified fifteenfold, requires a bivalent cation and DNA primer, denatured DNA priming the reaction ten times more effectively than native DNA.

b) Tumour Cells

The DNA nucleotidyltransferase of Ehrlich and Landschutz ascites-tumour cells has been systematically investigated over the last seven years (Davidson et al., 1958; Smellie et al., 1959; Smellie et al., 1960). Like those of normal tissues this enzyme requires a bivalent cation and is primed to a greater extent by denatured DNA than by native DNA (Keir, Binnie & Smellie, 1962). A sixfold purification of the enzyme has been achieved (Keir, 1962). The enzyme from Walker 256 carcinoma appears to be similar to the Landschutz enzyme in all respects (Furlong, 1965).

c) Virus-infected Cells

DNA nucleotidyltransferase activity has been studied in KB cells infected with vaccinia virus or with adenovirus (Green & Pina, 1962; Green, Pina & Chagoya, 1964), in L cells infected with vaccinia virus (Hanafusa, 1961), in Hela cells infected with vaccinia virus (Magee, 1962) and in baby hamster kidney cells infected with herpes simplex virus

(Keir & Gold, 1963). In vaccinia - and herpes-infected cells there is an increase in both DNA nucleotidyltransferase and DNaseI activities after infection. However, in adenovirus-infected cells there is no increase in the activity of either of these enzymes. Vaccinia virus replicates in the cytoplasm (Smadel & Hoagland, 1942), whereas both adenovirus and herpes simplex virus replicate in the cell nucleus (Green & Pina, 1963; Morgan, Ellison, Rose & Moore, 1954). It would be interesting to determine to what extent, if any, the site of replication of the animal virus within the cell relates to the mechanism by which its DNA is replicated. Unlike the situation in T_2 bacteriophage-infected E. coli system, in which a new DNA nucleotidyltransferase has been clearly demonstrated (Aposhian & Kornberg, 1962), there is as yet only limited evidence for the elaboration of a physically distinct enzyme in mammalian cells infected with a DNA-virus (Keir & Gold, 1963; Keir, 1965).

d) Other Systems

DNA nucleotidyltransferase has also been demonstrated in sea urchin embryos, whole nuclei from which are primed equally well by native and denatured DNA, and show an absolute requirement for a bivalent cation (Masia & Hinegardner, 1963).

3) Requirements of the Reaction

The reaction catalysed by all the mammalian enzyme systems mentioned above is very similar to that described in Fig. 7 for the E. coli enzyme. The presence of all four deoxyribonucleoside triphosphates is required

for optimal activity. The optimum pH seems to lie in the range 7.2 to 7.5 and there is an absolute requirement for a bivalent cation. There is no synthesis if primer DNA is omitted, but the nature of the primer requirement is still a matter of some uncertainty, for although it would seem valid to claim that most mammalian enzymes show a preference for denatured DNA, all of them use native DNA to some extent and the regenerating rat liver enzyme uses native DNA quite efficiently. There appears to be two types of primer activation, the first involving the action of denaturing agents such as heat, acid or alkaline treatment, all of which destroy the secondary and tertiary structures of the DNA molecule. This activation may be attributed to production of single-stranded regions in the primer (Bollum, 1959; Keir et al., 1962). The second type of primer activation, which involves the enzymic modification of the primer molecule, was investigated by Bollum (1962) and Keir (1962), who found that DNA partially treated with DNaseI was a more efficient primer than untreated DNA. This can be interpreted as due to the production of a greater number of 3'-hydroxy-terminal groups on the primer, for these groups are apparently required for the initiation of DNA replication (Richardson et al., 1963a). That these effects of nuclease in the in vitro system may not have physiological significance has been suggested by the work of Bollum (1963a) with an enzyme preparation which is free of DNaseI activity.

4) The Nature of the Product

The product of mammalian DNA nucleotidyltransferase activity, although not as well defined as that of the E. coli enzyme, appears to be double-stranded DNA of high molecular weight. In the Landschutz cell system it has been shown that the deoxyribonucleoside monophosphate residues are located in non-terminal regions of the product DNA (Smellie et al., 1960). Using the DNase - free DNA nucleotidyltransferase from calf thymus Bollum (1963a, b) has shown by nearest neighbour frequency analysis that the base composition of the product is determined by that of the primer. By separation of primer and product molecules using chromatography on MAK columns and countercurrent distribution in aqueous polymer systems, he has also shown that the product of the single-stranded DNA-primed calf thymus enzyme is double-stranded native DNA and that the reaction does not proceed beyond a doubling of the amount of primer originally present.

Mazia & Hinegardner (1963) have demonstrated that, in their sea urchin embryo system, the base composition of the product is very similar to that of the primer and that the product DNA has the same sedimentation properties as the primer.

C. Terminal Addition Reaction

In the early reports on DNA nucleotidyltransferase from both bacterial and mammalian sources, small amounts of incorporation of deoxyribonucleotide were observed when only one of the four deoxyribonucleoside triphosphates was present in the reaction mixture

(Adler, Lehman, Bessman, Simms & Kornberg, 1958; Bollum, 1960a). This was thought to be due to residual activity of DNA nucleotidyltransferase in the absence of its complementary substrates. However, the work of Krakow, Coutsogeorgopoulos & Canellakis (1962) showed that incorporation of this type in calf thymus nuclei was due to an enzyme separable from the well characterised DNA nucleotidyltransferase. This enzyme requires only one deoxyribonucleoside triphosphate, Mg^{2+} ions, cysteine and primer DNA for optimal activity, and if the complementary deoxyribonucleoside triphosphates are added to the reaction mixture, inhibition of the enzyme is observed. DNaseI treatment of the product and stimulation of incorporation on the addition of more primer DNA after the attainment of maximal activity, suggest that the enzyme acts by the addition of one or a few deoxyribonucleotides to the ends of primer chains. (In this section and in subsequent sections where both types of activity are being discussed together, the enzyme which requires only one deoxyribonucleoside triphosphate will be termed the <u>terminal</u> DNA nucleotidyltransferase and the one which catalyses the incorporation of all four deoxyribonucleoside monophosphates into DNA will be termed the <u>replicative</u> DNA nucleotidyltransferase). The <u>terminal</u> enzyme of Krakow <u>et al.</u>, (1962), which has been shown to catalyse the addition of either dCMP or dTMP residues to the ends of primer molecules, also has the ability to add terminally one ribonucleotide unit to a polydeoxyribonucleotide molecule, and both types of addition are strongly inhibited by pyrophosphate.

However, the terminal enzyme will add neither a ribonucleotide nor a deoxyribonucleotide unit to the ends of polyribonucleotide primer molecules. A DNA-dependent ribonucleotide terminal addition system, similar in this respect to the calf thymus terminal enzyme, has been observed in E. coli (Hurwitz, 1959). Krakow et al., (1962) achieved the separation of the terminal and replicative enzymes from calf thymus by chromatography on ion exchange celluloses.

Terminal DNA nucleotidyltransferase has also been detected in calf thymus nuclei prepared by a non-aqueous isolation technique, separation of the terminal and replicative enzymes in this case being achieved by ammonium sulphate fractionation (Keir & Smith, 1963). The terminal enzyme has the same nucleotide requirements as those of the enzyme of Krakow et al., (1962) in relation to the incorporation of dTMP residues. The incorporation of the other deoxyribonucleotides and of ribonucleotides has not been investigated. Cysteine, Mg^{2+} ions and primer DNA are also required for optimal activity of this terminal enzyme, which is most probably the same as that described by Krakow et al., (1962).

A terminal DNA nucleotidyltransferase has also been isolated by column chromatography on hydroxylapatite and by gel filtration on Sephadex G-100 of partially purified preparations of the replicative DNA nucleotidyltransferase from calf thymus tissue (Bollum, Groeniger & Yoneda, 1964). This enzyme is specific for deoxyribonucleoside triphosphates and oligodeoxyribonucleotide initiators and polymerises

dAMP fifty times more efficiently than dTMP, dCMP or dGMP. Homopolymers of deoxyadenylate have been prepared using this enzyme.

These three sources provide ample evidence for the existence in calf thymus of a terminal DNA nucleotidyltransferase physically separable from replicative DNA nucleotidyltransferase. Differences in experimental procedure do not permit a firm decision on whether the same terminal enzyme has been studied in each case, although the general observations indicate that this may be so. Up to the present there have been no reports of a separable terminal addition enzyme in other mammalian tissues.

D. Location of DNA Nucleotidyltransferase within the Mammalian Cell

The first reports of DNA nucleotidyltransferase in mammalian cells (Davidson et al., 1958; Bollum & Potter, 1958; Mantsavinos & Canellakis, 1959; Furlong, 1960) indicated that the enzyme was readily obtained from soluble supernatant fractions after high speed centrifugation of disrupted cell preparations. The apparent anomaly of the occurrence of DNA nucleotidyltransferase in a part of the cell other than the nucleus, where DNA is located and where its synthesis takes place, has led to further investigation of the intracellular location of the enzyme.

The work of the Glasgow group with non-aqueous nuclei, prepared by the method of Kay, Smellie, Humphrey & Davidson (1956), has direct bearing on the subject. Preparation of extracts in a non-aqueous medium ensures that the risk of passage of water-soluble cellular components across the nuclear membrane is minimised or eliminated. Smellie & Eason (1961)

failed to detect DNA nucleotidyltransferase in variously prepared nuclear samples, but subsequent work with regenerating rat liver (Keir, Smellie & Siebert, 1962) and calf thymus (Smith & Keir, 1963) non-aqueous preparations demonstrated that the enzyme is located in both the nuclear and cytoplasmic fractions. Previous failure to detect DNA nucleotidyltransferase activity in certain nuclear preparations was attributed, a) to interference by DNaseI and triphosphatase activities in the disrupted nuclei, and b) to probable leaching of the DNA nucleotidyltransferase from the nuclei during isolation in aqueous media (Smith & Keir, 1963).

It has also been shown that, in sea urchin embryo cells, the enzyme is found only in the nuclei if the nuclei are prepared in an aqueous medium containing Mg^{2+} ions and iso-osmolar sucrose (Mazia & Hinegardner, 1963; Mazia, 1963). The requirement of a bivalent cation for promoting retention of DNA nucleotidyltransferase in the nucleus during aqueous preparative procedures was clearly demonstrated by Main & Cole (1964), who showed that the proportion of enzyme retained in rat thymus nuclei prepared in Tris-HCl buffer is dependent on the Ca^{2+} ion concentration of the extraction medium.

The distribution of DNA nucleotidyltransferase in L cells, the growth of which was partially synchronised with 5-fluorodeoxyuridine, has been studied by Littlefield, McGovern & Margeson (1963) and by Gold & Helleiner (1964). Their results indicated that DNA nucleotidyltransferase is present in both the sediment and supernatant fractions derived from

centrifugation of disrupted cells, and that the activity of the enzyme increases in the sediment fraction and decreases in the supernatant fraction during the period of DNA synthesis. They suggest that the enzyme enters the nucleus in the early stages of DNA synthesis and becomes associated with DNA to form a particulate complex, which is not easily solubilised and which is difficult to assay for DNA nucleotidyltransferase activity. This seems to be an acceptable conclusion, for a DNA-DNA nucleotidyltransferase complex has been identified in E. coli cells (Billen, 1962; Kadoya et al., 1964).

Finally, the work of Keir & Gold (1963) with baby hamster kidney cells infected with herpes simplex virus, which replicates in the nucleus, has shown that there is a substantial increase in the amounts of DNA nucleotidyltransferase and DNaseI in the nuclear fraction of the cell. This supports the concept of Littlefield et al., (1963) in so far as there may be a localisation of the enzyme at the site of DNA synthesis when there is a stimulus for the cells to engage in this operation.

The apparent diversity among these various observations may not be as great as it seems on the first examination. It would be consistent with these results to postulate that the enzyme is firmly bound, in particulate form, within the nucleus during DNA synthesis and is distributed, in soluble form, throughout the cell at other times. The non-aqueous experiments substantiate this theory since, in calf thymus and regenerating rat liver, only a small proportion of the cells are synthesising DNA at any one instant, and one might reasonably expect the

enzyme to be predominantly located at non-chromosomal sites. This is an argument which can also be used in support of the theory concerning the observations on the aqueous extraction of rat thymus tissue. The work on partially synchronised L cells, of which a sufficiently large proportion are simultaneously synthesising DNA to allow the detection of a shift of DNA nucleotidyltransferase into the nucleus from the cytoplasm, lends positive support to this theory. Total location of the enzyme in the nuclei of sea urchin embryos is also consistent with this hypothesis, since the cells in this system are dividing so rapidly that they can be regarded as being almost continually engaged in DNA synthesis and, as a result, DNA nucleotidyltransferase would never leave the sites of synthesis.

The recent detection of small amounts of DNA in chicken embryo mitochondria (Nass & Nass, 1963a; Nass & Nass, 1963b) and in bovine heart, rat liver and rat kidney mitochondria (Schatz, Haselbrunner & Tuppy, 1964) may account for a small fraction of the cytoplasmic DNA nucleotidyltransferase, but probably cannot account for many of the above findings, which show that very often the greater part of the cell's total complement of DNA nucleotidyltransferase is located in the cytoplasm.

VI Replication of the Chromosome

It is intended in this section to discuss current knowledge on DNA replication at the level of the chromosome, rather than replication of simple primer molecules in an *in vitro* DNA-synthesising system. By virtue of its simpler structure, the bacterial chromosome has provided

more information on the *in vivo* replication of DNA than the more complex mammalian chromosome. For this reason it is convenient to discuss this problem under two headings A) Replication of the bacterial chromosome and B) Replication of the mammalian chromosome.

A) Replication of the Bacterial Chromosome

By labelling the *E. coli* cell with (^{3}H) thymidine, it has been shown by autoradiography and electron micrography that the chromosome of *E. coli* is circular, and measurements suggest that it consists of a single double-helix of DNA (Cairns, 1963a, b). Further isolation of a population of intact chromosomes from an asynchronous culture suggested that DNA replication is initiated at a well-defined point on the circular chromosome, proceeds along the double-helix and culminates in the production of two circular daughter chromosomes (Cairns, 1963a, b). Similar work on the detection of the growing point and direction of synthesis has been reported in thymine-requiring mutants of *E. coli* (Hanawalt & Ray, 1964). Confirmation of the idea that DNA synthesis in the bacterial chromosome is sequential and unidirectional from a fixed point, has been obtained from the work of Nagata (1963) with *E. coli*. and Yoshikawa & Sueoka (1963) with *B. subtilis*. These workers, using synchronized cells have shown that the appearance of genetic markers on newly synthesised DNA follows a fixed pattern during the period of replication. These findings are supported by studies on the effect of thymine and amino acid deprivation on subsequent DNA replication in thymine and amino acid auxotrophs of *E. coli* (Maaløe & Hanawalt, 1961;

Hanawalt, Maaløe, Cummings & Schaechter, 1961; Lark, Repko & Hoffman, 1963).

In considering the mechanisms of DNA replication, one can divide them into three classes, a) conservative b) semi-conservative and c) dispersive, according to the distribution of the parental molecule within the replica duplexes. Conservative DNA replication mechanisms are those which maintain the integrity of the entire parental DNA molecule so that of the two daughter double-helices, one is entirely parental DNA while the other is entirely new. The process of DNA replication is said to be semi-conservative when the parental molecule consists of two complementary strands each of which, on the first replication, becomes part of one of the two daughter duplexes so that, after an arbitrary number of replications, there are only two double-helices which are composed of part of the parental molecule. Finally, dispersive replication mechanisms are those in which the integrity of the parental chains is not conserved and, after several replications, the parental double-helix has been distributed in small pieces among several of the daughter duplexes. All three types of replication are described diagramatically in Fig. 9. That the mechanism of DNA replication in the bacterial chromosome is semi-conservative, a single strand of the parental duplex being the conserved unit, is strongly supported by ultracentrifugal evidence (Meselson & Stahl, 1958). Confirmation of this mechanism comes from <u>in vivo</u> and <u>in vitro</u> studies of bacterial (Sueoka 1960; Baldwin & Shooter, 1963; Cairns, 1963a, b)

FIGURE 9

Illustration of conservative, semi-conservative and dispersive mechanisms of DNA replication by distribution of parental material between the daughter duplexes.

(After Delbruck, M.& Stent, G. S. (1957). In "The Chemical Basis of Heredity", p. 709. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.

A. Conservative: One daughter duplex old, one new.

B. Semi-conservative: In each daughter duplex one chain is old and one is new.

C. Dispersive: The distribution of parental material is uniform through the four chains of the two daughter duplexes.

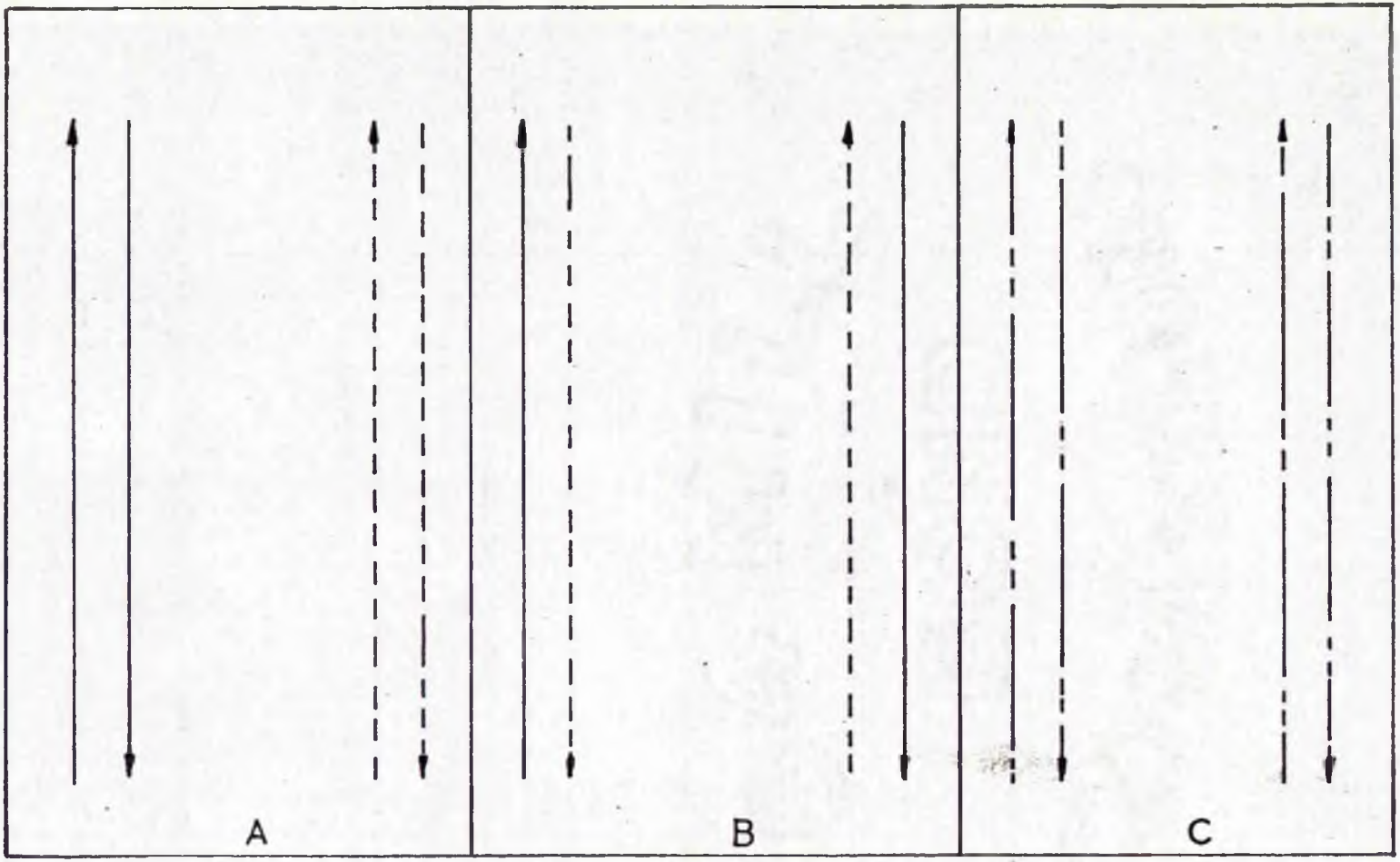


FIGURE 9

and viral systems (Kaplan & Ben-Porat, 1964), but a different interpretation has been given by Cavalieri and his co-workers to these, and to their own findings (Cavalieri & Rosenberg, 1961a, b,c; Cavalieri & Rosenberg, 1963). They propose that DNA replication is semi-conservative but that the conserved unit is a double-helix. This latter interpretation will be discussed later (see Discussion, section IIA in relation to the mechanism of action of DNA nucleotidyltransferase. Replication of the DNA of the T-even bacteriophages has been shown to be dispersive (Kosinski, 1961: Kosinski & Kosinski, 1963), but the fragmented parts themselves seem to be replicated semi-conservatively.

In vivo work of Maaløe (1963) and in vitro work of Gogol & Rosenberg (1964) indicate that there may be an inhibitor of DNA synthesis in extracts of non-dividing cells. Maaløe (1963) has also observed that the length of the DNA synthetic period is much less dependent on the nutritional content of the growth medium than is the generation time, and that de novo protein synthesis is required to initiate a new round of replication.

In conclusion, it can be said that the replication of DNA in the bacterial chromosome is a continuous process beginning at a fixed point and proceeding unidirectionally through the whole bacterial genome and recent evidence has strengthened the concept that the initiation and the termination of replication are unique and widely separated events in the division cycle (Pritchard & Lark, 1964; Lark & Lark, 1964).

B) Replication of the Mammalian Chromosome

The mode of DNA synthesis within the mammalian chromosome is more difficult to investigate than that of its bacterial counterpart for two main reasons. The first difficulty is that DNA synthesis in mammalian cells occurs during a very short part of a relatively long generation time, whereas DNA synthesis in rapidly-growing bacteria occupies a considerable, though variable, portion of a very short generation time (Maaløe, 1963). There are four well recognised stages in the life cycle of a mammalian cell between two successive mitotic sequences. These, in order of occurrence after telophase, are interphase, G_1, S and G_2 phases. In only one of these phases, the S phase, does DNA synthesis take place, the provision of a complete complement of DNA being required before the cell enters G_2 and subsequent prophase, and it has been shown in Hela cells that synthesis of RNA and protein in addition to that of DNA are prerequisites of cell division (Meuller, 1963). The second complication in the mammalian chromosome is the association of large amounts of histones and acidic proteins with DNA in the nucleus, which makes the study of chromosomal replication more difficult, because the structure of the chromosome itself is not well defined. Various models for mammalian chromosome structure have been proposed (Taylor, 1963a; Hsu, 1963; Dounce & Hilgartner, 1964), but no generally accepted structure has yet been established.

Radioautographic studies have shown that large chromosomes have

multiple growing points for DNA replication (Taylor, 1963b; Plaut, 1963), which together with the discovery of early and late replicating chromosomes (Hsu, 1964), suggests that there may be sequential replication of subunits within individual chromosomes. Using the techniques of radioisotope (Taylor, Woods & Hughes, 1958; Prescott & Bender, 1963) and heavy base analogue (Simon, 1961; Simon, 1963) labelling of chromosomal DNA during synthesis, it has been established that replication takes place in a semi-conservative manner.

Monod, Jacob & Gros (1962) have proposed the theory that histone-binding of DNA within the chromosome is responsible for repression of messenger RNA synthesis and hence of protein synthesis. It is thought that histone may fulfil this role by binding to the DNA at selected regions along the chromosome. For this reason, the inhibition of DNA-primed RNA nucleotidyltransferase (E C 2.7.7.6) by histones has been extensively investigated (Huang & Bonner, 1962; Allfrey, Littau & Mirsky, 1963; Barr & Butler, 1963). Similar studies with DNA nucleotidyltransferase preparations from regenerating rat liver (Gurley, Irvin & Holbrook, 1964; Lehnert, 1963) and calf thymus nuclei (Bazill & Philpot, 1963) have demonstrated that free histone inhibits DNA nucleotidyltransferase. However, intact nucleohistone (DNA-histone complex) does not inhibit the activity of the enzyme when added to a standard DNA-primed assay. Gurley et al., (1964) have also shown that the lysine-rich histone fraction inhibits the enzyme to a greater degree than does the arginine-rich histone fraction, but neither fraction inhibits DNA

nucleotidyltransferase to as great an extent as it inhibits DNA-primed RNA nucleotidyltransferase. Therefore, although histones appear to have a pronounced effect on transcription from DNA via RNA to protein, they may additionally have a function as regulators of DNA synthesis.

VII Degradation of DNA and Deoxyribonucleotides

In consideration of DNA biosynthesis, it was shown that the biosynthetic pathway could be divided into well defined stages (see Introduction, sections IV and V). Clearly, competing enzyme systems must also be taken into account e.g., hydrolysis of phosphodiester bonds in DNA (i.e., exo-and endonucleolytic action), and dephosphorylation of precursor nucleotides can interfere seriously in an *in vitro* DNA-synthesising system which measures the incorporation of deoxyribonucleoside triphosphates into DNA. Enzymes capable of carrying out these degradative operations are widely distributed in nature, and merit some consideration at this stage.

A) Hydrolysis of Deoxyribonucleotides

Phosphatases, which are specific for nucleoside monophosphates, are widely distributed among animals, plants, and bacteria (Schmidt & Laskowski, 1961; Heppel, 1961). They appear to be specific for either nucleoside 5'-monophosphates or nucleoside 3'-monophosphates; they express no preference between ribonucleotides and deoxyribonucleotides, and hydrolyse the substrate to the nucleoside and phosphoric acid.

Enzymes involved in the hydrolysis of the anhydride bonds of nucleoside di- and triphosphates are less well characterised. The

enzyme best known for hydrolysis of nucleoside triphosphates is myosin ATPase from muscle, which acts by hydrolysis of the bond between the β and γ phosphates of the nucleotide to form ADP and orthophosphate (Kielly, 1961a,b). Moreover, partially purified ATPases from other sources have shown hydrolytic activity towards the other ribonucleoside and deoxyribonucleoside triphosphates.

The existence of two different ATPases has been demonstrated in pig kidney cortex nuclei, cancer cell nuclei and regenerating rat liver nuclei (Siebert, 1960, 1963). Whether this is due to heterogeneity of the enzyme preparations or to low enzymic specificity has not so far been determined. Nucleoside diphosphates appear to be less susceptible to the action of these enzymes.

An enzyme which is specific for dCTP has been demonstrated in E. coli infected with T_2 bacteriophage (Kornberg, Zimmerman, Kornberg & Josse, 1959). The products of its action are dCMP and pyrophosphate; it has no effect on the other nucleoside triphosphates and has a much reduced activity towards dCDP.

Inorganic pyrophosphatases are widely distributed in living systems (Kunitz & Robbins, 1961). It is interesting to consider the possible role which these enzymes may play in ensuring the irreversibilty of DNA synthesis by removal of one of the products of the synthetic reaction (see Fig. 7). An obvious role for the other enzymes mentioned in this section would be in the control of DNA synthesis by regulating the concentrations of the deoxyribonucleotide precursor pools.

A phosphorolytic enzyme, which is a useful biochemical tool, is alkaline phosphatase from E. coli (Heppel, Harkness & Hilmoe, 1962) As the name suggests this enzyme has an alkaline optimum (pH of 9.5); it exhibits low specificity towards monoesterified hydroxyl groups and has been used to remove 3'-and 5'-phosphates from the terminal positions of primer DNA molecules in an in vitro DNA synthetic system (Keir, 1962).

B) Degradation of DNA

The sources and nature of enzyme which catalyse the hydrolysis of DNA have been reviewed by Laskowski (1961). These enzymes are sometimes called phosphodiesterases because they hydrolyse the phosphodiester bonds of the nucleic acids. DNases appear to be distributed among all subcellular compartments and are divided into two classes based on their site of attack on the substrate molecule. The first class is the exonucleases, which act by removal of mononucleotide units sequentially from one end of the DNA molecule. The second class is the endonucleases, which hydrolyse phosphodiester bonds situated at non-terminal sites in the polymer.

Two well characterised exonucleases, which are useful ancillary tools in nucleic acid research, are those of snake venom and calf spleen (Khorana, 1961). These enzymes hydrolyse the terminal phosphodiester bond of either a polyribonucleotide or a polydeoxyribonucleotide. The snake venom enzyme, a 5'-phosphodiesterase, starts at the 3'-hydroxyl end of the polynucleotide and proceeds along the polymer chain, splitting the terminal phosphodiester bond, sequentially producing nucleotide

5'-monophosphates. In contrast, the spleen enzyme, a 3'-phosphodiesterase, starts at the 5'-hydroxyl end of the polynucleotide and sequentially forms 3'-phosphoryl nucleosides as it moves along the chain. Phosphodiesterases with similar properties have been described in certain mammalian tissues (Razzell, 1961).

The endonucleases can be classified as 5'-phosphoryl ester oligonucleotide formers and as 3'-phosphoryl ester oligonucleotide formers. An endonuclease can also be classified as a DNaseI or a DNaseII. This classification is based on comparison of the DNases distributed in different tissues with pancreatic DNaseI (Kunitz, 1948; Kunitz, 1950), and thymic and spleen DNaseII (Mayer & Greco, 1949; Koerner & Sinsheimer, 1957a, b), in regard to their pH optima and activating and inhibiting agents. A generalisation often made is that the 5'-ester formers are synonomous with type I DNases and the 3'-ester formers are synonomous with type II DNases. One exception to this rule is micrococcal nuclease which produces 3'-phosphoryl nucleotides and has an alkaline pH optimum (Alexander, Heppel & Hurwitz, 1964). Very little is known about the specificity of endonucleases with regard to the bases on either side of the hydrolysed bond. The DNases of one biological system, the E. coli cell, have been most comprehensively studied by Lehman (1963).

The wide distribution and high activity of DNases in various cells has led to much speculation on their function. A role which they may play is to convert DNA within the somatic chromosome to an active form which will prime for DNA replication. A role more often assigned to them is

the destruction of foreign DNA within the cell; thus they may serve a protective function e.g., against viral infection.

A point which must always be remembered when attributing biological function to these enzymes, which degrade DNA and its precursors, is that they may not be permitted to come into contact with their substrates *in vivo*. DNA synthesis is very probably localised, at least in most instances, within the nucleus of the mammalian cell, and the various DNA-degrading enzymes appear to be widely distributed among the sub-cellular components. Therefore, many of these enzymes may fulfil no specific function *in vivo* until the death of the cell initiates hydrolytic action by this great battery of degrading enzymes.

VIII Current Problems of DNA Biosynthesis

Within recent years, biochemical research has indicated more and more clearly that DNA nucleotidyltransferases have the ability to catalyse the synthesis of a DNA molecule, which is either the complement or the copy of the DNA template, depending upon whether single-stranded or double-stranded DNA is used to prime the reaction. These DNA nucleotidyltransferases have been shown to be widely distributed among animal and bacterial cells. A great deal of effort has been expended in the establishment of this biological mechanism, which furnishes the biologist with the answer to the problem central in his mind for several decades, namely the molecular explanation for heredity. However, now that the flush of success has passed and time for reflection has become available, one realises that several aspects of DNA replication are still

unexplained. Furthermore, the inherent challenge of these problems is intensified by the probability that their solution will yield great benefit to mankind in the field of medicine.

In vitro studies have demonstrated DNA-primed direction of the enzymic product and one might think that, in so doing, their function has been fulfilled. However, if DNA biosynthesis is considered from the enzymic point of view, it can be seen that much knowledge may yet be derived from *in vitro* studies. The ultimate aim is to paint the picture of DNA synthesis *in vivo*, but to do this we must first be able, in the words of Lord Kelvin, "to measure that of which we speak, and express it in numbers". This will be done only by exploiting the *in vitro* system to the limit of its potential. It seems probable that the *in vitro* system will, from this point forward, be used to its fullest advantage if it is employed in the elucidation of the nature of the enzyme, and of the mechanisms by which it performs its task, rather than to concentrate the experimental effort on primer studies.

A role has been established for the *replicative* enzyme; one must now ask the question, "what is the function of the *terminal* enzyme and how is it related to the replicative enzyme?" The DNA nucleotidyltransferases of mammalian systems have been found, almost without exception, to display a preference for denatured DNA as primer. How can this fact be reconciled with the failure of many attempts to demonstrate the existence of single-stranded DNA *in vivo*? The nature of the *in vitro* reaction has so far suggested that the assembly of the complementary

bases on the template may be a random process. This seems to be slow, wasteful and uncharacteristic of a phenomenon, which, within the cell, appears to be rapid and well controlled. Perhaps elucidation of enzyme structure and specificity will account for various aspects of *in vivo* DNA synthesis, such as the constancy of the S phase. Lastly, *in vitro* studies on the effect of substances known to be present in close contact with the chromosome during replication may go part of the way in elucidating their biological significance.

The work described in this thesis was undertaken in an attempt to solve some of these problems.

EXPERIMENTAL

E X P E R I M E N T A L

I Biological Material

A) Landschutz Ascites - Tumour Cells

The Landschutz ascites-carcinoma, originally supplied by Dr. L. V. Crawford, Institute of Virology, University of Glasgow, was maintained by serial transplantation in 8 to 10 weeks-old albino mice, (Porton strain), reared in the departmental animal house. Tumour fluid was withdrawn by syringe, aseptically, under ether anaesthesia, from mice which had been inoculated seven to nine days previously, and 0.2 ml of this fluid was inoculated intraperitoneally into each mouse.

B) Calf Thymus Gland

Calf thymus glands, which were stored in ice or blast-frozen to -70° immediately after their removal from the animal, were routinely obtained from the city meat-market. Within several hours those stored in ice were either frozen to -70° or first lyophilised and then stored at -70° until required.

C) Other Tissues

Thymus, spleen, appendix and bone marrow were obtained from young rabbits, which were 2 to 3 months old and weighed approximately 1 Kg. The animals were killed by cervical dislocation and these tissues quickly removed from the carcase, shorn of contaminating material and lyophilised, care being taken at all stages to maintain the temperature at 0°. The tissues were stored at -70° until required for the preparation of soluble

extracts. Lyophilised rat liver and spleen preparations were obtained in a similar manner.

II Preparation of Enzyme Extracts

A) Landschutz Ascites-Tumour Cells

Soluble extracts of cells of the Landschutz ascites-carcinoma were prepared according to the method of Keir (1962) with some important modifications. All operations described in this section were carried out at 0°.

The ascitic fluid was withdrawn aseptically from mice six to eight days after inoculation, and transferred to a 0.9% (w/v) NaCl solution. The cells were washed several times with NaCl solution by sedimentation at 200-300 g and decantation. After the final wash, the cells were tightly packed by sedimentation at 700-800 g for 5 min. The resultant sediment was taken up in 12-15 volumes of 0.001 M-EDTA-0.005M - 2-mercaptoethanol, pH 7.5, and the cells were disrupted in a Potter-Elvehjelm homogeniser (Potter & Elvehjelm, 1936), 4-5 passes of the pestle being sufficient to effect complete lysis of the osmotically swollen cells without appreciable nuclear breakdown. Microscopic examination with the aid of crystal violet staining (1% (w/v) in 0.1M-citric acid) was used to confirm that complete disruption had been obtained. The cell homogenate was immediately made 0.01M with respect to potassium phosphate buffer, pH 7.5 and 0.15M with respect to KCl by the addition of 0.5M-potassium phosphate buffer, pH 7.5 and solid KCl. The enzymically active soluble extract used in this work was the supernatant

fluid obtained by centrifuging the disrupted cell suspension at 105,000 g for 30-60 min. in a Spinco Model L ultracentrifuge.

For the purpose of determining the DNA nucleotidyltransferase activity in the 105,000 g sediment, this fraction was on occasion, taken up in a small volume of 0.001-M-EDTA-0.005-M-2-mercaptoethanol, sonicated in a M.S.E. Ultrasonic sonicator for 2 min., then made 0.01M with respect to potassium phosphate buffer, pH 7.5, 0.15M with respect to KCl and centrifuged at 105,000 g as before. The supernatant fraction, which was termed the nuclear extract, was then assayed for DNA nucleotidyltransferase activity (see Experimental section, IV A).

In the earlier stages of this work EDTA and 2-mercaptoethanol were omitted from the preparation at the cell disruption step. Occasionally, for reasons that will become clear later, these preparative procedures were modified.

B) Calf Thymus Gland

Enzymically active soluble extracts from calf thymus tissue were prepared in a manner essentially similar to that described above for ascites-tumour cells with slight modification. The medium in which the tissue was homogenised was 0.25M-sucrose-0.001M-EDTA-0.005M-2-mercaptoethanol, pH 7.5, and the disrupted cell suspension was centrifuged for 90 min. at 105.000 g in the ultracentrifuge. In the earlier thymus preparations, EDTA and 2-mercaptoethanol were omitted from the disruption medium.

C) Other Tissues

Soluble extracts were prepared from thymus, spleen, bone marrow and appendix from young rabbits and from rat liver and spleen, as described before for ascites-tumour cells except that neither 2-mercaptoethanol nor EDTA was used in the preparative procedure.

D) Non-aqueous Preparations

In order to compare results obtained in this work with those of Smith & Keir (1963) and Keir & Smith (1963) soluble extracts of some tissues were prepared by incorporating a modification of the non-aqueous preparative procedure of Kay et al., (1956). The tissue was removed from the animal, minced finely with scissors and immediately frozen in a solid CO_2-ethanol mixture in preparation for lyophilisation. After complete drying, the tissue was weighed and ground to a powder in a mortar.

The powder (3-5 g.) was disintegrated in 30-60 ml. petroleum ether (b.p. 40-60°) in a Potter-type homogeniser, and centrifuged at 1000 g for 10 min. at -15°. After repeating this procedure twice, the final petroleum ether sediment was suspended in 30-60 ml. of a mixture of cyclohexane-CCl_4 (1 : 1, v/v), homogenised as before and centrifuged at 1000 g for 20 min. at -15°. At this stage the sediment, which contained the wat r soluble components of both cytoplasm and nucleus, was examined microscopically to check that maximum cell disruption had taken place, and finally dried in ether.

The dried powder was then taken up in 0.01M -potassium phosphate buffer, pH 7.5-0.15M-KCl (10 mg. dry powder/ml.), homogenised as above

and sonicated for 1 min. before centrifuging at 105,000 g in the Spinco Model L ultracentrifuge for 30 min. The 105,000 g supernatant fraction was used as the enzymically active soluble extract.

III Enzyme Fractionation Techniques

The techniques described in this section were used for fractionation of most soluble extracts, irrespective of their origins. As in the preparation of soluble extracts, during the earlier stages of this work EDTA and 2-mercaptoethanol were omitted from the preparative buffer systems employed. All operations were carried out at 0^{o}.

A) Acid Precipitation

A portion of a soluble extract preparation was taken to pH 5.0 by the addition of 0.2N-acetic acid (Keir, 1962). The solution was stirred with a magnetic follower throughout this operation, and the acetic acid was added in a slow dropwise manner to avoid formation of small pockets of low pH within the enzyme preparation. During this procedure the pH was continually observed at a pH meter (Model 23A E.I.L) previously adjusted to operate over the range pH 4.5-7.5. The resultant precipitate was sedimented without delay by centrifuging at 1000 g for 10 min., and was immediately dissolved by stirring for several hours in 0.001M-EDTA-0.005M-2-mercaptoethanol-0.2M-potassium phosphate buffer, pH 7.5. The volume of potassium phosphate buffer used to dissolve the precipitate was 0.25-0.35 times that of the soluble extract initially taken. On occasions when a less concentrated buffer e.g. 0.01M-potassium phosphate buffer, pH 7.5, was used to dissolve the precipitate, the resultant solution was

adjusted to pH 7.5 with 0.1N-KOH, if required. After about 12 hr. a small precipitate usually formed and was removed by centrifuging. The final supernatant fraction, termed pH5 precipitate fraction, was stored at 0°.

B) Ammonium Sulphate Fractionation

Saturated $(NH_4)_2\ SO_4$ used in the procedure was prepared by dissolving Analar $(NH_4)_2\ SO_4$ to saturation level in H_2O at 75° and allowing the solution to come to room temperature. The pH of the resulting solution was adjusted to 7.5 by the addition of 10N-NH_4OH and made 0.005M with respect to 2-mercaptoethanol and 0.002M with respect to EDTA. EDTA at this concentration was sufficient to chelate the known cationic impurities in the $(NH_4)_2\ SO_4$.

The following operations were carried out at 0°. To a measured portion of pH5 precipitate fraction was added (in a controlled dropwise fashion to avoid localised concentration) the volume of saturated $(NH_4)_2\ SO_4$ required to bring the $(NH_4)_2\ SO_4$ saturation to 20%. After stirring for 10 min., the preparation was centrifuged at 12,000 g for 10 min. in the SS-34 rotor of the Servall centrifuge. The sediment was discarded and to the supernatant fraction was added, slowly, with stirring, the volume of saturated $(NH_4)_2SO_4$ required to bring the saturation to 45%. This fraction was allowed to stand for 10 min. before centrifuging exactly as described for the previous step. The precipitate was dissolved in 0.005M-2-mercaptoethanol - 0.001 M-EDTA-0.001M-potassium phosphate buffer, pH 7.5 (10% of the volume of the pH5 precipitate fraction

originally taken) and stored at 0° until required.

c) Chromatography on DEAE-cellulose

The purpose of this fractionation step was to remove nucleic acid from the enzyme preparations. The 20-45% saturated $(NH_4)_2SO_4$ preparations were used at this stage, which is a modification of the method used by Bollum (1960).

DEAE-cellulose (Whatman DE11) was washed by repeated decantation to remove fine particles and stored in 2M-NaCl. A column 6 cm. x 2 cm. of DEAE-cellulose was then prepared under slight pressure (15 cm. Hg) to ensure uniform packing. After packing the column was washed under gravity flow with 2M-NaCl until the extinction of the column eluate at 260 and 280 mu. was zero (at least 2l.), after which the column was washed with 2l. of distilled H_2O and finally with approximately 2l. of 0.15M-potassium phosphate buffer, pH 7.5 containing 0.001M-EDTA and 0.005M-2-mercaptoethanol. The column was then allowed to equilibrate at 4°. Potassium phosphate buffer at 0.15M (ionic strength 0.45) prevents retention of the enzyme on the DEAE-cellulose while allowing the adsorption of DNA and the various forms of RNA.

A 20-45% saturation $(NH_4)_2SO_4$ fraction, containing approximately 35 mg. of protein was made 0.15M with respect to potassium phosphate buffer, pH 7.5 and applied to the DEAE-cellulose column. Protein was eluted with 0.005M-2-mercaptoethanol-0.001M-EDTA-0.15M-potassium phosphate buffer, pH 7.5, and collected in measured volumes, each fraction being assayed for protein content and enzymic activity (see Experimental.

sections IX B and IV A). The removal of nucleic acid from the enzyme fraction was followed by observing the increase in the ratio $E_{280}:E_{260}$ as measured in the Cary Recording Spectrophotometer Model II.

D) Gel Filtration

Dry Sephadex powder (G200, particle size 40-120u) was allowed to swell in 0.001M-potassium phosphate buffer, pH 7.5 for 48 hr. during which time stirring and decantation was regularly carried out to remove fine slowly-sedimenting gel particles.

Columns ranging in size from 60 cm. x 1.5 cm. to 130 cm. x 1.5 cm. were packed carefully under gravity flow in 0.001M-potassium phosphate buffer, pH 7.5, ensuring even distribution of gel particles and absence of air bubbles. Packing was done at room temperature, but the columns were equilibrated at 4° in the cold room and washed with several column volumes of 0.001M-potassium phosphate buffer, pH 7.5 before use. The column surface was covered with a disc of glass-fibre paper to protect it against mechanical disturbance during application of the sample. The flow rate of these columns was in the region of 6 ml/hr. The void volume (Vo) of each column was determined by measuring the volume of eluate emerging in advance of a band of native DNA.

20-45% saturation $(NH_4)_2SO_4$ fractions, containing 8-16 mg. of protein were applied to these columns in 0.001M-potassium phosphate buffer, pH 7.5 (N.B. 2-mercaptoethanol and EDTA were absent during almost all experiments which employed the gel filtration technique). The volumes of the applied fractions never exceeded 3 ml. and were preferably no greater

than 2 ml. The sample was layered with great care on the bed surface with a Pasteur pipette after the removal of the supernatant buffer. The outlet tap was then opened and the sample allowed to enter the column bed until the sample surface had just disappeared below the bed level, when a small amount of eluting buffer (0.001M-potassium phosphate buffer, pH 7.5) was added to the bed surface to wash in the sample. This having been done, a larger volume of buffer was added to start elution and the column eluate was collected in measured volumes and assayed for protein content and enzymic activity (see Experimental, sections IX B and IV A and B).

Because of changes in void volume and flow rate due to tight packing on standing, columns were freshly prepared and characterised before each fractionation.

E) Chromatography on Hydroxylapatite

Hydroxylapatite, a modified form of calcium phosphate gel (Tiselius, Hjerten & Levin, 1956), was used in the present investigations for the purpose of enzyme fractionation.

Hydroxylapatite (Hypatite-C) was supplied in a form equilibrated in 0.001M-potassium phosphate buffer, pH 6.8. The pH was raised to 7.5 by the addition of very dilute KOH accompanied by vigorous stirring. On standing for several days the pH gradually fell below 7.5, because of the release of phosphate groups from the crystalline lattice. On each occasion before use the pH of the stock hydroxylapatite was adjusted to 7.5.

A 10 cm. x 1 cm. column of hydroxylapatite equilibrated in 0.001M - EDTA-0.005 M-2-mercaptoethanol - 0.001M-potassium phosphate buffer, pH 7.5 was packed at room temperature under a pressure of 20-25 cm. of mercury from an air pump, care being taken to ensure that the packing of the column was uniform. The column was removed to the cold room (4°) for overnight equilibration and subsequent operation. While packing and applying the sample to hydroxylapatite columns, the same precautions as observed while using G-200 Sephadex were taken to ensure the best possible fractionation.

A 20-45% saturation $(NH_4)_2SO_4$ fraction containing approximately 30 mg. of protein was applied under pressure to the column. Protein was eluted from the column under pressure either by batchwise washings with increasing concentrations of potassium phosphate buffers, pH 7.5, or by a gradient elution technique similar to that described by Richardson et al., (1964a). In the gradient elution method the buffer quantities and strengths used were as follows.

1) Mixing Vessel

120 or 150 ml. 0.005 M-2-mercaptoethanol-0.001M-EDTA-0.05M-potassium phosphate buffer, pH 7.5.

2) Reservoir Vessel

120 or 150 ml. 0.005M-2-mercaptoethanol-0.001 M-EDTA-0.35M-potassium phosphate buffer, pH 7.5.

The mechanics of the system were such that the levels in the mixing and reservoir vessels equilibrated without application of pressure. This

system gave a linear gradient from 0.05M to 0.30M-potassium phosphate and the flow rate under a pressure of approximately 25 cm. of mercury was 8-10 ml./hr.

The column eluate was collected in 2 ml. fractions which were assayed for protein content and enzymic activity (see Experimental sections IXB & IV A & B). In an effort to stabilise the enzyme on elution 300 µg. heat-denatured DNA was present in each collection tube.

IV Enzyme Assays

A) DNA Nucleotidyltransferase

DNA nucleotidyltransferase was assayed according to the method of Keir et al., (1962) with certain modifications.

1) Incubation Conditions

i) Replicative DNA Nucleotidyltransferase

Replicative DNA nucleotidyltransferase assays were carried out in 3 ml. stoppered test tubes in a total volume of 0.25 ml. at 37° for variable time intervals. The standard assay conditions are listed below. Departure from these conditions, for specific purposes, will be described in the Results section.

tris-HCl buffer, pH 7.5	20mM
KCl	60mM
EDTA	0.4mM
$MgSO_4$ or $MgCl_2$	4mM
2-mercaptoethanol	5mM
DNA	50 µg.

dATP	0.20-0.30 μM
dGTP	0.20-0.30 μM
dCTP	0.20-0.30 μM
dTTP	0.20-0.30 μM
DNA nucleotidyltrasferase fraction	15-150 μg. of protein

In almost all assays at least one of the deoxyribonucleoside triphosphates, usually dTTP was labelled with (^{32}P) in the α phosphate group. In a few assays the radioactive deoxyribonucleoside triphosphate was (8-^{14}C)dATP. The nature of the DNA used to prime the reaction varied according to the purpose of the assay.

In earlier stages of the work 2-mercaptoethanol was not included in the incubation mixture and EDTA was present at a concentration of 0.32mM. In assays designed to investigate the effect of varying the amount of one of the constituents of the reaction mixture, the concentrations of the others were kept constant, usually at the levels indicated above. In all assays relevant control tubes were incubated to measure non-enzymic incorporation levels of radioactivity.

In certain experiments the reaction mixture containing the enzyme, DNA, KCl, EDTA, tris-HCl buffer, pH 7.5, $MgSO_4$ and 2-mercaptoethanol, but lacking the deoxyribonucleoside triphosphates, was submitted to a prior incubation at temperatures ranging between 37° and 50°, before addition of the four deoxyribonucleoside triphosphates and incubation at 37°. Occasionally the procedure was modified so that preincubation of the enzyme was carried out in the presence of 75 μg. heat-denatured

DNA, pH 7.0, before addition of the standard assay components, including more DNA, and subsequent incubation at 37°.

After incubation the enzymic reaction was stopped by freezing the tubes in solid CO_2-ethanol mixture and storing at -10° until required.

ii) Terminal DNA Nucleotidyltransferase

The conditions for assay of terminal DNA nucleotidyltransferase were the same as those for the replicative enzyme with one major exception. Only one (^{32}P)-labelled deoxyribonucleoside triphosphate, usually (α-^{32}P)-dTTP, was present, the three complementary deoxyribonucleoside triphosphates being omitted. Occasionally in terminal DNA nucleotidyltransferase assays the concentrations of K^+ and Mg^{2+} ions were altered to 40mM and 10mM respectively.

2) Analytical Techniques

Both replicative and terminal DNA nucleotidyltransferase activites were assayed by the measurement of incorporation of a (^{32}P)-or-(^{14}C)-deoxyribonucleoside monophosphate from an (α-^{32}P)-or(β-^{14}C)-deoxyribonucleoside triphosphate into acid-insoluble polydeoxyribonucleotide material using the disc assay technique described below.

The frozen incubation mixtures were thawed and 50 µl. of a solution of bovine serum albumin (2 mg./ml. in H_2O) were added to each tube as a co-precipitant. After mixing thoroughly a 50 µl. portion from each tube was pipetted on to a disc of Whatman No. 1 (2.5 cm. diam.) filter paper. The paper discs were immediately plunged into a measured volume of 5% (w/v) trichloroacetic acid (15 ml./disc). Each set of discs was washed 4 to 6

times with 5%(w/v) trichloroacetic acid depending on the specific activity of the radioactive substrate, the duration of each wash being approximately 10 min. After the trichloroacetic acid washes the discs were dried by rinsing successively in ethanol and ether, which was evaporated under compressed air. The discs were placed on stainless steel planchettes and counted in a Nuclear Chicago gas flow counter (see Experimental, section IX F). The activity of the enzyme is expressed as mμ moles (^{32}P)-deoxyribonucleoside monophosphate incorporated/mg. of protein/unit time.

B) DNaseI

1) Incubation Conditions

Although the primary purpose of this work was to investigate the properties of DNA nucleotidyltransferase from several mammalian tissues, it was often found necessary to measure the activity of DNaseI in order to interpret many of the results obtained during the fractionation of DNA nucleotidyltransferase. However, no attempt was made to measure DNaseI activity under optimal conditions since only the nature and the magnitude of this activity in the environment of DNA nucleotidyltransferase assays were relevant to the problem. Therefore, all DNaseI assays were carried out under conditions similar in most respects to DNA nucleotidyltransferase assays.

The total reaction volume for DNaseI assays was scaled up 5 times to 1.25 ml. and the concentrations of $MgSO_4$, KCl, tris-HCl buffer, pH 7.5, EDTA and 2-mercaptoethanol were exactly the same as those in the DNA

nucleotidyltransferase assay. Deoxyribonucleoside triphosphates, however, were omitted from the reaction mixture, because they would have interfered with the analytical procedures used in the assay. Approximately 300 µg. of heat-denatured DNA was present as substrate in each assay. In the early stages of the work the protein content of each assay was often as high as 300 µg., but latterly it was confined to the range of 20-200 µg./assay. Incubations were carried out at 37° for varying time intervals, and after incubation the assay tubes were frozen in a solid CO_2-ethanol mixture and stored at -10° until required.

2) Analytical Procedures

DNaseI activity was measured by the quantity of deoxyribonucleotide rendered acid-soluble by a given amount of protein. Thus the purpose of the analytical techniques was to remove by precipitation any acid-insoluble polydeoxyribonucleotide material and estimate the extinction at 260 mµ. of the remaining acid-soluble material.

The assay tubes were thawed and 0.25 ml. of a solution of bovine serum albumin (2 mg./ml. in H_2O) was added to each tube as a co-precipitant, followed by 1.5 ml. of 2.1 N-perchloric acid. After shaking vigorously to ensure even distribution of acid, the tubes were allowed to stand for 10 min. before being centrifuged at 700 g for 15 min. to sediment precipitated DNA and protein. The supernatant fractions were decanted into fresh tubes, and their extinctions at 260 mµ. measured in a Unicam SP-500 spectrophotometer (see Experimental, section IX A). All operations before the decantation stage were carried out at 0° to prevent acid hydrolysis of

undigested DNA and of protein. Control tubes, with enzyme alone in the absence of DNA, were run in parallel to compensate for E_{260} extinction content of the enzyme preparations, and control tubes containing DNA but no enzyme were assayed to compensate for acid-soluble ultraviolet-light absorbing material arising non-enzymically from the substrate DNA. Where practicable, all assays and controls were done in duplicate.

Extinction at 260 mμ. was related to acid-soluble nucleotide content by the use of the average molar extinction coefficient at 260 mμ. of the four deoxyribonucleotides commonly found in DNA (10.5×10^3). Enzymic activity was expressed as μmoles or μg. of deoxyribonucleotide rendered acid-soluble/mg. of protein/unit time.

C) Phosphatase Assays

1) Incubation Conditions

For the reasons given above regarding the measurement of DNaseI activity, phosphatase activity in enzyme fractions was estimated only under conditions optimal for the assay of DNA nucleotidyltransferase activity. Thus, the incubation conditions for phosphatase assays were the same as those for DNaseI assays with respect to tris-HCl buffer, pH 7.5, KCl, $MgSO_4$, EDTA and 2-mercaptoethanol, the reaction volume also being 1.25 ml. DNA was omitted from the reaction mixture and dATP, dGTP, dCTP or dTTP was present at a concentration of 500 mμmoles/assay. Protein content of the assays was in the range, 20-200 μg. Incubation was carried out at 37° for varying time intervals, and the reaction was

stopped by freezing the tubes in a solid CO_2-ethanol mixture. The tubes were stored at -10° until required.

2) Analytical Procedures

Phosphatase activity was measured by the quantity of inorganic orthophosphate released from a deoxyribonucleoside triphosphate during the period of incubation with a given amount of protein. The protein was precipitated from the assay mixtures by addition of bovine serum albumin and 2.1N perchloric acid exactly in the manner described for DNaseI assays, and the supernatant fraction was taken for measurement of inorganic orthophosphate by a modification of the method of Allen (see Experimental, section IX C). All operations before the phosphate assay were carried out at 0° to prevent acid hydrolysis of phosphate from the deoxyribonucleotides and from protein. Control tubes were run in parallel to compensate for the inorganic orthophosphate content of the enzyme preparations and of the substrate deoxyribonucleoside triphosphate solutions. Enzymic activity was expressed as μmoles inorganic orthophosphate released/assay/unit time.

V Chemical Preparation of (^{32}P)-Labelled Deoxythymidine 5'-phosphates

(α-^{32}P)-deoxythymidine 5'-triphosphate ((α-^{32}P)dTTP) was prepared from (^{32}P)-orthophosphoric acid using modifications of the methods of Tener (1961) and Smith & Khorana (1958), which involved the intermediate synthesis of (^{32}P)-2-cyanoethylphosphate ((^{32}P)-CEP) and (^{32}P)-labelled deoxythymidine 5'-monophosphate ((^{32}P)-dTMP). 2-cyanoethylphosphate was either obtained commercially or prepared in the laboratory from

carrier-free (^{32}P)-orthophosphoric acid and 2-cyanoethanol in the presence of dicyclohexylcarbodiimide (DCC). The whole reaction sequence is outlined in Fig. 10, stages I, II and III describing the synthesis of (^{32}P)-CEP, (^{32}P)-dTMP and (^{32}P)-dTTP respectively.

A) Preparation of (^{32}P)-CEP

A solution containing 100 mC. of carrier-free (^{32}P)-orthophosphate was added to 1 m mole of(^{31}P)-orthophosphoric acid in dilute acid solution to permit randomisation of (^{32}P). Traces of HCl were removed by evaporation to dryness *in vacuo* in a rotary evaporator at 40°. 10 ml. of anhydrous pyridine (freshly distilled and stored over calcium hydride) and 1 ml. of 2-cyanoethanol were added to the resultant (^{32}P)-orthophosphate residue, and the mixture was evaporated to an oil *in vacuo* at 40°. A further 10 ml. portion of anhydrous pyridine was added to the oil and removed by evaporation at 40°. This procedure was repeated once more, and the resultant oil was dried in a rotary evaporator for at least 30 min. after the removal of pyridine to ensure the complete absence of water from the reaction mixture.

The oil from the final evaporation was dissolved in 5 ml. of anhydrous pyridine with the subsequent addition of 2.1 g. of DCC. The mixture was carefully shaken to permit uniform distribution of the reactants and allowed to stand overnight at 25° in a securely stoppered reaction flask. The reaction was terminated by the addition of 5 ml. of distilled water, and the resultant mixture was heated in a boiling water bath for 30 min., before being evaporated to dryness *in vacuo* at 40°.

FIGURE 10

Outline of the chemical synthesis of deoxyribonucleoside 5'-polyphosphates.

(After Grav, H. J. (1964) Ph. D. Thesis, The University, Glasgow).

Stage I — The formation of 2-cyanoethylphosphate from 2-cyanoethanol and orthophosphate.

Stage II — The synthesis of deoxyribonucleoside 5'-monophosphates by phosphorylation of deoxyribonucleosides with 2-cyanoethyl-phosphate.

Stage III — The formation of deoxyribonucleoside 5'-triphosphates from the corresponding 5'-monophosphates and orthophosphate.

The symbols α, β and γ illustrate the nomenclature employed in describing radioactive isomers of deoxyribonucleoside 5'-polyphosphates.

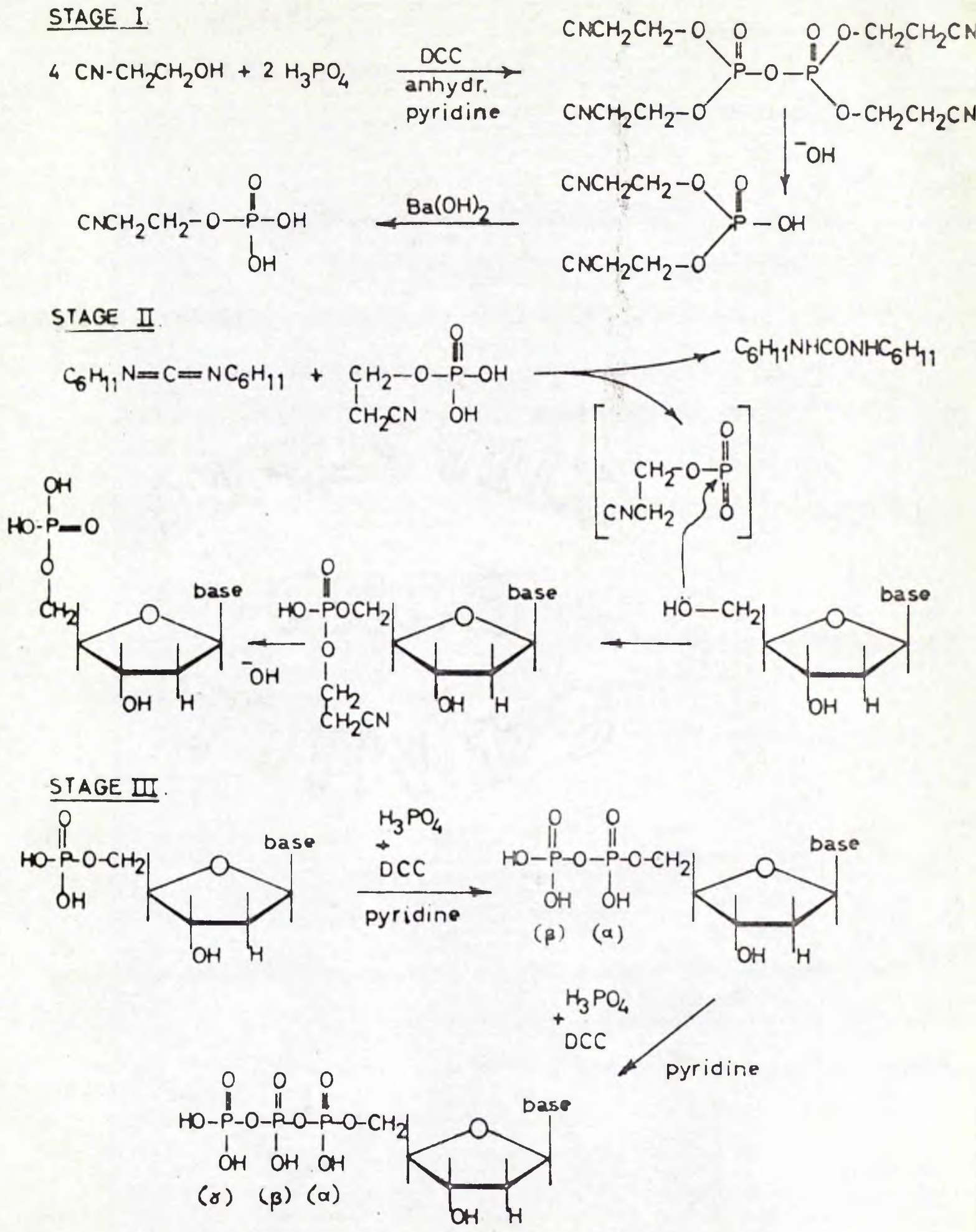

STAGE I
4 CN-CH2CH2OH + 2 H3PO4
DCC
anhydr.
pyridine
Ba(OH)2
STAGE II
C6H11N=C=NC6H11
C6H11NHCONHC6H11
base
STAGE III
H3PO4
DCC
pyridine
(β) (α)
(γ) (β) (α)

FIGURE 10

10 ml. portions of water and saturated barium hydroxide were added to the residue, which was then allowed to stand for 10 min. at room temperature before adjusting the pH of the solution to 7.5 with glacial acetic acid. The precipitated dicyclohexylurea (DCU) and barium phosphate were removed from the mixture by filtration. The filtered precipitate was liberally washed with distilled water, the washings being added to the bulk of the solution. Two volumes of 98% (v/v) ethanol were added to the resultant solution to precipitate the (^{32}P)-barium 2-cyanoethylphosphate. The solution was swirled vigorously and allowed to crystallise for 1 hr. at 0°. After precipitation the CEP crystals were collected by centrifugation at 700 g., the ethanol supernatant fraction being discarded. The crystals were redissolved in 5 ml. of distilled water, a few drops of dilute acetic acid being added to facilitate their solution. The resultant solution was neutralised with saturated barium hydroxide and any undissolved material was removed by centrifugation. The supernatant fraction containing the barium-CEP in solution was transferred to a ready-weighed centrifuge tube in which the CEP was reprecipitated by the addition of 2 volumes of 98% (v/v) ethanol. The CEP was then sedimented by centrifugation and washed sucessively in ethanol, acetone and ether before drying at room temperature. The centrifuge tube was then reweighed and the amount of CEP (barium salt, monohydrate) obtained was usually of the order of 400 mg. which represents a yield of 65%. The reaction sequence for the formation of (^{32}P)-BaCEP is outlined in Fig. 10 stage I.

The barium salt of CEP was converted to the free acid before use in the synthesis of dTMP. This was done by dissolving the crystals in a small volume (10 ml.) of distilled water with the help of a little dilute acetic acid. The solution was then passed through a 10 cm. x 1 cm. Dowex-50-H^+ column (see Experimental, section V E), elution with water being continued until most of the radioactivity appeared in the effluent. The effluent, which contained the free acid of CEP, was finally evaporated to dryness *in vacuo* at 40° to remove contaminating acetic acid, and dissolved in pyridine.

B) Preparation of (^{32}P)-dTMP

The following reaction mixture was set up for the preparation of (^{32}P)-dTMP from 1 mmole of (^{32}P)-CEP prepared as described above or obtained commercially (see Fig. 10, stage II).

Deoxythymidine was dissolved in pyridine and 1 mmole of (^{32}P)-CEP, also dissolved in pyridine, was added. (In all preparations the molar proportion of thymidine : CEP was 2 : 1). The mixture was concentrated to an oil *in vacuo* at 40°, and 10 ml. anhydrous pyridine was added. The solution was again concentrated to an oil at 40°, this procedure being repeated once more when the evaporation process was continued for at least 30 min. to ensure the complete removal of water. The final residue was dissolved in 10 ml. of anhydrous pyridine to which was added 620 mg. of DCC. The flask was shaken to dissolve all the DCC, then securely stoppered and allowed to stand for 12-15 hr. at 25°.

To terminate the reaction, 5 ml. of distilled water was added to the

reaction mixture, and, after allowing it to stand for 30 min. at room temperature, 10 ml. of concentrated NH_4OH (specific gravity 0.880) was added. The solution was then heated at 60° for 1 hr., after which it was evaporated to dryness *in vacuo* at 40° and subjected to a 10 min. hydrolysis at 100° in 0.1N-HCl to split any deoxythymidine dinucleotides formed during the reaction. The acid-hydrolysed solution was evaporated to dryness *in vacuo* at 40°. The residue was then extracted with 20 ml. distilled water and the precipitated DCU removed by filtration, and washed liberally with distilled water. The DCU washings were combined with the original filtrate.

At this point a sample of the filtrate was taken for descending chromatography on paper as described in Experimental, section IX E, to determine approximately the relative proportion of dTMP formed. This was done by elution of all the ultraviolet-light absorbing spots by capillary flow in water and measuring their respective extinctions at 267 mμ. (pH2). The percentage of dTMP relative to the total extinction could thus be determined.

The combined filtrate and washings were then applied to a 20 cm. x 2 cm. column of Dowex-1-Cl^- (see Experimental, section V E). Unreacted deoxythymidine was eluted from the column by washing with water until the extinction at 267 mμ. in the effluent approached zero. The dTMP was eluted from the column by gradient elution with 1l. of distilled water in the mixing vessel and 0.10N-HCl in the reservoir, fractions of 12-15 ml. being collected at a rate of approximately 1.5 ml./min. The gradient employed was non-linear and similar to that

described by Hurlbert, Schmitz, Brumm & Potter (1954). Two completely separated radioactive peaks containing extinction at 267 mu. were obtained by this procedure. The first peak contains dTMP, and it is believed that the second peak contains deoxythymidine 3'-monophosphate (Tener, 1961) together with residual deoxythymidine dinucleotides which are at present unidentified (T. P. Abbiss personal communication). The dTMP peak was pooled and evaporated to dryness at 40° *in vacuo*. The amount of dTMP at this stage was of the order of 300 μmoles which represents a yield of approximately 30%. The purity of the preparation was checked by paper chromatography in the ammonium isobutyrate solvent system, and the location of free orthophosphate and bound phosphate on the chromatogram was determined (see Experimental, section IX E). The specific activity of the product (^{32}P)-dTMP, which was determined in a Nuclear Chicago gas-flow counter, was of the order of 10^8 counts/min./μmole.

C) Preparation of (α-^{32}P)-dTTP

The formation of (α-^{32}P)-dTTP from (^{32}P)-dTMP and orthophosphoric acid is outlined in Fig. 10, stage III. The method used was essentially that of Smith & Khorana (1958) with minor, but important modifications.

(α-^{32}P)dTTP was prepared by condensation of (^{32}P)-dTMP with (^{31}P)-orthophosphoric acid in the presence of DCC. The following reactants:

1.2 ml. of tri-n-butylamine,

6.0 ml. of pyridine, (non-anhydrous),

0.2 ml. of 85% (v/v) orthophosphoric acid,

3.0 g. of DCC

were added to the flask containing the dTMP residue in the order shown, thorough mixing being performed on each addition. (These proportions relate to 100 μmoles of dTMP). The flask was then securely stoppered and allowed to stand at 25° for 48 hr., during which time a thick precipitate of DCU formed. The reaction was finally terminated by the addition of an equal volume of distilled water, with shaking to disperse the aggregated DCU precipitate. After 1 hr. at 0°, the precipitate was filtered off and liberally washed several times with distilled water, the washings being combined with the original filtrate. Pyridine was removed from the combined filtrates by repeated extraction with small volumes of ether. The combined ether extracts were in turn extracted with a small volume of distilled water to recover any nucleotide material which may have passed into the ether phase. This final water fraction was combined with the ether-extracted filtrate. The ether extracts were discarded, and the aqueous solution was evaporated to dryness in vacuo at 40°.

The residue was dissolved in a small volume of distilled water and passed through a 10 cm. x 2 cm. column of Dowex-50-Na^+ (see Experimental, section V E) which removed tri-n-butylamine and pyridine, and converted the product deoxyribonucleotides from the pyridinium to the sodium salts. The column was washed with water until the effluent contained almost negligible ultraviolet-light absorbing material and very little radioactivity. The total extinction at 267 mμ. of the combined column eluate was estimated at this point and usually accounted for the recovery

of around 90% of the deoxythymidine nucleotides originally present in the reaction mixture.

This solution was then applied to a Dowex-1-Cl^--column (25 cm. x 2 cm.) which was washed with distilled water to remove contaminating deoxyribonucleoside material. A linear gradient was constructed using 21.0.01N-HCl in the mixing vessel and 21.0.01N-HCl-0.5M-LiCl in the reservoir. The column effluent was collected in 10 ml. fractions at a rate of approximately 7 min./fraction. The first few tubes collected were tested for Cl^- with silver nitrate in concentrated acid in order to establish the position of the gradient front. This system gave good resolution of the applied sample into dTMP, dTDP and dTTP which were eluted from the column at 0.09, 0.22 and 0.34M-LiCl respectively. All the tubes in the (α-^{32}P)-dTTP peak were combined and the amount of the triphosphate estimated from the extinction at 267 mμ.

In order to remove contaminating inorganic polyphosphates from the product, the dTTP fraction was applied to a 10 cm. x 1 cm. charcoal column (see Experimental, section V E), which was washed with a large volume of distilled water until the effluent was free of chloride ions. Inorganic ortho- and pyrophosphates were then removed by washing the column with 0.01M-$NaHCO_3$ for about 10 column volumes or until ultra-violet-light absorbing material appeared in the effluent. On termination of bicarbonate elution the column was allowed to drain and was then washed with two separate column volumes of distilled water. After each wash the

column was allowed to drain to ensure the removal of the last traces of bicarbonate. Elution of the dTTP with 0.14N-NH_4OH in 70% (v/v) ethanol was then commenced and continued until the extinction at 267 mμ. of the effluent fell below 0.5 units/ml. A recovery of 90-95% of the absorbed dTTP was routinely achieved by this method.

The combined charcoal column eluate was taken to dryness <u>in vacuo</u> at 25°. The dTTP residue, which was in the form of the ammonium salt, was then converted to the sodium salt by solution in a small volume of water and passage through a 5 cm. x 2 cm. Dowex-50-Na^+ column. The eluate was evaporated to dryness at 25° and the (α-^{32}P)-dTTP (sodium salt) was dissolved in a suitable volume of water and stored at -10°.

A sample of the final preparation was chromatographed in the ammonium isobutyrate solvent system (see Experimental section IX E) and showed a single ultraviolet-light absorbing spot corresponding to dTTP. Subsequent scanning of the chromatogram for radioactivity and staining for free and bound phosphate (see Experimental section IX E) revealed that all the radioactivity and phosphate present in the preparation were associated with the dTTP spot. The final yield of dTTP was of the order of 240 μmoles when starting with 400 μmoles of (^{32}P)-dTMP, which represents a yield of the order of 60%. The specific radioactivity of (α-^{32}P)-dTTP prepared over a period of 2 weeks from commercially prepared (^{32}P)-CEP was of the order of 6×10^7 counts/min./μmole, when measured in a Nuclear Chicago gas-flow counter (see Experimental, section IX F

Starting from (^{32}P)-orthophosphate the final specific radioactivity of the (α-^{32}P)-dTTP was of the order of 2.5-3.0 x 10^7 counts/min./µmole.

D) Preparation of (α-^{32}P)-dTDP from (α-^{32}P)-dTTP

10 µmoles of (α-^{32}P)-dTTP and 0.04 ml. of 5N-HCl in a total volume of 1 ml. were incubated at 50° for 45 min., the incubation being terminated by cooling in ice. Part of this reaction mixture was then chromatographed, with several applications, on Whatman 3MM paper in the ammonium isobutyrate solvent system (see Experimental, section IX E) along with dTTP and dTDP markers. This resulted in the resolution of (^{32}P)-dTMP, -dTDP and -dTTP as the major products of hydrolysis. The (α-^{32}P)-dTDP spots were cut out and eluted by capillary flow in water (0.5 ml. water for each spot), and the combined eluates estimated for dTDP by measurement of the extinction at 267 mµ. The total yield of (α-^{32}P)-dTDP obtained was 2.02 µmoles of specific activity 1.71 x 10^6 counts/min./µmole. A sample of the final combined eluate was rechromatographed in the same solvent system and found to be free of both dTMP and dTTP contamination.

E) Pretreatment of Adsorbents used in Radioactive Preparations

1) Preparation of Charcoal Adsorbent

Animal charcoal (20-60 mesh) was obtained commercially, sieved to 40-60 mesh, suspended in 2 volumes 5N-HCl and allowed to stand overnight. The HCl was decanted and the charcoal was washed with distilled water until the pH of the supernatant fluid became neutral. Ultraviolet-light absorbing and coloured materials were then removed from the charcoal by washing liberally with large volumes of spectrophotometrically pure ethanol.

The charcoal was then resuspended in distilled water and washed until free of ethanol.

At this stage the charcoal was ready to be packed into a column and all subsequent treatment was directed towards its use in the preparation of dTTP (see Experimental section V C). The charcoal was packed into a 10 cm. x 1 cm. column and washed with 10-12 column volumes of 1.0N-HCl, followed by distilled water until the effluent pH was 7.0. The column was then washed with 0.01M-$NaHCO_3$ until the effluent was completely free of ultraviolet-light absorbing material, after which the pH of the effluent was brought back to neutrality by washing with distilled water. The column was then washed with 0.14N-NH_4OH in 70% (v/v) ethanol until the extinction at 220, 260 and 290 mμ. approached zero, in which condition it was used for the adsorption of the dTTP peak of the dTTP synthetic reaction. After use the column was regenerated by further washing with the ethanol-ammonia solvent until all the residual adsorbed material had been removed. The column was then treated, in turn, in a manner exactly similar to that described above with water, 1.0N-HCl, water, 0.01M$NaHCO_3$, water and ethanol-ammonia before it was again ready for use.

2) Pretreatment of Dowex-1-Cl^- for Column Chromatography

Dowex AG1-X8 (chloride form) of 100-200 mesh was suspended in 2N-HCl, allowed to stand overnight and packed into a Quickfit and Quartz column, fitted at the bottom with a sintered glass disc. The ion-exchange resin was then washed with distilled water until the pH of the effluent was 7.0,

after which it was ready for use. The resin could be regenerated by washing the column with approximately 10 column volumes of 0.5M-LiCl-0.1N-HCl to remove all the residual adsorbed material, followed by washing with distilled water until the pH of the effluent returned to 7.0.

3) Pretreatment of Dowex-50-Na^+ for Column Chromatography

Dowex AG50W-X8 (hydrogen form) of 100-200 mesh was suspended overnight in 2.0N-NaOH and then packed into a column. This treatment converted the resin from the hydrogen form to the sodium form. Before use the column was washed with distilled water until the pH of the eluate was neutral. When required in the hydrogen form the resin was suspended in 2.0N-HCl before packing and subsequent washing to remove acid. The columns were regenerated by washing with approximately 10 column volumes of 2.0N-HCl, followed by water to neutral pH. Reconversion to the sodium form was achieved by washing the column with 10 volumes of 2.0N-NaOH and then with distilled water until the pH was 7.0.

VI Preparation and Characterisation of DNA

A) Preparation of DNA

DNA was prepared from Landschuts ascites-tumour cells by the method of Kay, Simmons & Dounce (1952). The procedure described below is suitable for 5×10^9 cells, but may be scaled up or down according to the cell number.

About 5×10^9 cells were harvested and washed in 0.9% (w/v) NaCl (see Experimental, section II A) before being disrupted osmotically by the addition of 10 volumes of ice-cold water followed by homogenisation

in a Potter-type homogeniser. The cell lysate was then centrifuged at 1,000 g for 15 min. at 0°, the supernatant fluid being discarded while the sediment was homogenised in 0.9% (w/v) NaCl-0.01M-trisodium citrate, made up to 200 ml. with the same solution, and centrifuged at 900 g for 10 min. The supernatant fluid was discarded and the sediment was treated twice more in the same fashion with 0.9% (w/v) NaCl-0.01M-trisodium citrate. The final sediment was homogenised in 200 ml. 0.9% (w/v) NaCl and 18 ml. 0.5% (w/v) sodium dodecyl sulphate in 45% (v/v) aqueous ethanol were added, after which the preparation was stirred for 2 hr. at room temperature. Solid NaCl was added with stirring to 1.0M and the preparation was then centrifuged at 20,000 g for 30-60 min. in the 6 x 100 ml. angle head No. 9610 rotor of the M.S.E. Major centrifuge. The opalescent supernatant fluid was decanted and to it were added 2 volumes of redistilled ethanol to precipitate the DNA, which was collected on a glass rod, washed three times with redistilled ethanol, three times with acetone and allowed to dry.

The DNA thus obtained was purified by solution in 200 ml. distilled water at room temperature, adding 18 ml. 0.5% (w/v) sodium dodecyl sulphate in 45% (v/v) aqueous ethanol and stirring the preparation for a further 60 min. NaCl was then added to 1.0M and the solution centrifuged at 20,000 g as before. The DNA was precipitated from the 20,000 g supernatant fluid, washed and dried as described previously. A final purification step was carried out by dissolving the DNA in 200 ml. distilled water and adding 18 ml. 0.5% (w/v) sodium dodecyl sulphate in

45% (v/v) aqueous ethanol as before. NaCl was added to 1.0M and, in the absence of a precipitate the centrifugation step was omitted. The DNA was finally precipitated and collected as described above.

DNA prepared in this way was dissolved in distilled water at a concentration of 2 mg./ml., which using the conversion factor, 40 µg. DNA/ml. = 1 extinction unit/ml. at 260 mµ., represented an absolute DNA concentration of 1.5 mg./ml. DNA in this form was denatured for routine use in DNA nucleotidyltransferase assays by heating in a boiling water bath for 10 min. at 100°, followed by rapid cooling. When it was necessary to specify the DNA concentration more accurately, DNA was measured and expressed in terms of DNA phosphorus (see Experimental, section IX C & D3). Calf thymus DNA was prepared in a similar fashion.

B) DNaseI Treatment of DNA

In order to stimulate DNA nucleotidyltransferase activity in certain enzyme preparations DNA partially hydrolysed by commercially prepared bovine pancreatic DNase was used as primer in the DNA nucleotidyltransferase assay system. This treatment creates more 3'-hydroxyl groups in the primer which therefore has more sites available for the initiation of DNA synthesis (Keir et al., 1962).

Native Landschutz or calf thymus DNA (4 ml. of approximately 1.3-1.5 mg./ml.) was incubated at 37° for 60 min. with 45 µmoles of $MgCl_2$, 400 µmoles of tris-HCl buffer, pH 7.5 and 40 mµg. of crystalline DNase in a total volume of 10 ml. After incubation the DNA was thermally denatured as described above and used in DNA nucleotidyltransferase assays when required.

C) Characterisation of DNA

1) Methylated Albumin - Kieselguhr (MAK) Column Chromatography

DNA, prepared and purified as described in the previous section, was characterised by chromatography on MAK columns (Mandell & Hershey, 1960; Sueoka & Cheng, 1962). Methylated albumin and protein coated kieselguhr were prepared according to the method of Mandell & Hershey (1960).

To 180 µg. of a stock (1.5 mg./ml.) Landschutz DNA solution, prepared as described above was added, 0.2 ml. 10 x SSC and the volume was made up to 2 ml. with distilled water making the solution standard with respect to saline citrate. This solution was applied to a 3 cm. x 1 cm. MAK column (capacity 200 µg. DNA), which had been thoroughly washed with 0.4M-NaCl-0.05M-potassium phosphate buffer, pH 6.8.

The applied DNA was eluted from the column with the following gradient:

Mixing Vessel:	200 ml. of 0.5M-NaCl-0.05M-potassium phosphate buffer, pH 6.8.
Reservoir:	200 ml. of 2.0 M-NaCl-0.05M-potassium phosphate buffer, pH 6.8.

The gradient was passed through the column under pressure from a small peristaltic pump. The column effluent was collected in 2 ml. fractions whose extinctions at 260 mµ. were measured in order to detect the elution of DNA from the column. The elution pattern obtained from such an experiment is shown in Fig. 11, which gives the expected picture for MAK column chromatography of native DNA. A peak of DNA was observed in the region 0.65-0.75 M-NaCl, which is native DNA, while there was no peak in the region of 0.9 M-NaCl, where denatured DNA would be expected to come off

FIGURE 11

Chromatography of native DNA from Landschutz ascites-tumour cells on methylated albumin-kieselguhr.

See test for details.

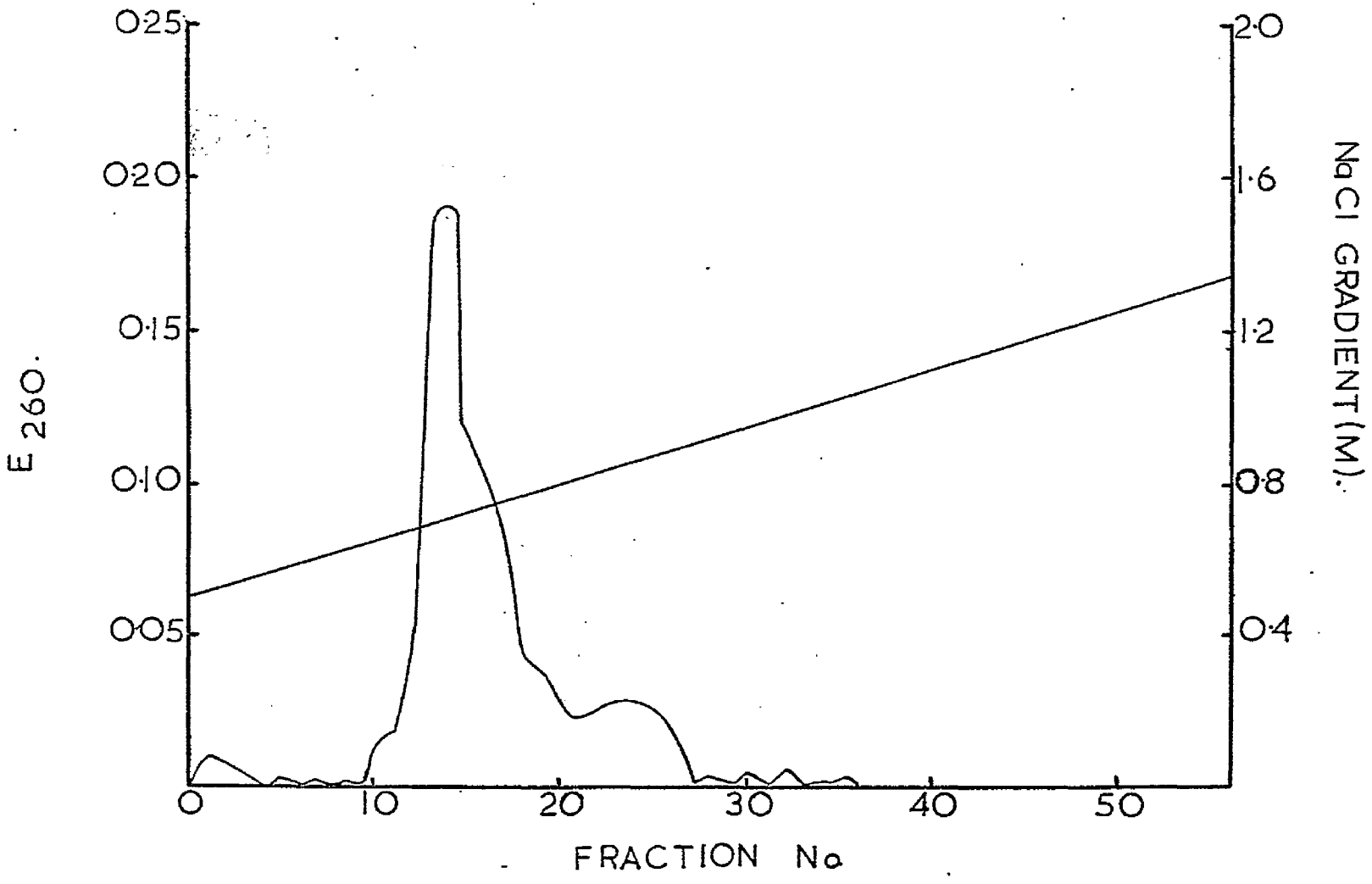

0·25
0·20
0·15
0·10
0·05
E 260.
2·0
1·6
1·2
0·8
0·4
NaCl GRADIENT (M).
0
10
20
30
40
50
FRACTION No

FIGURE II

(Sueoka & Cheng, 1962). Therefore the DNA preparations obtained as described in section A above, by the criterion of MAK column chromatography were free of single-stranded material.

2) Analytical Ultracentrifuge Studies

Solutions of stock preparations of both Landschuts and calf thymus DNA in SSC were centrifuged in the Spinco Model E analytical Ultracentrifuge equipped with both Schlieren and ultraviolet optical systems. Sedimentation coefficients of these preparations were found to be in the range 18-25S using both types of optical system for their determination.

VII Preparation of Buffer Solutions

A) Tris-HCl Buffer

Tris-HCl buffer was prepared by adjusting the pH of 0.4 and 0.8M solutions of 2-amino-2-(hydroxymethyl)-propane-1, 3 diol to the desired level with concentrated HCl. The buffer was generally used at pH values in the range 6.8-7.5. Tris-HCl buffers of lower ionic strength were prepared by dilution of the concentrated buffer, with distilled water. Crystalline tris-HCl mixtures, which gave a specific pH and ionic strength on the addition of a defined volume of distilled water, were obtained commercially.

B) Phosphate Buffers

Phosphate buffers were prepared by adding 0.5M-KH_2PO_4 to 0.5M-K_2HPO_4. $2H_2O$ until the required pH was obtained. The buffer was almost exclusively used at pH 7.5.

VIII Ultracentrifugal Techniques

Sedimentation analyses of DNA preparations in caesium chloride density gradients were performed according to the method of Meselson, Stahl & Vinograd (1957).

Dilute Analar caesium chloride was purified by passage through a charcoal column and recrystallised by evaporating to dryness *in vacuo*.

Solid purified caesium chloride (approx., 2.7 g) was added to 2 ml. portions of DNA solutions containing 40-80 ug. of DNA, in SSC to give a density of 1.73 g./ml. in a total volume of 3-4 ml. The density was determined by refractometry using an Abbe refractometer. This preparation was transferred to a 5 ml. cellulose centrifuge tube, which was filled to the top by carefully layering 1 ml. of liquid paraffin on the caesium chloride solution, to prevent collapse of the tube during centrifugation.

Ultracentrifugation was carried out in the SW39 rotor of the Spinco Model L ultracentrifuge at 33,000 rev./min. at 24° for 60 hr. Fractions were collected from the resultant gradient by puncturing the base of the tube with a hypodermic needle (size 12) and allowing the contents to drop out into collecting tubes under slight pressure. Three drops were collected in each tube, diluted by the addition of 1 ml. of distilled water and measured for extinction at 260 mμ. using micro-cells (see Experimental, section IX A) and for radioactivity (see Experimental, section IX F).

IX General Analytical Procedures

A) Spectrophotometric Analysis

Extinction measurements in visible and ultraviolet light, were taken in Unicam SP-600, Unicam SP-500 and Beckman DB spectrophotometers. Where the volume of DNA fractions from MAK columns or caesium chloride density gradients was less than 3 ml., the extinction was determined using micro-cells in a Unicam SP-500 spectrophotometer fitted with a micro-cell attachment.

B) Estimation of Protein

Estimation of protein was carried out by the method of Lowry, Rosebrough, Farr & Randall (1951).

The reagents were:

i) Reagent A : 2% (w/v) Na_2CO_3 in 1N-NaOH.

ii) Reagent B : 0.5% (w/v) $CuSO_4.5H_2O$ in 1% (w/v) sodium or potassium tartrate.

iii) Reagent C : Alkaline copper solution (1 ml. of reagent B in 50 ml. reagent A).

iv) Reagent E : Folin-Ciocalteau phenol reagent, 1N in acid.

To 1 ml. of protein solution was added 5 ml. of reagent C, and, after 10 min., 0.5 ml. of reagent E was added, rapidly with vigorous shaking. The mixture was allowed to stand for 30 min., and the extinction at 750 mμ. was read in a Unicam SP-600 spectrophotometer. The assay was calibrated using a standard solution of bovine serum albumin.

C) Estimation of Phosphorus

Phosphorus was measured by the method of Allen (1940) which involves the conversion of all phosphorus to inorganic orthophosphate by digestion

with sulphuric acid and hydrogen peroxide, followed by the formation of a phosphomolybdate complex by reaction with ammonium molybdate, and reduction of this complex to a blue pigment with amidol (2:4 diaminophenol hydrochloride).

A sample of the solution to be assayed, containing between 20 and 150 µg. phosphorus, was pipetted into a microkjeldahl digestion flask together with 1.2 ml. 10N-H_2SO_4. A glass bead was then added to the flask which was transferred to a digestion rack and heated over a small flame until the contents were dark brown. At this point the flask was removed from the rack and two drops of hydrogen peroxide added, after which, digestion of the contents was continued until they became clear and fuming. The neck of the flask was then washed down with a small volume of distilled water and the contents once again digested to the fuming stage to remove any traces of hydrogen peroxide, which might have interfered with the reduction stage. The flask was then cooled and 21.65 ml. of water added, followed by 1 ml. 8.3% (w/v) ammonium molybdate and 2 ml. of 1% (w/v) amidol in 20% (w/v) potassium (or sodium) metabisulphate to a total volume of 25 ml. The mixture was then allowed to stand for 10 min. (but not longer than 30 min.) before measuring the extinction at 639 mµ in a Unicam SP-600 spectrophotometer against a reagent blank. When estimating the inorganic orthophosphate content of a solution, no digestion was required and, for small amounts of phosphorus, the estimations were occasionally performed using quantities scaled down to one fifth of those described above. A standard phosphate solution containing

1-150 µg. phosphorus was used for the construction of a calibration curve.

D) Estimation of Nucleotide Material

1) Estimation of DNA Spectrophotometrically

DNA was estimated by measuring the extinction of a preparation at 260 mµ. as described in the Experimental, section IX A. The conversion factor used was, 40 µg. DNA/ml. = 1 extinction unit at 260 mµ. This was based on total phosphorus estimations of samples of the DNA.

2) Estimation by Determination of Pentose Content

a) Estimation of DNA

DNA was estimated by the Ceriotti method (Ceriotti, 1952, 1955). To 2 ml. of a DNA solution (containing from 2.5 to 15 µg. DNA/ml.) was added 1 ml. of 0.04% (w/v) indole and 1 ml. of concentrated HCl. The tube was immersed in a boiling water bath for 10 min., and then cooled under running water. The cooled solution was then extracted three times with 4.0 ml. of chloroform, the chloroform layer being discarded each time. On the final extraction the tubes were centrifuged at 700 g for 10 min. to ensure complete removal of the chloroform. The extinction of the yellow colour remaining in the aqueous phase was measured at 460 mµ. in a Unicam SP-600 spectrophotometer against a reagent blank and the quantity of DNA in the sample was read from a standard calibration curve.

b) Estimation of Ribose

Ribose was estimated by the orcinol method (Kerr & Seraidarian, 1945). 3.0 ml. of ribose-containing solution was mixed with 3.0 ml. orcinol reagent

(60 mg. of orcinol dissolved in 10 ml. 0.02% $FeCl_3.6H_2O$, (w/v) in concentrated HCl), and heated in a boiling water bath for 30 min. The colour developed was measured in a Unicam SP-600 spectrophotometer at 665 mμ. A standard ribose solution containing 1-10 μg. ribose/ml. was used for calibration.

3) Estimation of DNA Phosphorus

DNA phosphorus was estimated by the Allen procedure for the determination of organically bound phosphorus (see Experimental, section IX C).

E) Paper Chromatography

Acid-soluble nucleotide material was routinely characterised by chromatography on paper. Neutralised solutions containing nucleotide material (0.05-2 μmoles) were applied to sheets of Whatman No. 1 chromatography paper which were subjected to descending chromatography in the ammonium isobutyrate solvent system (66 ml. isobutyric acid, 33 ml. water and 1.0 ml. ammonia) for 18 hr. at 25°. The paper was dried and the nucleotides located by virtue of their ability to absorb ultraviolet-light emitted at 254 mμ. from a low pressure mercury lamp. This procedure provided satisfactory resolution of the mono-, di-and triphosphates of each of the four deoxyribonucleosides. The various deoxyribonucleotides were identified either by their position relative to markers of known composition or by their Rf values.

During the preparation of (α-^{32}P)-dTTP it was found desirable, in order to establish the purity of the product, to locate inorganic ortho- and pyrophosphate and ester-linked phosphate in the chromatograms of the product (^{32}P)-dTMP and (α-^{32}P)-dTTP (see Experimental sections V B & C).

This was achieved by dipping the dried chromatogram in a solution of the following composition; 1.0 g. of ammonium molybdate dissolved in 8 ml. of distilled water to which were added 3 ml. 70% (v/v) perchloric acid, 3 ml. concentrated HCl and 86 ml. acetone. The paper was dried in air and spots of inorganic phosphate could be detected as light green coloured areas. If the paper was then exposed for 30 min. to ultraviolet-light emitted from a low pressure mercury lamp phosphorus containing areas showed up as blue spots. Therefore, it was possible in this way to distinguish between inorganic and ester-linked phosphate on paper chromatograms. The blue colour so obtained tended to fade rather quickly, but could be made to reappear by exposing the paper to ammonia vapour.

F) Radioactive Counting Procedures

The emission of β-particles from the radioactive phosphorus used in this work was recorded in a Nuclear Chicago windowless gas-flow counter. Occasionally the counting procedure was modified by fitting a thin end-window into the counter. The efficiency of these counters with (^{32}P) was approximately 50%, the introduction of an end-window only fractionally reducing this figure.

Paper discs, previously treated as described in the Experimental, section III A, were put on to stainless steel planchettes and counted for (^{32}P) radioactivity under conditions which approached infinite thinness, no correction for self absorption being necessary. Liquid samples which required to be assayed for (^{32}P) radioactivity were pipetted on to stainless steel planchettes together with several drops of ethanol to ensure even spreading of the fluid. The samples were then evaporated to dryness under an infra-red lamp and counted in the standard way. The specific radioactivity

of (α-^{32}P)-dTTP samples and the radioactive content of caesium chloride density gradient fractions were determined in this manner. When calculating the activities of DNA nucleotidyltransferase preparations, allowance was always made for the radioactive decay of the (^{32}P)-dTMP incorporated during the enzymic reaction.

Radioactivity on paper chromatograms was assayed by scanning 2.5 cm. strips cut from them, in a Nuclear Chicago Actigraph, coupled to a chart recorder which moved synchronously with the paper strip.

G) Materials

(^{32}P)-CEP and carrier-free (^{32}P)-orthophosphoric acid were purchased from the Radiochemical Centre, Amersham, Bucks, England. Deoxythymidine and the deoxyribonucleoside mono-di-and triphosphates of thymine, guanine, cytosine and adenine were purchased from Pabst Laboratories, Milwaukee, Wisconsin, U.S.A, the Sigma Chemical Company, St. Louis, Missouri, U.S.A and Schwarz Bioresearch Incorporated, Mount Vernon, New York, U.S.A. (α-^{32}P)-labelled dATP and dGTP were purchased from the International Chemical and Nuclear Corporation, Yorktown, California, U.S.A. Commercial preparations of calf thymus DNA were obtained from Seravac Laboratories Ltd., Bucks, England and crystalline bovine pancreatic DNase was purchased from the Sigma Chemical Company.

Hydroxylapatite was purchased from the Clarkson Chemical Company, Williamsport, Pennsylvania, U.S.A. and Sephadex G200 from Pharmacia Ltd., Uppsala, Sweden. Dowex ion-exchange resins were purchased from Bio-Rad Laboratories, Richmond, California, U.S.A, Whatman DEAE-cellulose from

RESULTS

W. & R. Balston, England and charcoal from British Charcoals, McDonalds Ltd., Grangemouth, Scotland.

DCC was purchased from the Aldrich Chemical Company, Milwaukee, Wisconsin, U.S.A, 2 cyanoethanol, iodoacetamide and GSH from British Drug Houses Ltd., Poole, Dorset, England, caesium chloride from Hopkins & Williams Ltd., Chadwell Heath, Essex, England and 2-mercaptoethanol from L. Light & Company Ltd., Bucks, England. The sodium salt of sarkomycin (2-methylene-3-oxo-cyclopentane carboxylic acid, - Hooper et al, 1955) was kindly gifted by the Banyu Pharmaceutical Company, Tokyo, Japan and sodium p-hydroxymercuribenzoate and tris-HCl buffer preparations were obtained from the Sigma Chemical Company. Actinomycin D was a gift from Merck, Sharp and Dohme Inc., Rahway, New Jersey, U.S.A.

RESULTS

I Enzyme Fractionation

A) General Considerations

The results contained in parts B) and C) of this section have been obtained with enzyme fractions prepared by a routine fractionation procedure. Indeed, fractionation of both Landschuts and thymus enzymes by the soluble extraction, pH 5 precipitation and ammonium sulphate steps has been repeated many times. As can be readily appreciated, enzyme activities of initial extracts of both tissues varied within relatively wide limits depending on such factors as the condition of the cells at the time of preparation and the duration of the fractionation procedure. Therefore, the activities and purification factors quoted for both enzymes were chosen because they give a good representation of the average fractionation. As far as possible throughout this work, it was attempted to standardise preparative procedures but, on occasion, they were modified for particular purposes.

B) DNA Nucleotidyltransferase from Landschutz Ascites-Tumour Cells

1) Routine Fractionation Procedure

The purification and activities achieved for DNA nucleotidyltransferase from Landschuts cells after a typical fractionation by soluble extract preparation, pH 5 precipitation, ammonium sulphate fractionation and hydroxylapatite chromatography (see Experimental, sections II & III) are given in Table 1.

On occasions, a pH 5.9 precipitate fraction was initially removed from

TABLE 1

Routine fractionation procedure for DNA nucleotidyltransferase from Landschutz ascites-tumour cells.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH7.5; 15 µmoles of KCl; 0.10 µmole of EDTA; 1 µmole of $MgSO_4$; 1.25 µmoles of 2 mercaptoethanol; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP; dGTP, dCTP and (α-^{32}P) -dTTP (4.5×10^6 counts/min./µmole) and the indicated enzyme fraction, in a total volume of 0.25 ml.

Protein concentration/assay for all fractions except the hydroxylapatite eluate was in the region, 100-150 µg. Protein concentration/assay for the hydroxylapatite fraction was in the range, 20-30 µg. Incubation was carried out at 37° for 60 min.

TABLE 1

Fraction	(^{32}P)-dTMP incorporated (mμmoles/mg. of protein/hr.)	% Recovery of Enzyme	Purification (over Soluble Extract)
Soluble Extract	4.43	-	-
pH 5 Precipitate	11.18	76	2.6
20 - 45% $(NH_4)_2SO_4$ Fraction	18.72	48	4.2
Hydroxylapatite Fraction	48.03	-	10.8

the soluble extract before preparation of a pH 5.9 - 5.0 precipitate fraction. However, as this procedure removed very little protein and effected negligible purification, it was discontinued. On standing for 10-12 hr. a precipitate formed in the solubilised pH 5 precipitate fraction; although it usually contained some DNA nucleotidyltransferase activity, it was removed by centrifugation as it was desirable to work with a completely soluble fraction.

Ammonium sulphate fractionation was originally carried out with calf thymus DNA nucleotidyltransferase preparations. In these experiments it was found that the best purification achieved with various permutations of ammonium sulphate saturation bands was of the order of 3-fold in the 40-50% saturation fraction, whereas the bulk of the enzyme precipitated in the 20-45% fraction with a purification of the order of 1.7-fold. It was therefore, decided on this basis to take an ammonium sulphate cut of 20-45%, thus compromising to obtain a fraction, which was less pure but contained a greater amount of enzyme. In later stages of the work the same procedure was used with the Landschuts enzyme and yielded a purification of the same order. The value of the ammonium sulphate fraction seems to lie more in its much greater solubility when compared to the pH 5 precipitate fraction, than in its purification properties.

The data in Table 1 for hydroxylapatite chromatography were calculated for the fraction with the highest activity and do not represent pooled fractions of the enzyme.

The hydroxylapatite column work yielded results which were primarily

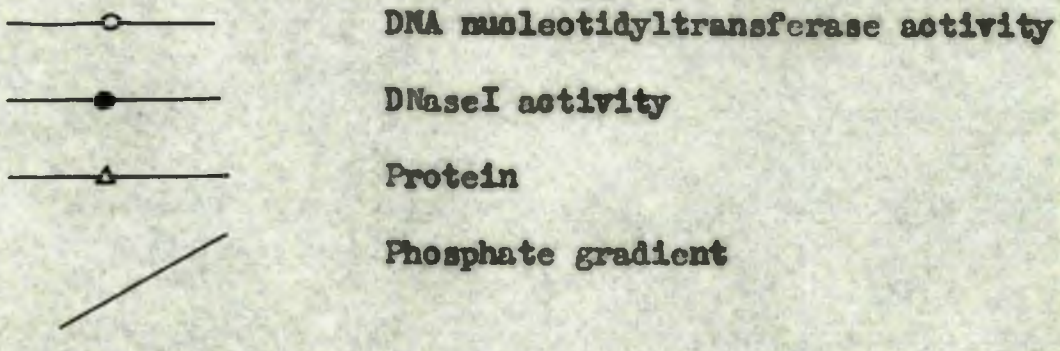
of academic interest, although it unquestionably offers a firm basis for future provision of DNA nucleotidyltransferase in large quantities. An almost total separation of DNA nucleotidyltransferase from DNaseI was achieved by the hydroxylapatite chromatographic procedure (see Experimental, section III & Fig., 12). 35.5 mg. of protein, approximately 50% of which was recovered, was added to such a 10 cm. x 1 cm. column. The orders of recovery of DNA nucleotidyltransferase and DNaseI from the column were 50% and 90% respectively. The enzymes recovered were distributed among several fractions of varying activity and, for this reason, no overall yield of enzyme for the hydroxylapatite chromatography step is given in Table 1.

By means of exploratory experiments with standard hydroxylapatite columns, a linear phosphate gradient was constructed (see Experimental, section IX C). In the analytical hydroxylapatite column the DNaseI was eluted in the region 0.02-0.11M phosphate and the DNA nucleotidyltransferase in the region 0.05-0.18M phosphate. Two approaches were adopted to scale up the process in order to obtain enough DNaseI-free DNA nucleotidyltransferase for experimental purposes. In the first of these, the column size was increased to 10 cm. x 2 cm., but it was found that on application of protein under pressure to such a column, the hydroxylapatite bed cracked forming channels through which the eluting fluid percolated in a non-uniform fashion. The second method investigated was a stepwise fractionation of the 20-45% ammonium sulphate fraction on hydroxylapatite. The enzyme was adsorbed to a measured quantity of hydroxylapatite equilibrated in

FIGURE 12

Hydroxylapatite chromatography of DNA nucleotidyltransferase extracts of Landschutz ascites-tumour cells.

For experimental details see Experimental, section III and Results section I B. The conditions for DNA nucleotidyltransferase assays were as described in Fig. 16, and those for DNaseI assays as described in Fig. 14 Incubation was carried out at 37° for 60 min.



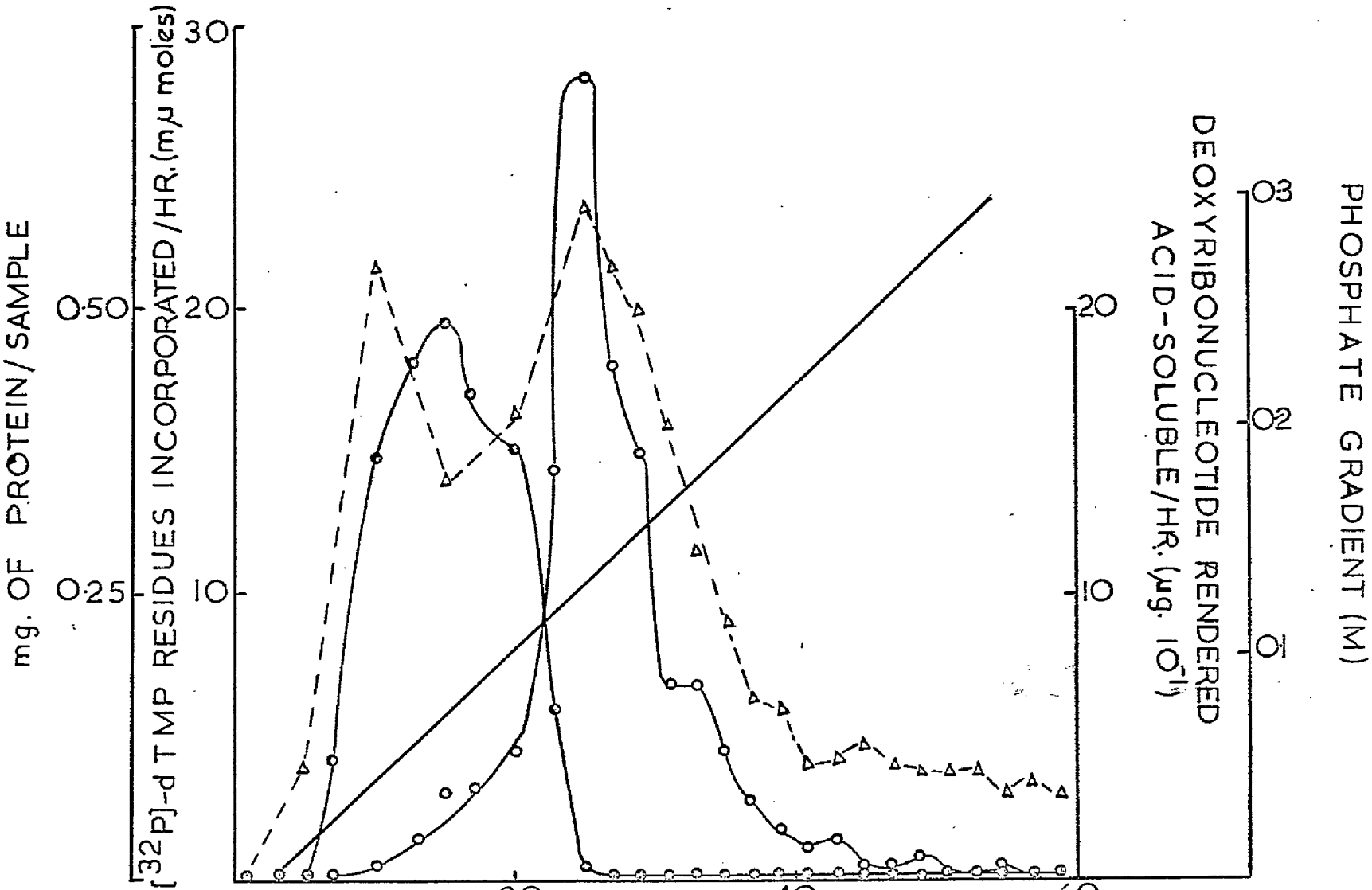

mg. OF PROTEIN/SAMPLE
0·50
0·25
[32P]-d TMP RESIDUES INCORPORATED/HR.(mμ moles)
30
20
10
20
40
60
FRACTION No.
DEOXYRIBONUCLEOTIDE RENDERED ACID-SOLUBLE/HR. (μg. 10-1)
20
10
PHOSPHATE GRADIENT (M)
0·3
0·2
0·1

FIGURE 12

0.001M-EDTA-0.005M-2-mercaptoethanol-0.001M-potassium phosphate buffer, pH 7.5, by stirring for 10 min. at 0°. The supernatant fraction was removed by centrifugation and the hydroxylapatite was sequentially eluted with phosphate buffers of increasing ionic strength in an attempt to separate the enzymes. However, although a separation was achieved, this method yielded neither significant purification nor good recovery of enzymes.

The overall purification achieved for the whole fractionation procedure was of the order of 10-12 fold.

The following calculation expresses the amount of DNA synthesised by the 20-45% ammonium sulphate fraction (see Table 1) as a percentage of the primer DNA:

Activity of the 20-45% ammonium sulphate fraction =

= 18.72 mμmoles of (^{32}P)-dTMP incorporated/mg. of protein/hr.

Amount of protein/assay = 150 μg.

∴ Synthesis/assay = 18.72 x 0.15

= 2.81 mμmoles of (^{32}P)-dTMP incorporated/assay/hr.

= 2.81 x 4 mμmoles of all 4 deoxyribonucleotides incorporated/assay/hr.

= 11.24 mμmoles of deoxyribonucleotide incorporated/assay/hr.

Amount of primer/assay = 50 μg.

= 50/329 μmoles (329 is the average molecular weight of the 4 deoxyribonucleotides)

= 152 mμmoles deoxyribonucleotide

∴ Percentage primer replicated = $\frac{11.24}{152}$ = 7.4%/hr./assay

2) DEAE-Cellulose Chromatography

While the quantities of both DNA and RNA in the 20-45% ammonium sulphate fractions, as determined by the Ceriotti and orcinol methods (see Experimental, section IX D,2), were only of the order of 4 µg./mg. of protein, for the purpose of obtaining enzyme preparations completely free of nucleic acid, chromatography of these fractions on DEAE-cellulose was employed.

This technique described in Experimental, section III C, was essentially that employed by Bollum (1960). The enzyme was applied to the column in a solution of ionic strength 0.45, which allowed retention of most species of cellular nucleic acids while the enzyme passed through unadsorbed. Removal of nucleic acid from the preparation was followed by the increase in the ratio of the extinction at 280 mµ. to that at 260 mµ in the fractions obtained from the column. The column eluate was collected in measured volumes, whose absorption spectra were read in the Cary Recording spectrophotometer and compared with the spectrum of the preparation applied to the column. Fig. 13 shows the effect of DEAE-cellulose chromatography on a 20-45% ammonium sulphate preparation of Landschutz DNA nucleotidyltransferase. The $E_{280}:E_{260}$ ratio has increased on chromatography from 0.57 to 1.50, which indicates that almost all the nucleic acid has been removed from the preparation. The column eluates were then dialysed, assayed for protein content, DNA nucleotidyltransferase and DNaseI activities and the fraction containing the highest DNA nucleotidyltransferase activity used in experiments designed to demonstrate a requirement for primer DNA. As only 25% of the applied protein and

FIGURE 13

Spectra of the 20 - 45% ammonium sulphate fraction from Landschutz ascites-tumour cells before and after DEAE-cellulose chromatography, measured between 240 and 320 mμ.

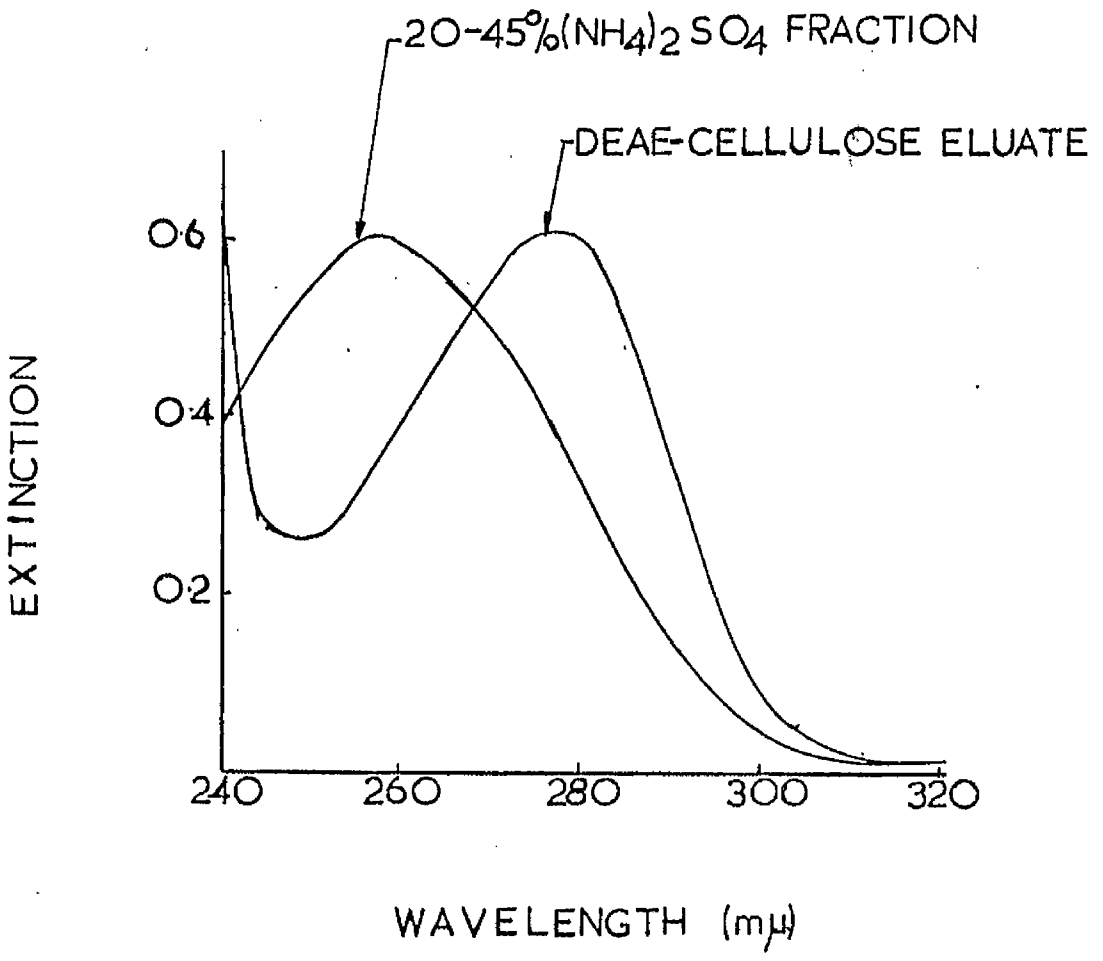

20-45% $(NH_4)_2SO_4$ FRACTION
DEAE-CELLULOSE ELUATE
EXTINCTION
0·6
0·4
0·2
240
260
280
300
320
WAVELENGTH (mμ)

FIGURE 13

20% of the applied DNA nucleotidyltransferase were recovered from the column and negligible purification obtained, this technique was not routinely employed in enzyme fractionation.

3) Nuclease Content of the Soluble Extract, pH5 Precipitate and 20-45% Ammonium Sulphate Fractions

DNaseI assays were routinely carried out on the fractions obtained at the various stages of DNA nucleotidyltransferase fractionation described in section 1 above. These assays were performed under conditions optimal for DNA nucleotidyltransferase activity (see Experimental, section IV B). The DNaseI activities of the soluble extract, pH5 precipitate and 20-45% ammonium sulphate fractions, shown in Table 2, are expressed as μmoles of deoxyribonucleotide rendered acid-soluble/mg. of protein/hr. During this fractionation procedure the activity tends to diminish at each succeeding stage indicating that DNaseI is being removed.

Fig. 14 shows a plot of DNaseI activity against protein concentration for a Landschutz 20-45% ammonium sulphate fraction. There is a very short lag phase at low protein concentrations, where the quantity of enzyme may not be sufficient to produce an amount of acid-soluble nucleotide material proportional to that produced by higher concentrations of protein during the same incubation period. Above 150 μg. of protein/assay the slope of the activity curve becomes much less steep indicating a decrease in the reaction rate. Therefore, all DNaseI measurements were carried out, whenever possible, with approximately 100 μg. of protein/assay.

TABLE 2

DNaseI activities of the fractions obtained during the purification of DNA nucleotidyltransferase from Landschutz ascites-tumour cells.

The reaction mixture contained: 25 μmoles of tris-HCl buffer, pH7.5; 75 μmoles of KCl; 0.50 μmoles of EDTA; 5 μmoles of $MgSO_4$; 7.25 μmoles of 2-mercaptoethanol; 300 μg. of heat-denatured DNA and the indicated enzyme fraction, in a total volume of 1.25 ml. Protein concentration/assay for all fractions was in the region, 80-120 μg. Incubation was carried out at 37° for 60 min.

TABLE 2

Fraction	Deoxyribonucleotide Rendered Acid-Soluble (μmoles/mg. of protein/hr.)
Soluble Extract	0.284
pH 5 Precipitate	0.254
20 - 45% $(NH_4)_2SO_4$ Fraction	0.217

FIGURE 14

DNaseI activity of extracts of Landschutz ascites-tumour cells as a function of protein concentration of the fraction in the assay.

The reaction mixture for the DNaseI assay contained: 25 µmoles of tris-HCl buffer, pH 7.5; 5 µmoles of $MgSO_4$; 75 µmoles of KCl; 0.5 µmole of EDTA; 7.25 µmoles of 2-mercaptoethanol and 300 µg. of heat-denatured DNA. The protein concentration/assay was as indicated and incubation was carried out at 37° for 60 min.

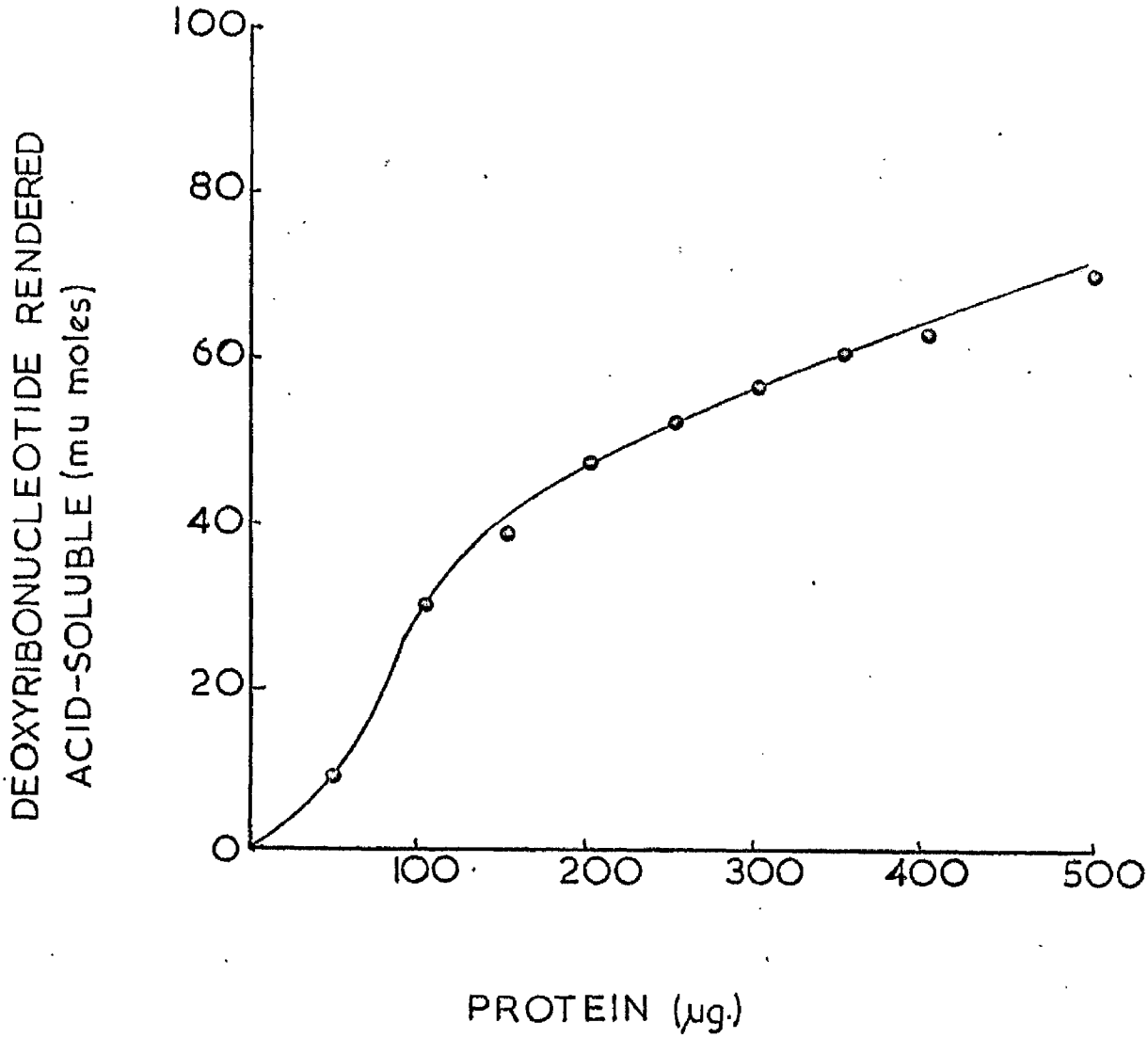

DEOXYRIBONUCLEOTIDE RENDERED
ACID-SOLUBLE (mu moles)
100
80
60
40
20
0
100
200
300
400
500
PROTEIN (µg.)

FIGURE 14

The following calculation quantitates the effect of DNaseI activity in the DNA nucleotidyltransferase assay system in terms of the percentage of primer rendered acid-soluble.

Amount of DNA/assay = 300 µg.

= 910 mµmoles (average molecular weight of 4 deoxyribonucleotides = 329)

Average activity of DNaseI activity (Table 2)

= 250 mµmoles of acid-soluble deoxyribonucleotide/ mg. of protein/hr.

= 25 mµmoles of deoxyribonucleotide acid-soluble/ 100 µg. of protein/hr.

∴ % Primer rendered acid-soluble/hr./assay = 25/910 = 2.75%

At this stage it seems appropriate to point out that there may well be a divergence between the DNA nucleotidyltransferase assay and the DNaseI assay with respect to the length of the deoxyribonucleotide molecule which is acid-soluble. Since, in the DNA nucleotidyltransferase assay, precipitation takes place in the cellulose matrix of the paper disc, whereas in the DNaseI assay acid-precipitation takes place in free solution, it is possible that short molecules which are not precipitated in the DNaseI assay will tend to be precipitated when they are caught up in the cellulose fibres of the paper disc in the DNA nucleotidyltransferase assay. Therefore, it may well be that the figure of 2.75% of the primer DNA rendered soluble by DNaseI activity during assay may not be as great when considered in the context of the DNA nucleotidyltransferase assay.

4) Deoxyribonucleoside Triphosphatase Activities of the Soluble Extract, pH 5 Precipitate and 20-45% Ammonium Sulphate Fractions

Soluble extract, pH 5 precipitate and 20-45% ammonium sulphate fractions were assayed for deoxyribonucleoside triphosphatase activity under conditions optimal for DNA nucleotidyltransferase activity as described in Experimental, section IV C. Triphosphatase activity towards all four deoxyribonucleoside triphosphates, dATP, dGTP, dCTP and dTTP was investigated. In all four cases and for all enzyme fractions there was found to be little or no triphosphatase activity as measured by the production of inorganic orthophosphate over a 2 hr. period of incubation (see Experimental, section IX C for phosphate estimation method).

5) Time Course of DNA Nucleotidyltransferase Preparations

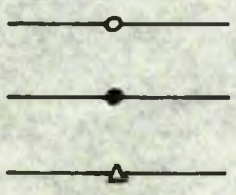
Time courses, covering a period of 120 min., for DNA nucleotidyltransferase activities of soluble extract, pH 5 precipitate and 20-45% ammonium sulphate fractions are shown in Fig. 15. Both the soluble extract and pH 5 precipitate activities were linear over the whole 2 hr. period, the 20-45% ammonium sulphate fraction, however, was linear only for 60 min., levelling off during the second half of the incubation period. In other experiments, linearity over a 90 min. incubation period was observed for the 20-45% ammonium sulphate fraction, before activity began to fall off. For uniformity 60 min. was routinely employed as the incubation period for DNA nucleotidyltransferase assays for all enzyme fractions, except where practical or experimental considerations determined the use of different incubation times.

FIGURE 15

Time courses of DNA nucleotidyltransferase activities of extracts of Landschutz ascites-tumour cells.

The reaction mixture for DNA nucleotidyltransferase contained: 5 µmoles of tris-HCl buffer, pH 7.5, 0.1 µmole of EDTA; 1 µmole of $MgSO_4$; 15 µmoles of 2-mercaptoethanol; 50 µg. of heat-denatured DNA; 50 mµmoles each of dGTP, dATP, dCTP and (α-^{32}P) - dTTP (11.2 x 10^6 counts/min./µmole) and enzyme in a total volume of 0.25 ml. The protein content/assay for the soluble extract, pH 5 precipitate and 20 - 45% ammonium sulphate fractions were 102, 107 and 154 µg. respectively.

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



soluble extract

pH 5 precipitate

20 - 45% ammonium sulphate fraction

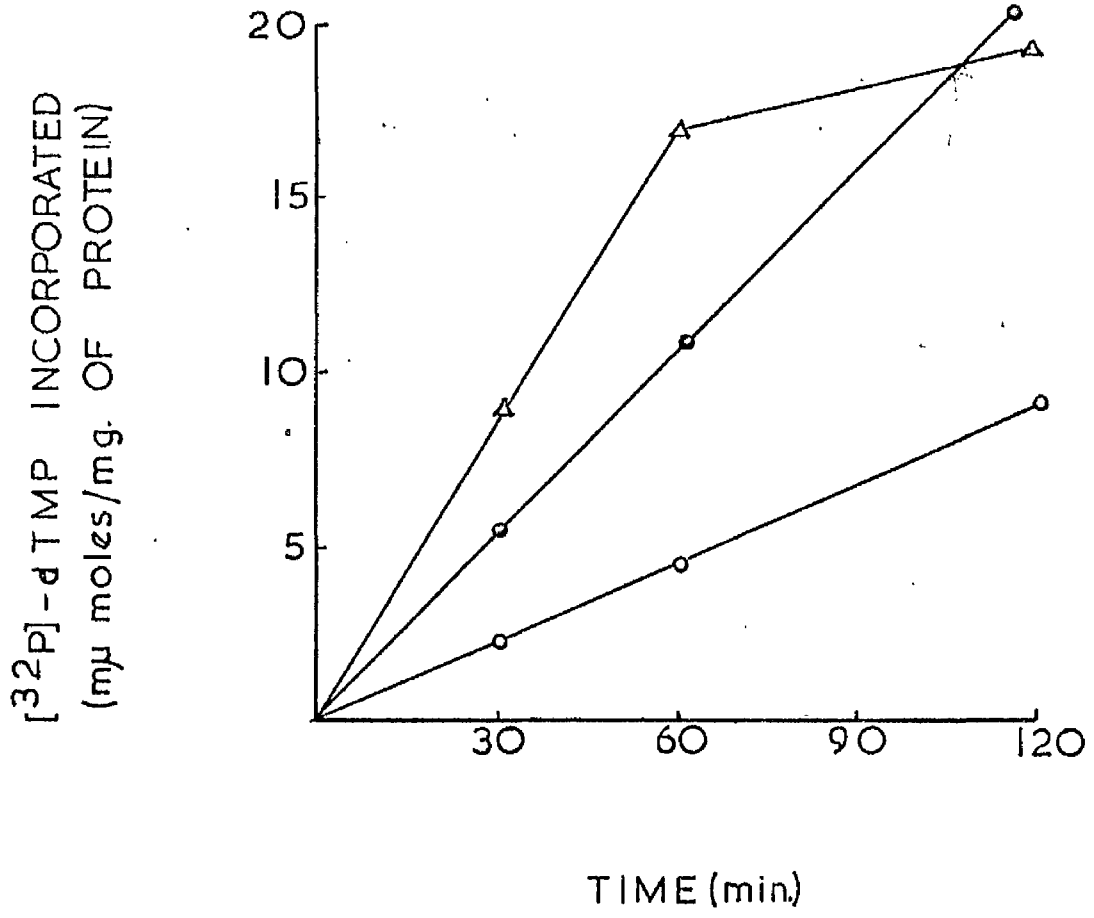

[32P]-d TMP INCORPORATED
(mμ moles/mg. OF PROTEIN)
20
15
10
5
30
60
90
120
TIME (min.)

FIGURE 15

6) The Effect of Enzyme Concentration on DNA Nucleotidyltransferase Preparations

The effect of increasing the protein content in DNA nucleotidyltransferase assays of soluble extract, pH 5 precipitate and 20-45% ammonium sulphate fractions is shown in Fig. 16, which indicates that the assay system is linear for protein concentrations up to 120 µg./assay and deviates only very slightly from linearity between 120 and 200 µg./assay. Other experiments have indicated that linearity can extend as far as 240 µg. of protein/assay (Keir, 1962). Therefore, where ever possible, enzyme activity was measured in the region of 100-150 µg. of protein/assay.

C) DNA Nucleotidyltransferase from Calf Thymus Gland

1) Routine Fractionation Procedure

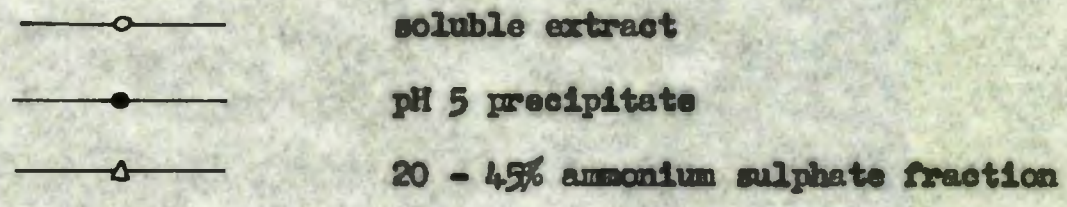
Soluble extract, pH 5 precipitate and 20-45% ammonium sulphate fractions were prepared from cell-free preparations of calf thymus tissue as described in Experimental, section III and in the Results section which describes a similar fractionation of Landschutz extracts. The quantitative aspects of the fractionation are given in Table 3. Fractionation of DNA nucleotidyltransferase from calf thymus gland through the stages of pH 5 precipitation and 20-45% ammonium sulphate precipitation led to enzyme yields and purifications similar to those obtained for the DNA nucleotidyltransferase from Landschutz cells. The yield of enzyme at the 20-45% ammonium sulphate fraction stage was approximately 50% with a purification of 5-6-fold.

Experiments involving chromatography of a 20-45% ammonium sulphate fraction from calf thymus on hydroxylapatite columns, using a batchwise

FIGURE 16

Incorporation of (^{32}P)-dTMP into product in the DNA nucleotidyltransferase assay system at various protein concentrations of the enzyme fraction by extracts of Landschuts ascites-tumour cells.

The reaction mixtures contained: 5 µmoles of tris-HCl buffer pH 7.5: 15 µmoles of KCl; 0.1 µmole of EDTA; 1.0 µmole of $MgSO_4$; 1.25 µmoles of 2-mercaptoethanol; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)- dTTP (2.8×10^6 counts/min./µmole) and the indicated amount of protein in a total volume of 0.25 ml. Incubation was carried out at 37° for 60 min.



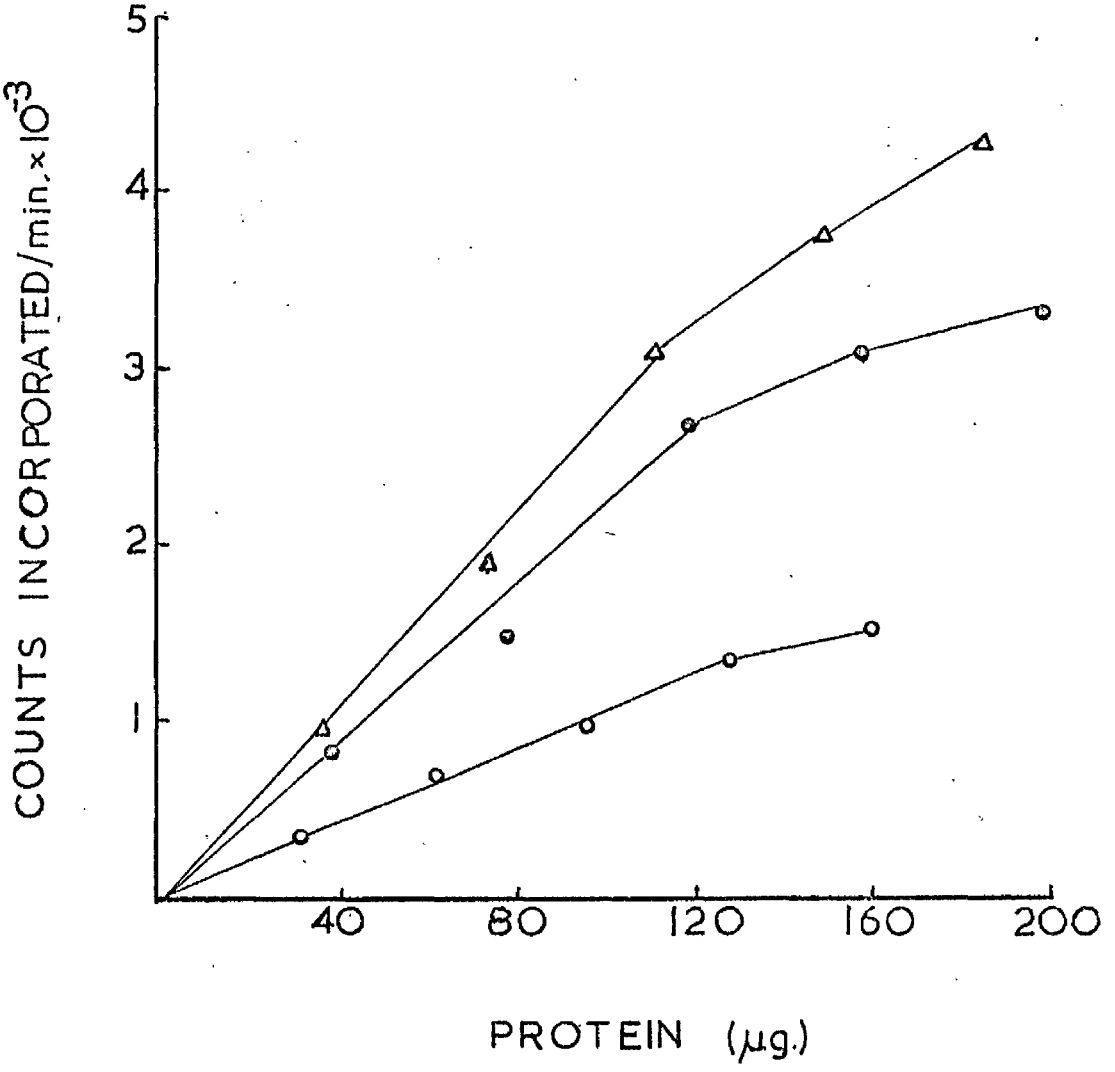

COUNTS INCORPORATED/min.×10⁻³
5
4
3
2
1
40
80
120
160
200
PROTEIN (μg.)

FIGURE 16

TABLE 3

Routine fractionation procedure for DNA nucleotidyltransferase from calf thymus tissue.

The reaction mixture contained: 5 μmoles of tris-HCl buffer, pH 7.5; 15 μmoles of KCl; 0.10 μmole of EDTA; 1 μmole of $MgSO_4$; 1.25 μmoles of 2-mercaptoethanol; 50 μg. of heat-denatured DNA; 50 mμmoles each of dATP, dGTP, dCTP, and (α-^{32}P)-dTTP (6.2×10^6 counts/min./μmole) and the indicated enzyme fraction, in a total volume of 0.25 ml. Protein concentration for all fractions was in the region of 100 - 150 μg. Incubation was carried out at 37° for 60 min.

TABLE 3

Fraction	(^{32}P)-dTMP Incorporated (mμmoles/mg. of protein/hr.)	% Recovery of Enzyme	Purification over Soluble Extract
Soluble Extract	7.10	-	-
pH 5 Precipitate	17.71	79	2.5
20 - 45% $(NH_4)_2SO_4$ Fraction	38.78	53	5.5

elution technique indicated that DNaseI and DNA nucleotidyltransferase activities were eluted at 0.175M and 0.225M-phosphate respectively. During this procedure, a purification of 2.3-fold was achieved for DNA nucleotidyltransferase.

2) Gel Filtration on Sephadex G-200

Chromatography of a 20-45% ammonium sulphate fraction from calf thymus was carried out on Sephadex G-200 as described in Experimental, section III D. Each fraction was assayed for protein content, DNA nucleotidyltransferase and DNaseI activities, the results of which are shown in Fig. 17. A partial separation of DNA nucleotidyltransferase and DNaseI activities was achieved, the DNaseI accompanying the bulk of the protein with the DNA nucleotidyltransferase following slightly behind. This resulted in a 1.5-fold purification of DNA nucleotidyltransferase. Collection of fractions began 2 ml. before the void volume of eluting buffer had passed through the column. Elution of DNA nucleotidyltransferase was first detected at 1.4 void volumes of the column and the peak of enzymic activity was located at 1.75 - 1.90 void volumes. Recoveries of protein, DNA nucleotidyltransferase and DNaseI were 68%, 41% and 83% respectively.

3) Other Properties of Calf Thymus Enzyme Fractions

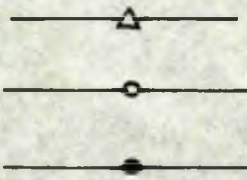
As the main object of this work was the investigation of DNA synthesis in Landschutz ascites-tumour cells, the characteristics of the thymus enzymic preparations were not thoroughly investigated. Preliminary experiments indicated that such properties as DNaseI activity, time course

FIGURE 17

Sephadex G-200 chromatography of DNA nucleotidyltransferase extracts of calf thymus.

The dimensions of the column were 60 cm. x 1.5 cm.; the volume of each fraction was 3 ml.; the void volume of the column, as determined by the elution volume of native DNA was 28 ml. and the quantity of protein applied to the column was 12.4 mg. in a total volume of 1.5 ml.

The conditions for the DNA nucleotidyltransferase assays were as described in Fig. 16 and those for the DNaseI assays as described in Fig. 14. Incubation was carried out at 37° for 90 min.



protein content

DNA nucleotidyltransferase activity

DNaseI activity

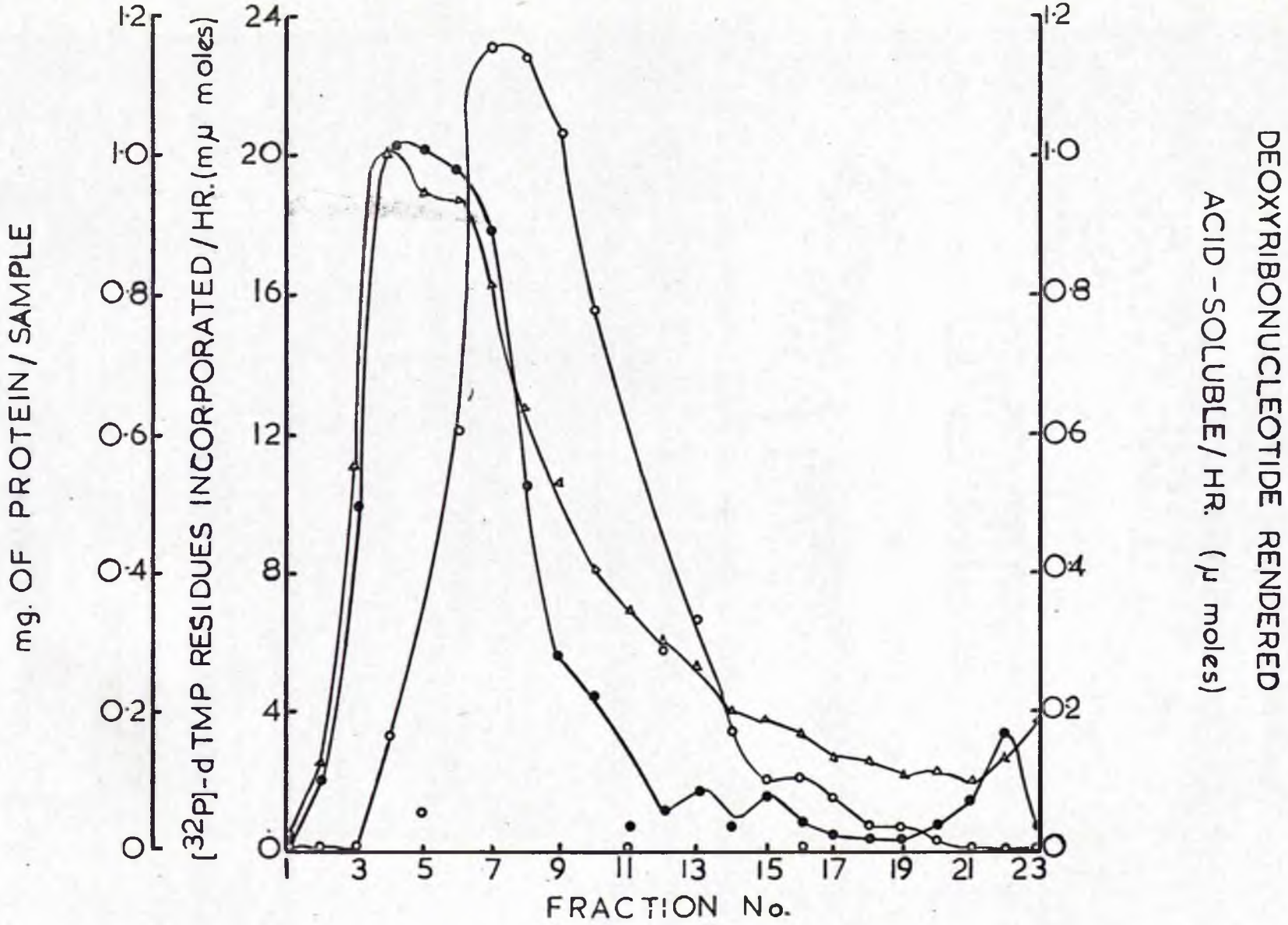

mg. OF PROTEIN / SAMPLE
[32P]-d TMP RESIDUES INCORPORATED / HR. (mμ moles)
DEOXYRIBONUCLEOTIDE RENDERED ACID-SOLUBLE / HR. (μ moles)
FRACTION No.
0
0·2
0·4
0·6
0·8
1·0
1·2
4
8
12
16
20
24
1
3
5
7
9
11
13
15
17
19
21
23

FIGURE 17

and DNA nucleotidyltransferase activity versus protein concentration relationship were very similar to those in the corresponding Landschutz preparations. Therefore, whenever possible assay conditions with respect to these variables were made the same as those chosen as optimal for the Landschutz enzyme (see Results, section I A)

D) DNA Nucleotidyltransferase Activity in Other Tissues

Evidence for DNA nucleotidyltransferase in certain tissues of the young rabbit (bone marrow, thymus, spleen and appendix) and in two tissues of the rat (liver and spleen) has been obtained under conditions optimal for the Landschutz enzyme. The activities of pH 5.0 precipitate fractions obtained from these tissues are shown in Table 4.

The figure for rabbit bone marrow was much lower than that quoted by Gray et al. (1960) for a less purified fraction and that for rabbit spleen is much lower than the figure for rat spleen. These figures were so unusual that several attempts were made to detect greater activity in rabbit bone marrow and spleen. However, they did not meet with success and it may be that some difference in the fractionation procedure or assay conditions inactivates the enzyme in some way.

II Requirements for DNA Nucleotidyltransferase Activity

A) DNA Nucleotidyltransferase from Landschutz Ascites-Tumour Cells

1) General Considerations

The sub-sections which follow describe the investigation of the various requirements of DNA nucleotidyltransferase. The enzyme has been shown to require primer DNA, a bivalent cation, four deoxyribonucleoside

TABLE 4

DNA nucleotidyltransferase activities of various rodent tissues.

The reaction mixture contained: 10 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.08 µmole of EDTA; 1 µmole of $MgCl_2$; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (20.5 - 8.4 x 10^6 counts/min./µmole) and 90 - 160 µg. of protein from the appropriate pH 5 precipitate fraction, in a total volume of 0.25 ml. Incubation was carried out at 37° for 60 min.

TABLE 4

Tissue	(^{32}P-)-dTMP Incorporated (mμmoles/mg. of protein/hr.)
Rabbit Bone Marrow	0.56
Rabbit Thymus	6.96
Rabbit Appendix	8.96
Rabbit Spleen	0.81
Rat Liver	0.68
Rat Spleen	3.39

triphosphates, dATP, dGTP, dCTP and dTTP for activity at pH 7.5, and to be stimulated by a univalent cation and by EDTA.

2) The Effect of pH on DNA Nucleotidyltransferase Activity

The effect of pH on the DNA nucleotidyltransferase activity of 20 - 45% ammonium sulphate fractions is shown in Fig. 18. The pH of these buffers was measured both at 25° and 37°. The optimal pH for the enzyme during incubation (i.e., at 37°) lies in the range 7.2 - 7.4. As the pH of these commercially prepared tris-HCl buffers was shown to fall approximately 0.2 pH units on heating from 25° to 37° the enzyme was routinely assayed with 5-10 μmoles of tris-HCl buffer, pH 7.5, prepared in the laboratory as described in Experimental VII A.

3) Requirement for Primer DNA

The fraction from the DEAE-cellulose column (see Results, section I B2) which contained the highest activity of DNA nucleotidyltransferase was assayed for DNA nucleotidyltransferase with increasing concentrations of DNA-primer. The results are shown in Fig. 19, which indicates that the enzyme has an absolute requirement for primer DNA, and in its absence no incorporation of radioactivity into acid-insoluble product can be detected.

The information in Fig. 19 suggests that for the 20 - 45% ammonium sulphate fraction 50 μg. of DNA/assay lies on the plateau of enzymic activity under standard assay conditions. Fig. 20 shows that the same property applies to the soluble extract and pH 5 precipitate fractions. Therefore, 50 μg. of heat-denatured DNA were routinely included in assay of DNA nucleotidyltransferase under optimal conditions.

FIGURE 18

The effect of pH on DNA nucleotidyltransferase activity of extracts of Landschutz ascites-tumour cells.

The reaction mixture contained: 10 µmoles of tris-HCl buffer at the indicated pH; 1 µmole of $MgSO_4$; 15 µmoles of KCl; 0.10 µmole of EDTA; 1.25 µmoles of 2-mercaptoethanol; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (4.4×10^6 counts/min./µmole) and 0.05 ml. of a 20 - 45% ammonium sulphate fraction (125 µg. of protein) in a total volume of 0.25 ml. Incubation was carried out at 37° for 60 min.

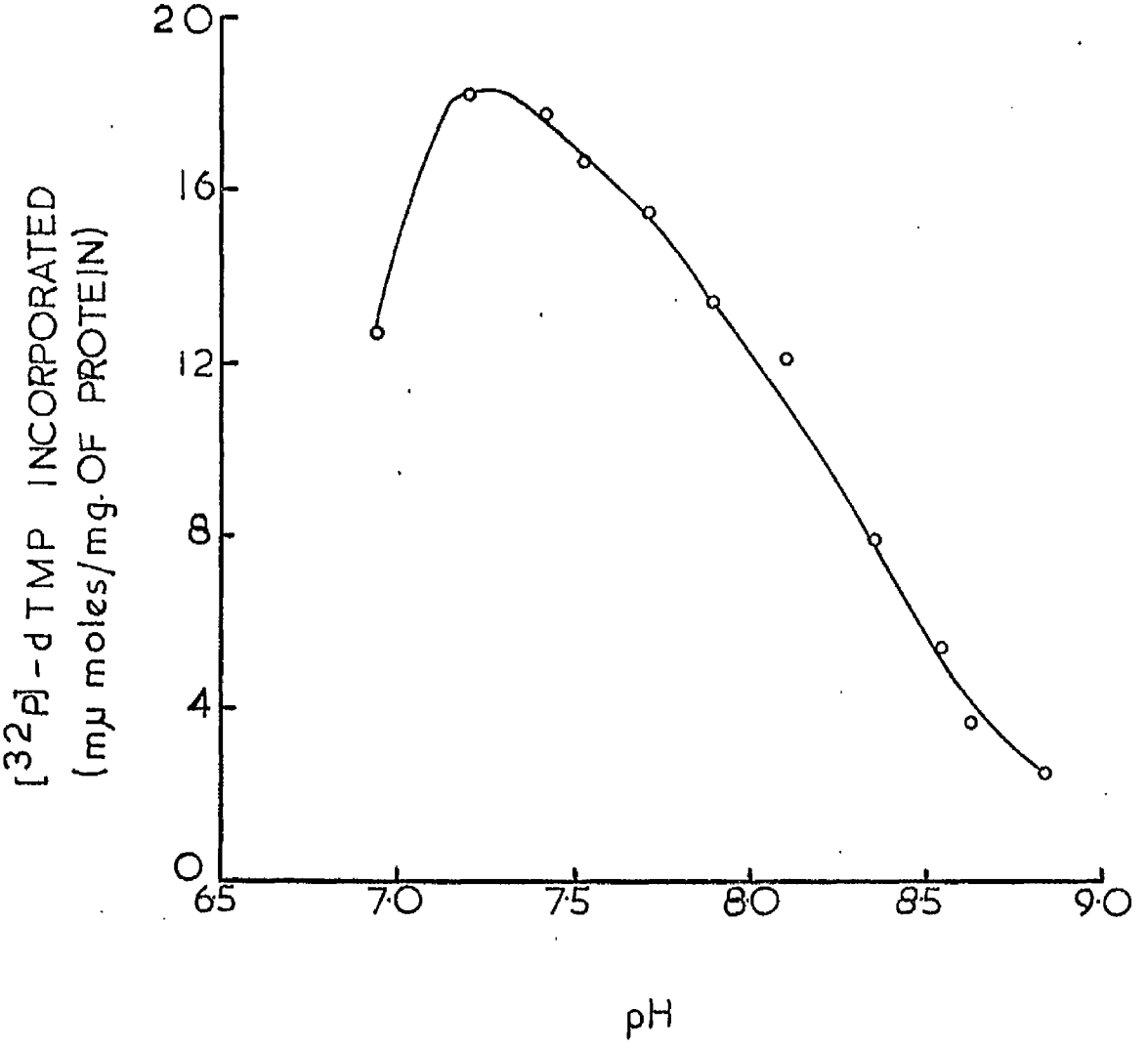

20
16
12
8
4
0
6·5
7·0
7·5
8·0
8·5
9·0
[32P]-dTMP INCORPORATED
(mμ moles/mg OF PROTEIN)
pH

FIGURE 18

FIGURE 19

The effect of primer-DNA concentration on the DNA nucleotidyltransferase activity of a 20 - 45% ammonium sulphate fraction from Landschutz ascites-tumour cells previously chromatographed on DEAE-cellulose.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.10 µmole of EDTA; 1 µmole of $MgSO_4$; 1.25 µmoles of 2-mercaptoethanol; the indicated amount of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (2.4×10^6 counts/min./µmole) and 0.10 ml. (61 µg. of protein) of the DEAE-cellulose column eluate in a total volume of 0.25 ml. Incubation was carried out at 37° for 60 min.

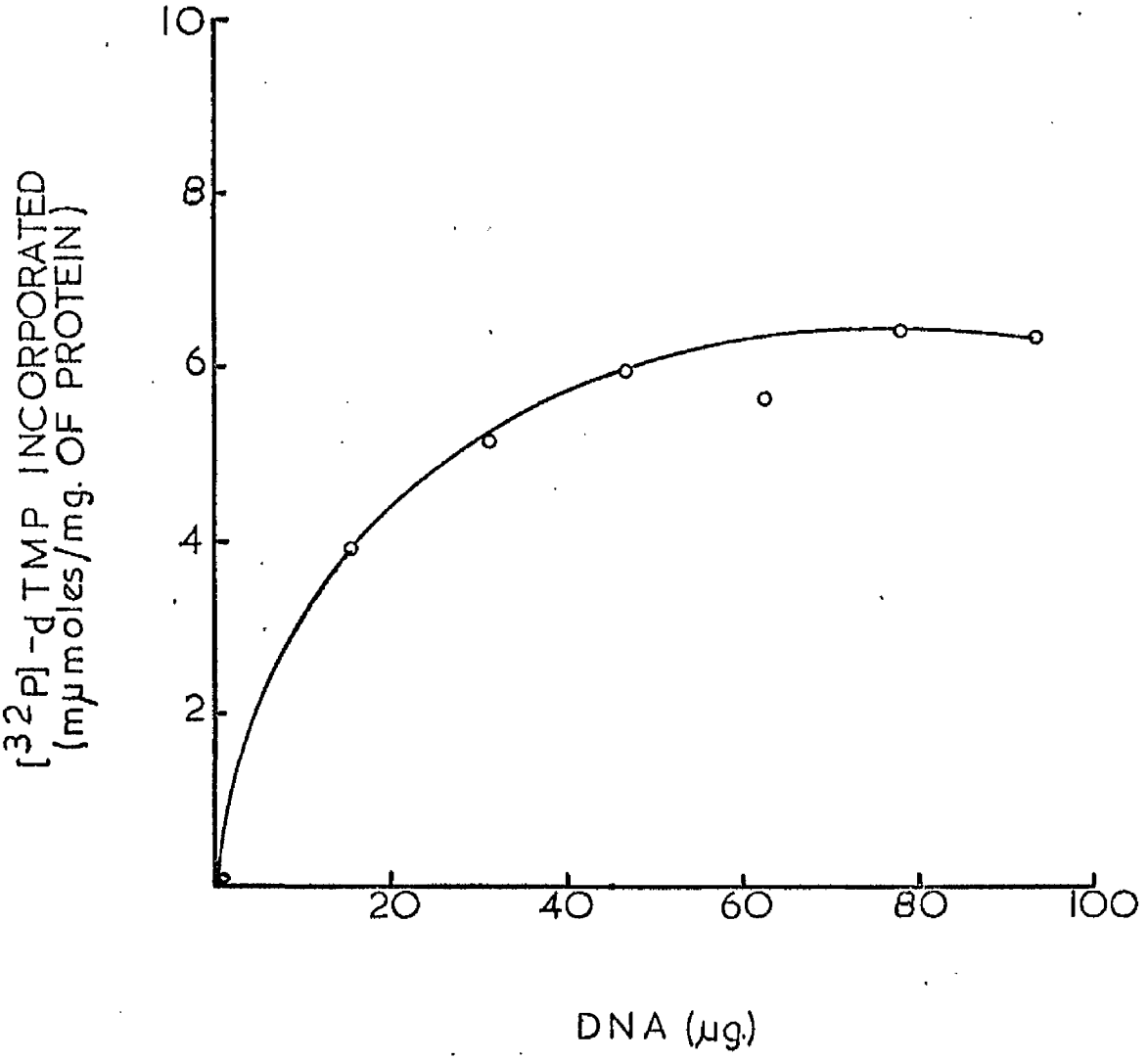

[32P]-d TMP INCORPORATED
(mμmoles/mg. OF PROTEIN)
10
8
6
4
2
20
40
60
80
100
DNA (μg.)

FIGURE 19

FIGURE 20

The effect of primer-DNA concentration on the DNA nucleotidyltransferase activity of extracts of Landschuts ascites-tumour cells.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.10 µmole of EDTA; 1 µmole of $MgSO_4$; 1.25 µmoles of 2-mercaptoethanol; the indicated amount of heat-denatured DNA; 50 nmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (9.6×10^6 counts/min./µmole) and 0.05 ml. of enzyme preparation in a total volume of 0.25 ml. Incubation was carried out at 37° for 60 min.

The protein concentrations were 126 and 149 µg. of protein/assay respectively for the soluble extract and pH 5 precipitate fractions.

—o— soluble extract

—•— pH 5 precipitate

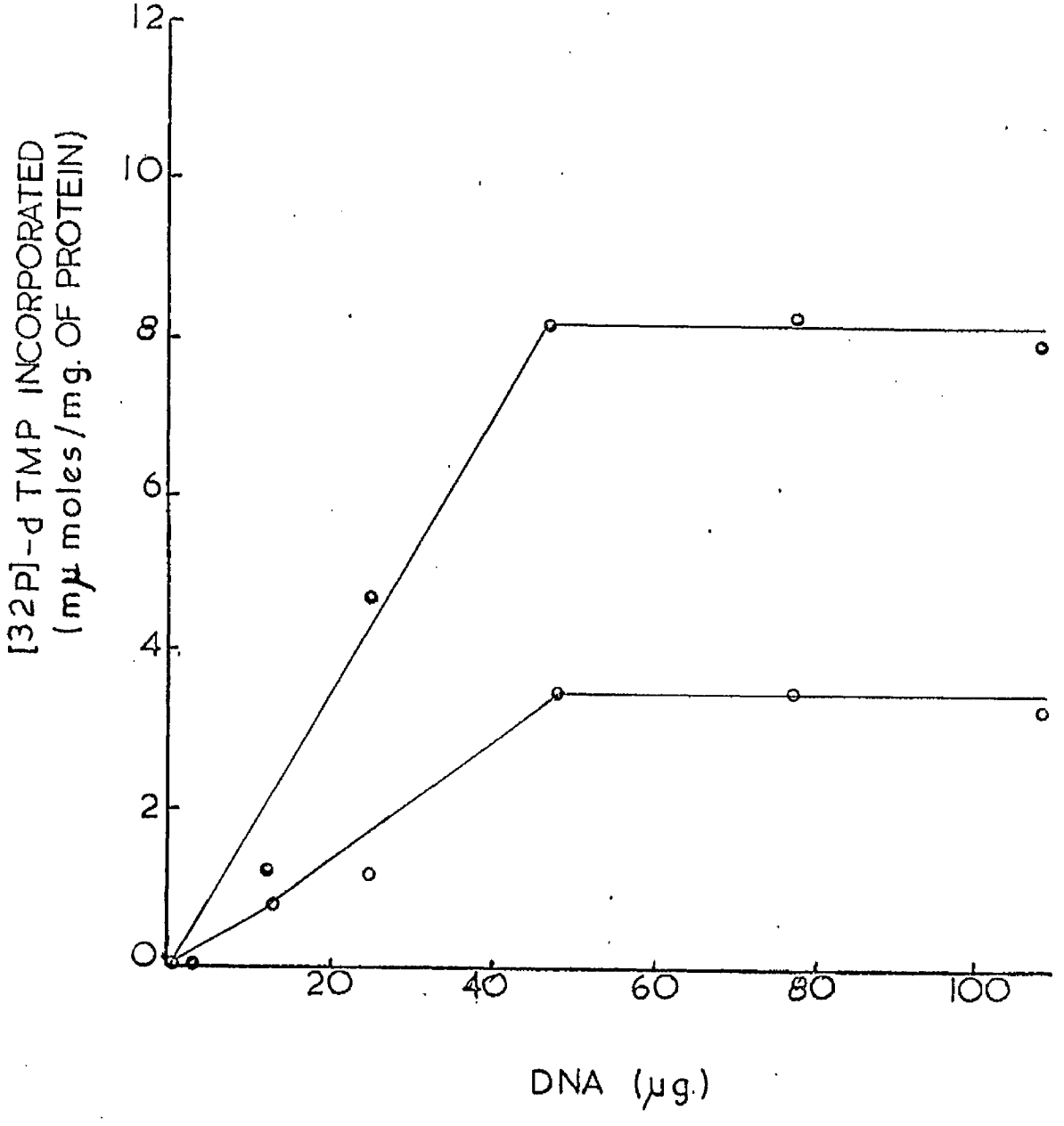

[32P]-d TMP INCORPORATED
(mμ moles/mg. OF PROTEIN)
12
10
8
6
4
2
0
20
40
60
80
100
DNA (μg.)

FIGURE 20

Heat-denatured DNA has been shown previously (Keir et al., 1962) to prime the Landschutz enzyme to a greater extent than does native DNA. The priming ratios, denatured DNA : native DNA and the priming capacity of RNA, for soluble extract, pH 5 precipitate and 20 - 45% ammonium sulphate fractions are shown in Table 5. For comparison, the ability of native DNA, heat-denatured DNA and RNA to prime the activity of DNA nucleotidyltransferase in 20 - 45% ammonium sulphate fractions from calf thymus are also included.

4) Requirement for a Bivalent Cation

The effect of varying concentrations of Mg^{2+} Mn^{2+} and Ca^{2+} ions on the DNA nucleotidyltransferase activity of 20 - 45% ammonium sulphate fractions is shown in Fig. 21. In the absence of a bivalent cation no enzymic activity can be detected. Mg^{2+} ions support activity to a much greater degree than do Mn^{2+} ions, while Ca^{2+} ions are completely inactive. Zn^{2+} ions have been shown to inhibit the enzyme in the presence of standard amounts of Mg^{2+} ions. Optimal activity of the enzyme is achieved in the range 3 - 8 mM Mg^{2+} ions therefore in DNA nucleotidyltransferase assays 1 µmole of Mg^{2+} ions was routinely included in the reaction mixture.

5) Requirement for a Univalent Cation

Certain univalent cations were found to stimulate DNA nucleotidyltransferase from rat thymus gland (Walwick & Main, 1962). The results of investigation of the effects of K^+ and Na^+ ions on the DNA nucleotidyltransferase activity of 20 - 45% ammonium sulphate fractions from Landschutz cells are shown in Fig. 22. K^+ ions over the range 0-60 mM appear to stimulate enzymic activity up to twofold, while Na^+ ions only stimulate by about 30% within this range. The K^+ ion optimum is at 50 mM and the Na^+ optimum is at

TABLE 5

Relative abilities of various polynucleotide preparations to prime DNA nucleotidyltransferase activities in Landschutz ascites-tumour cell and calf thymus enzyme fractions.

The reaction mixture contained: 5 μmoles of tris-HCl buffer, pH 7.5, 15 μmoles of KCl; 0.08 μmole of EDTA; 1 μmole of $MgSO_4$ or $MgCl_2$; 50 μg. of primer as indicated; 50 mμmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (4.0 or 2.8 x 10^6 counts/min./μmole) and enzyme as indicated (90-170 μg. of protein), in a total volume of 0.25 ml. Incubation was carried out at 37° for time intervals between 60 and 90 min. Enzyme activity is expressed as percentage of the activity with denatured DNA as primer.

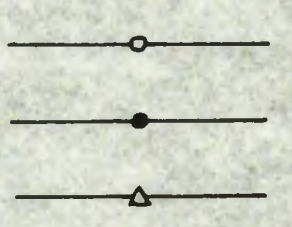
TABLE 5

Fraction	Primer	(^{32}P)-dTMP Incorporated (% of that obtained with Denatured DNA)
Landschutz Cells		
Soluble Extract	Native DNA	35.9
pH 5 Precipitate	Native DNA	25.3
20 - 45% $(NH_4)_2SO_4$ Fraction	Native DNA	9.6
	Yeast RNA	2.7
	Krebs II RNA	1.5
Calf Thymus		
20 - 45% $(NH_4)_2SO_4$ Fraction	Native DNA	11.3
	Yeast RNA	1.5
	Krebs II RNA	0.9

FIGURE 21

Differential effects of bivalent cations on the DNA nucleotidyltransferase activity of extracts of Landschutz ascites-tumour cells.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.10 µmole of EDTA; the indicated amounts of Mg^{2+}, Mn^{2+} or Ca^{2+} ions; 1.25 µmoles of 2-mercaptoethanol; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (1.1×10^6 counts/min./µmole) and 0.01 ml. of a 20 - 45% ammonium sulphate fraction (148 µg. of protein) in a total volume of 0.25 ml. Incubation was carried out at 37° for 60 min.



Mg^{2+} ion concentration

Mn^{2+} ion concentration

Ca^{2+} ion concentration

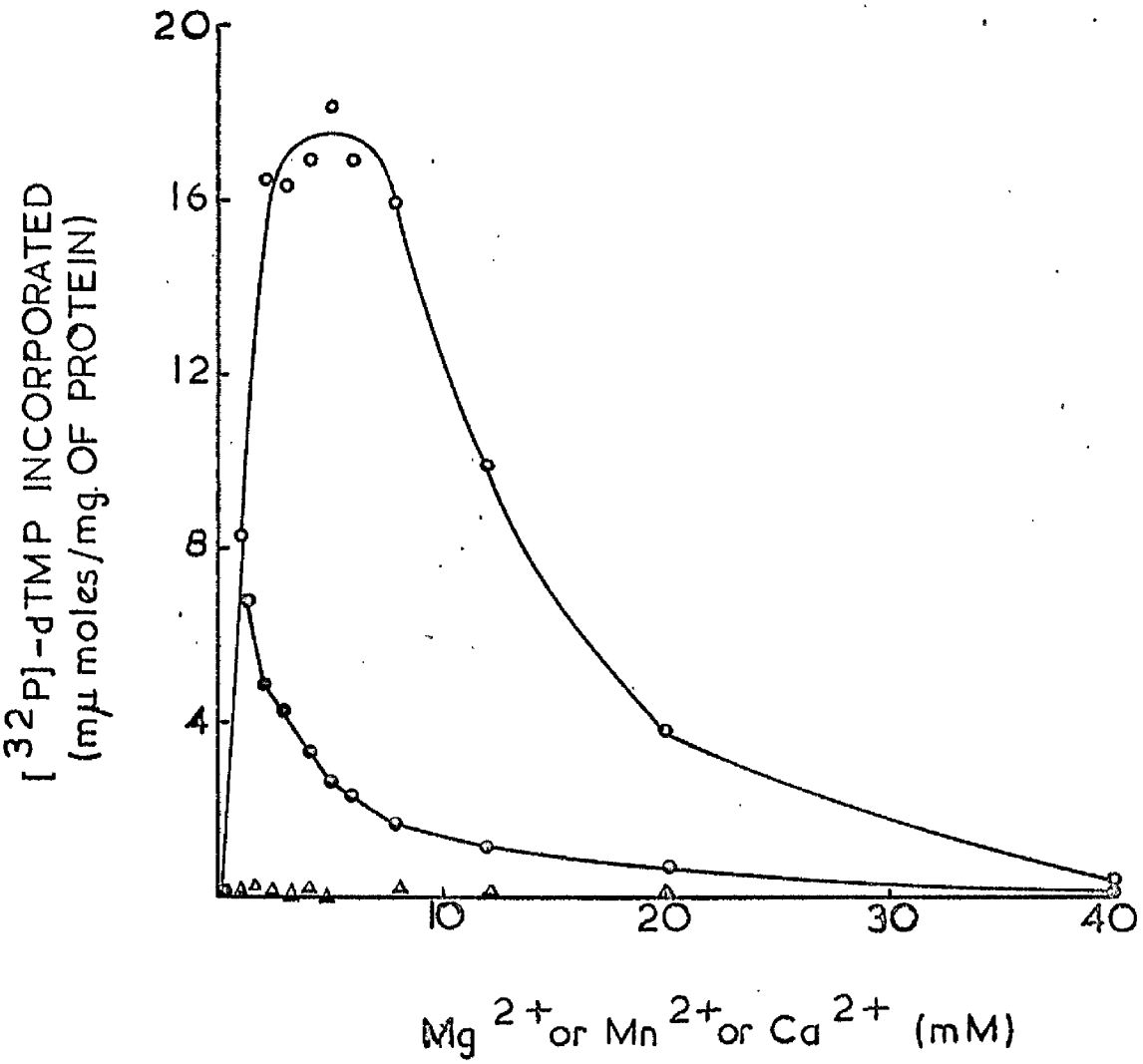

20
16
12
8
4
10
20
30
40
[32P]-dTMP INCORPORATED
(mμ moles/mg. OF PROTEIN)
Mg 2+ or Mn 2+ or Ca 2+ (mM)

FIGURE 21

FIGURE 22

The effect of K^+ and Na^+ ion concentration on DNA nucleotidyltransferase activity of extracts of Landschutz ascites-tumour cells.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; the indicated amount of K^+ or Na^+ ions; 0.08 µmole of EDTA; 1 µmole of $MgCl_2$; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP, and (α-^{32}P)-dTTP (15.7×10^6 counts/min./µmole) and 0.01 ml. of a 20 - 45% ammonium sulphate fraction (94 µg. of protein) in a total volume of 0.25 ml. Incubation was carried out at 37° for 45 min.

—o— K^+ ion concentration

—•— Na^+ ion concentration

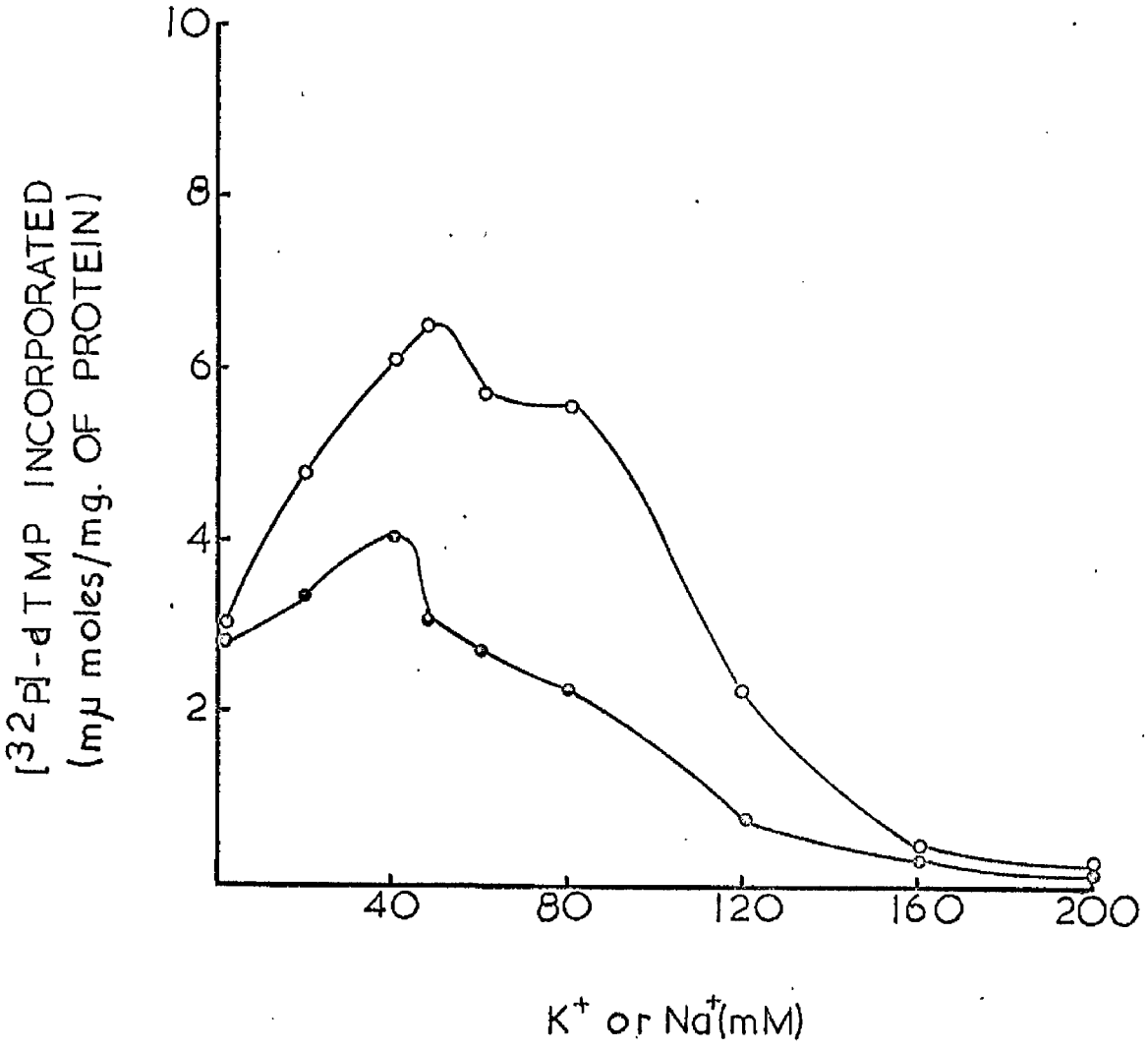

[32P]-dTMP INCORPORATED
(mμ moles/mg. OF PROTEIN)
10
8
6
4
2
40
80
120
160
200
K+ or Na+(mM)

FIGURE 22

40mM. For this reason and from results obtained using DNA nucleotidyltransferase preparations from calf thymus (see Results, section VII C), 15 µmoles of K^+ions were routinely included in DNA nucleotidyltransferase assays.

6) EDTA Requirement

The enzyme has been shown to have a requirement for EDTA for optimal activity. In some cases this requirement was absolute. The effect of EDTA concentration on the DNA nucleotidyltransferase activity of a 20 - 45% ammonium sulphate fraction is shown in Fig. 23. In this case no activity was observed when EDTA was absent from the reaction mixture and a broad peak of enzymic activity was found in the region 0.3 - 0.6 mM, beyond which activity declined rapidly. Therefore, 0.08 - 0.12 µmoles of EDTA were routinely included in DNA nucleotidyltransferase assays.

7) Deoxyribonucleoside Triphosphate Requirement

Enzymic activity of 20 - 45% ammonium sulphate fractions was investigated using deoxyribonucleoside mon- and diphosphates in place of the deoxyribonucleoside triphosphates. The results of these experiments which involve the incorporation of (^{32}P)-dTMP from three precursor molecules, $(\alpha\text{-}^{32}P)$-dTTP, $(\alpha\text{-}^{32}P)$-dTDP and (^{32}P)-dTMP, are shown in Table 6. The enzyme activities under the various conditions are expressed as percentages of the activity under standard assay conditions. The results indicate that the best incorporation of (^{32}P)-dTMP is attained when all four deoxyribonucleoside triphosphates are present. In other experiments where dGTP was replaced by dGMP, or dCTP by dCMP, or dATP by

FIGURE 23

The effect of EDTA concentration on the DNA nucleotidyltansferase activity of extracts of Landschutz ascites-tumour cells.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; the indicated amount of EDTA; 1 µmole of $MgCl_2$; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (8.8×10^6 counts/min./µmole) and 0.02 ml. of a 20 - 45% ammonium sulphate fraction (141 µg. of protein) in a total volume of 0.25 ml. Incubation was carried out at 37° for 60 min.

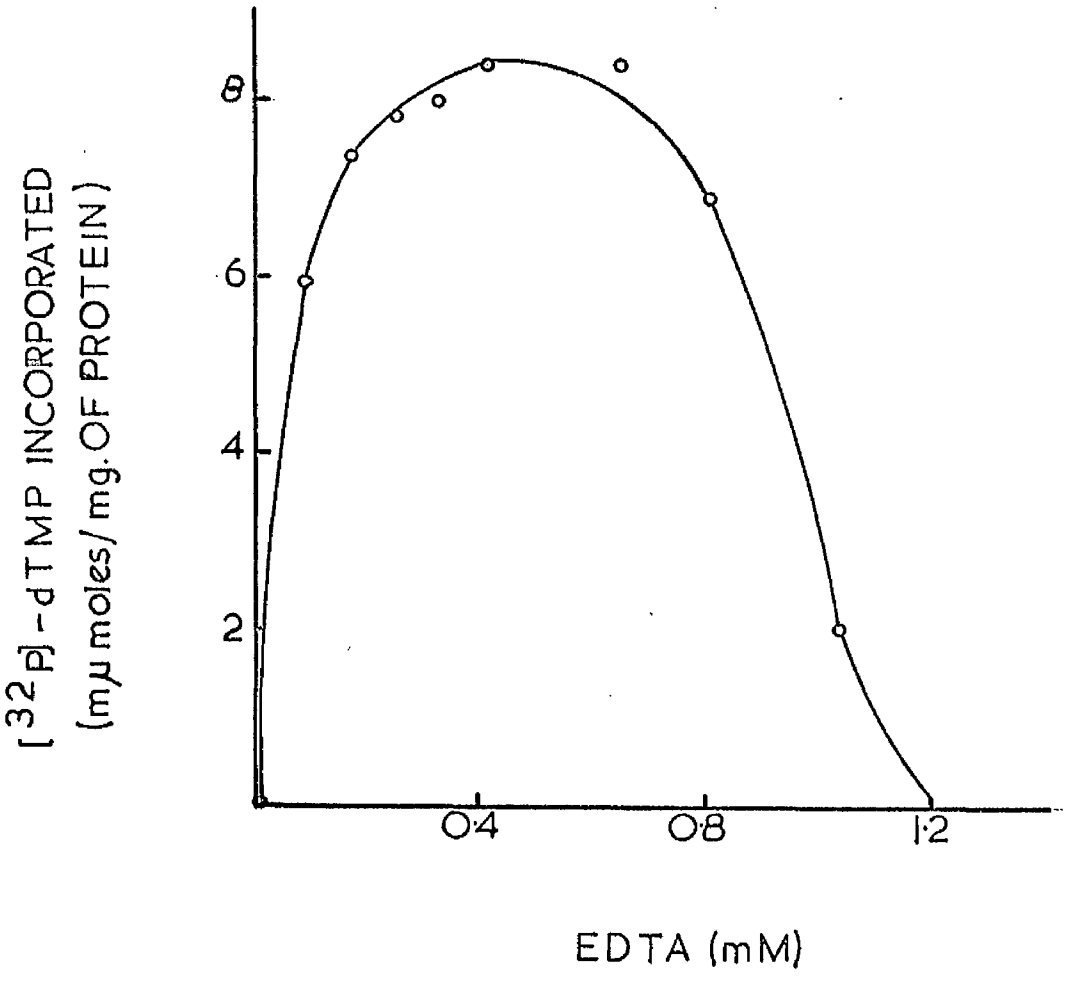

[32P]-dTMP INCORPORATED
(mμ moles/mg. OF PROTEIN)
8
6
4
2
0·4
0·8
1·2
EDTA (mM)

FIGURE 23

TABLE 6

The activity of DNA nucleotidyltransferase from Landschutz ascites-tumour cells towards substrate deoxyribonucleosides at all levels of phosphorylation.

All reaction mixtures contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.08 - 0.10 µmole of EDTA; 1 µmole of $MgSO_4$ or $MgCl_2$ 50 µg. of heat-denatured DNA; 50 mµmoles each of the indicated deoxyribonucleoside 5'-mono-, di-or triphosphates and 20 - 45% ammonium sulphate fraction, in a total volume of 0.25 ml. The specific radioactivites of (^{32}P)-dTMP, - dTDP and - dTTP were respectively 15.4, 1.7 and 6.2x 10^6 counts/min/µmole. The assays involving deoxyribonucleoside 5'-monophosphates contained no 2-mercaptoethanol; their protein content was 94 µg. and their incubation was carried out at 37° for 90 min. The assays involving deoxyribonucleoside 5'-di-and triphosphates contained 1.25 µmoles of 2-mercaptoethanol and 154 µg. of protein, incubation being carried out at 37° for 60 min. Enzyme activity is expressed as a percentage of the activity when all four deoxyribonucleoside 5'-triphosphates were present.

TABLE 6

Substrates	(^{32}P)-dTMP Incorporated (% of that obtained with Standard Assay)
dAMP, dGMP, dCMP, (α-^{32}P)-dTMP	0
dADP, dGDP, dCDP, (α-^{32}P)-dTDP	9.2
dAMP, dGMP, dCMP, (α-^{32}P)-dTTP	24.7
dADP, dGDP, dCDP, (α-^{32}P)-dTTP	41.6
(α-^{32}P)-dTTP	1.8

dAMP, or (α-^{32}P)-dTTP by (^{32}P)-dTMP, incorporations, expressed as percentage of standard assay, were 45.8, 50.4, 28.3 and 0.3% respectively.

DNA nucleotidyltransferase activity as a function of deoxyribonucleoside triphosphate concentration is plotted in Fig. 24. All four deoxyribonucleoside triphosphates were present in equal amounts, the total nucleotide range covered being 0-1,200 µM. The activity levels off in the region of 600 µM, therefore, 50 - 75 mµmoles of each of the four deoxyribonucleoside triphosphates were used in a standard assay of DNA nucleotidyltransferase activity.

8) Storage Properties, and 2-Mercaptoethanol Requirement

In the early stages of this work attempts were made to demonstrate stimulation of DNA nucleotidyltransferase with increasing concentrations of 2-mercaptoethanol, but little or no increase in activity was initially achieved by the addition of 2-mercaptoethanol to the reaction mixture. At this point pH 5 precipitate fractions prepared in the standard fashion, but in the absence of 2-mercaptoethanol, could only be stored for 10 - 14 days at 0°, during which time they lost two thirds of their activity. A typical storage-inactivation curve is shown in Fig. 25 for a pH 5.0 precipitate fraction prepared from Landschutz cells. This experiment suggested that stability might be conferred upon the enzyme if 2-mercaptoethanol were present during preparation and storage. From this point all enzyme fractionation procedures and all enzyme assays were carried out in solutions which were 0.005M with respect to 2-mercaptoethanol

FIGURE 24

The effect of deoxyribonucleoside triphosphate concentration on the DNA nucleotidyltransferase activity of extracts of Landschutz ascites-tumour cells.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.08 µmole of EDTA; 1 µmole of $MgCl_2$; 50 µg. of heat-denatured DNA; the indicated amount of each of the deoxyribonucleoside triphosphates, dATP, dGTP, dCTP and (α-^{32}P)-dTTP (3.8×10^6 counts/min./µmole) and 0.02 ml. of a 20 - 45% ammonium sulphate fraction (142 µg. of protein) in a total volume of 0.25 ml. Incubation was carried out at 37° for 60 min.

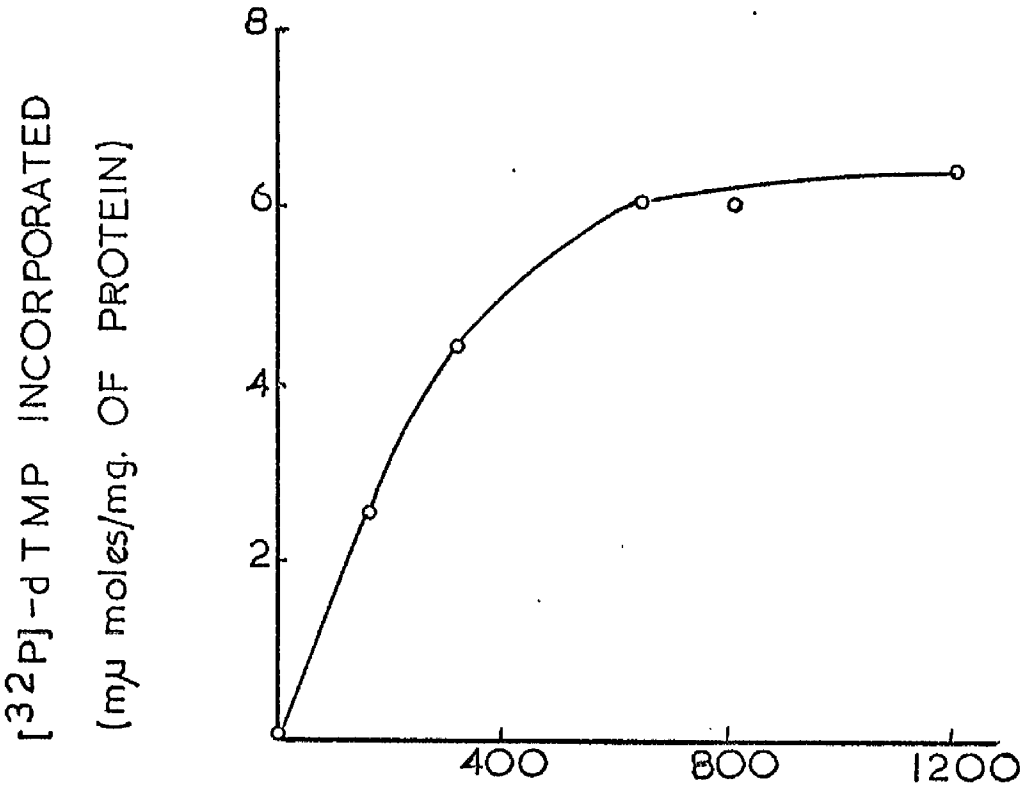

[32P]-d TMP INCORPORATED
(mμ moles/mg. OF PROTEIN)
8
6
4
2
400
800
1200
DEOXYRIBONUCLEOSIDE TRIPHOSPHATES (μM)

FIGURE 24

FIGURE 25

The effect of storage at 0° in the absence of EDTA and 2-mercaptoethanol on the DNA nucleotidyltransferase activity of extracts of Landschuts ascites-tumour cells.

The reaction mixture contained: 5 µmoles of tris-HCl buffer pH 7.5; 15 µmoles of KCl; 0.08 µmole of EDTA; 1 µmole of $MgCl_2$; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (ranging from 26.0 - 11.4 x 10^6 counts/min./µmole) and 0.05 ml. of a pH 5 precipitate fraction (150 µg. of protein) in a total volume of 0.25 ml. Incubation was carried out at 37° for 120 min.

The enzyme fraction was assayed in an identical manner at 2 to 3 day intervals for a period of 16 days.

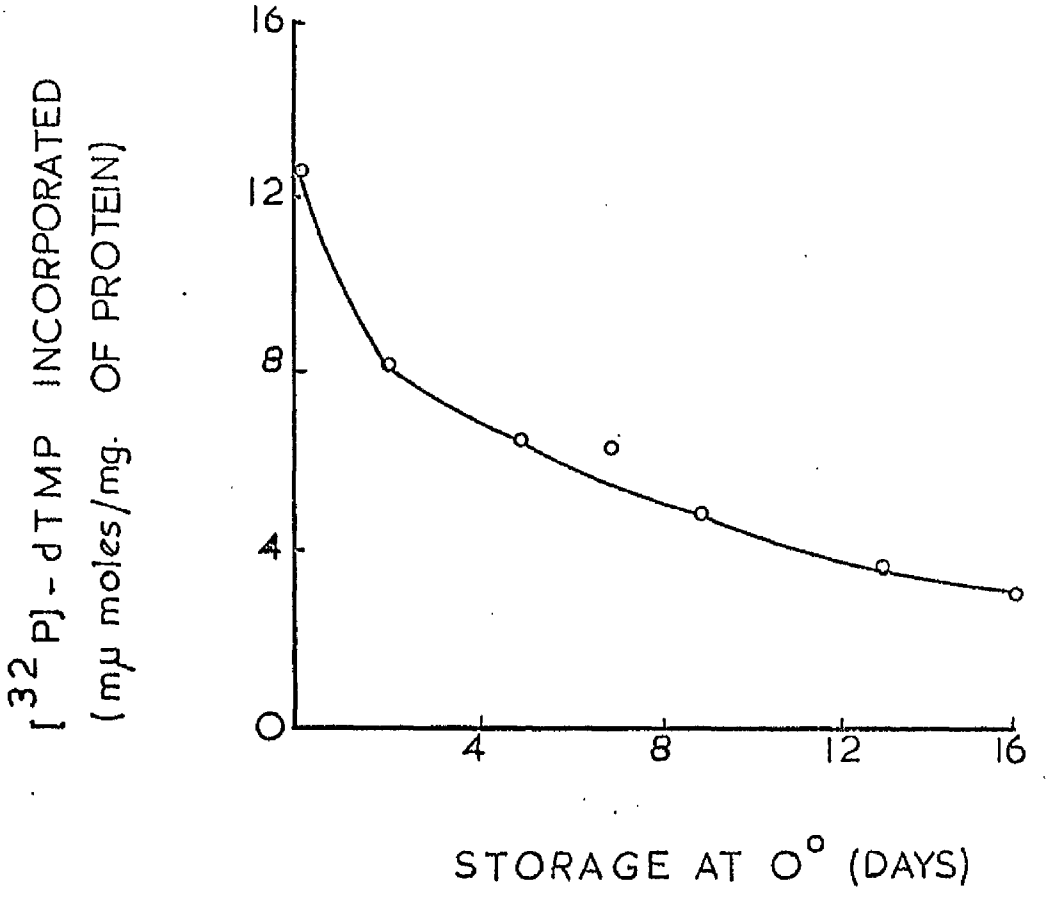

[32P]-dTMP INCORPORATED
(mμ moles/mg. OF PROTEIN)
16
12
8
4
0
4
8
12
16
STORAGE AT 0° (DAYS)

FIGURE 25

This resulted in two great improvements with regard to DNA nucleotidyltransferase activities obtained from Landschutz cells. The first of these was that enzyme preparations extracted in a 5-component buffer (0.005M-2-mercaptoethanol-0.001M-EDTA-0.15M-KCl-0.004M-$MgSO_4$-0.02M-tris-HCl buffer, pH 7.5) could be stored for two months at 0° without detectable loss of activity. The storage properties of enzyme fractions prepared exactly as before (see Experimental, sections II & III) but with 2-mercaptoethanol at 0.005M and EDTA at 0.001M present at all stages, were enhanced in a similar manner. The second consequence of introducing 2-mercaptoethanol and EDTA into all enzyme preparative procedures was that the activities of the various intermediate fractions in the purification were increased nearly threefold in some cases. Table 7 compares the activities of various fractions prepared in the presence and absence of 2-mercaptoethanol and EDTA. This explains some of the low activities of 20 - 45% ammonium sulphate fractions in some of the sub-sections of this set of results, as these experiments were done before the introduction of 2-mercaptoethanol and EDTA during fractionation.

B) <u>DNA Nucleotidyltransferase from Calf Thymus Gland</u>

The fractionation procedure employed for the purification of DNA nucleotidyltransferase from both calf thymus gland and Landschutz ascites-tumour cells is a modification of the method used by Bollum (1960) for the purification of calf thymus DNA nucleotidyltransferase. Therefore, it seems not unreasonable to conclude that the enzymic activity in these various calf thymus fractions is the same as that investigated and

TABLE 7

Comparison of the DNA nucleotidyltransferase activities of extracts of Landschutz ascites-tumour cells prepared in the presence and absence of 0.005M-2-mercaptoethanol and 0.001M-EDTA.

The reaction mixtures were those routinely used for the assay of DNA nucleotidyltransferase activity in the presence and absence of 2-mercaptoethanol. The ranges of data quoted were gathered in each case from 5 to 10 experiments.

TABLE 7

Fraction	0.005 M-2-Mercaptoethanol-0.001M-EDTA	(^{32}P)-dTMP Incorporated (mμmoles/mg. of protein/hr.
Soluble Extract	+	4 - 8
Soluble Extract	-	2 - 4
pH 5 Precipitate	+	10 - 20
pH 5 Precipitate	-	4 - 8
20 - 45% $(NH_4)_2SO_4$ Fraction	+	15 - 33
20 - 45% $(NH_4)_2SO_4$ Fraction	-	4 - 12

characterised by Bollum (1960, 1963a). It would be possible to reproduce in detail results concerning primer, Mg^{2+} ion, EDTA, deoxyribonucleoside triphosphate requirement etc. Instead sufficient information will be selected to establish the identity of the enzyme activity of these fractions with that of Bollum (1960), and the similarity of its requirements with those of the Landschutz DNA nucleotidyltransferase.

Table 8 describes the loss of enzyme activity when Mg^{2+} ions, EDTA, and dATP, dGTP and dCTP were, in turn, omitted from the reaction mixture. The effect of replacing various components of the standard reaction mixture by other similar molecules e.g., native DNA for denatured DNA, is shown in Table 9. Together with similarity of the preparative procedure, the results shown in Tables 8 & 9, obtained from calf thymus 20 - 45% ammonium sulphate fractions, indicate that the nature of the enzymic activity being investigated was the same as that characterised by Bollum (1960).

In addition, using the conditions optimal for the Landschutz DNA nucleotidyltransferase with respect to primer DNA, protein content, Mg^{2+} ions, K^{+} ions, EDTA, tris-HCl buffer, pH 7.5, deoxyribonucleoside triphosphates and 2-mercaptoethanol, linearity of enzymic activity with time up to 60 min. at least has been obtained for soluble extract, pH 5 precipitate and 20 - 45% ammonium sulphate fractions. This is shown in Fig. 26 which is very similar to the corresponding figure for the Landschutz enzyme fractions (Fig. 15). It has been shown that 0.4 mM EDTA and

TABLE 8

Loss of DNA nucleotidyltransferase activity by extracts of calf thymus tissue on omission of certain compounds from the reaction mixture.

Enzyme activities are expressed as percentages of the activity obtained with the complete reaction mixture which contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.08 µmole of EDTA; 1 µmole of $MgCl_2$; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (2.0 - 3.0 x 10^6 counts/min./µmole) and 0.05 ml. (100 - 150 µg. of protein) of a 20 - 45% ammonium sulphate fraction, in a total volume of 0.25 ml. Incubation was carried out at 37° for 45 or 90 min.

TABLE 8

Reaction Mixture	(^{32}P)-dTMP Incorporated (% of Standard Assay)
Complete	100
Minus Mg^{2+} ions	0
Minus EDTA	0.08
Minus DNA	0
Minus dATP, dGTP, and dCTP	39.2

TABLE 9

The effect of substitution of various components of the reaction mixture on the DNA nucleotidyltransferase activity of extracts of calf thymus tissue.

Enzymic activities are expressed as percentages of the activity obtained under standard assay conditions which were: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.08 µmole of EDTA; 1 µmole of $MgCl_2$; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (4.0 - 6.2 x 10^6 counts/min./µmole) and 0.01 ml. (114 - 171 µg. of protein), in a total volume of 0.25 ml. Incubation was carried out at 37° for 90 min.

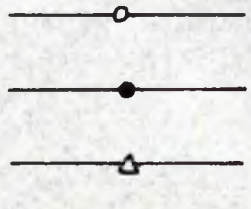
TABLE 9

Substitution	(^{32}P)-dTMP Incorporated (% of Standard Assay)
Standard Reaction Mixture	100
dAMP, dGMP, dCMP and (^{32}P)-dTMP for dATP, dGTP, dCTP and (α-^{32}P)-dTTP	0
dAMP, dGMP and dCMP for dATP, dGTP and dCTP	12.0
dAMP for dATP	24.5
dGMP for dGTP	73.0
dCMP for dCTP	37.3
(^{32}P)-dTMP for (α-^{32}P)-dTTP	0
Native DNA for heat-denatured DNA	11.5
Yeast RNA for heat-denatured DNA	1.0

FIGURE 26

Time courses of DNA nucleotidyltransferase activity of extracts of calf thymus tissue.

The reaction mixtures contained: 5 μmoles of tris-HCl buffer, pH 7.5; 15 μmoles of KCl; 0.10 μmole of EDTA, 1.0 μmole of $MgSO_4$; 1.25 μmoles of 2-mercaptoethanol; 50 μg. of heat-denatured DNA; 50 mμmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (4.3×10^6 counts/min./μmole) and varying volumes of soluble extract, pH 5 precipitate or 20 - 45% ammonium sulphate fractions in a total volume of 0.25 ml. The protein content/assay was 82, 100 and 121 μg. respectively for soluble extract, pH 5 precipitate and 20 - 45% ammonium sulphate fractions. Incubation was carried out at 37° for the indicated time interval.



soluble extract

pH 5 precipitate

20 - 45% ammonium sulphate fraction

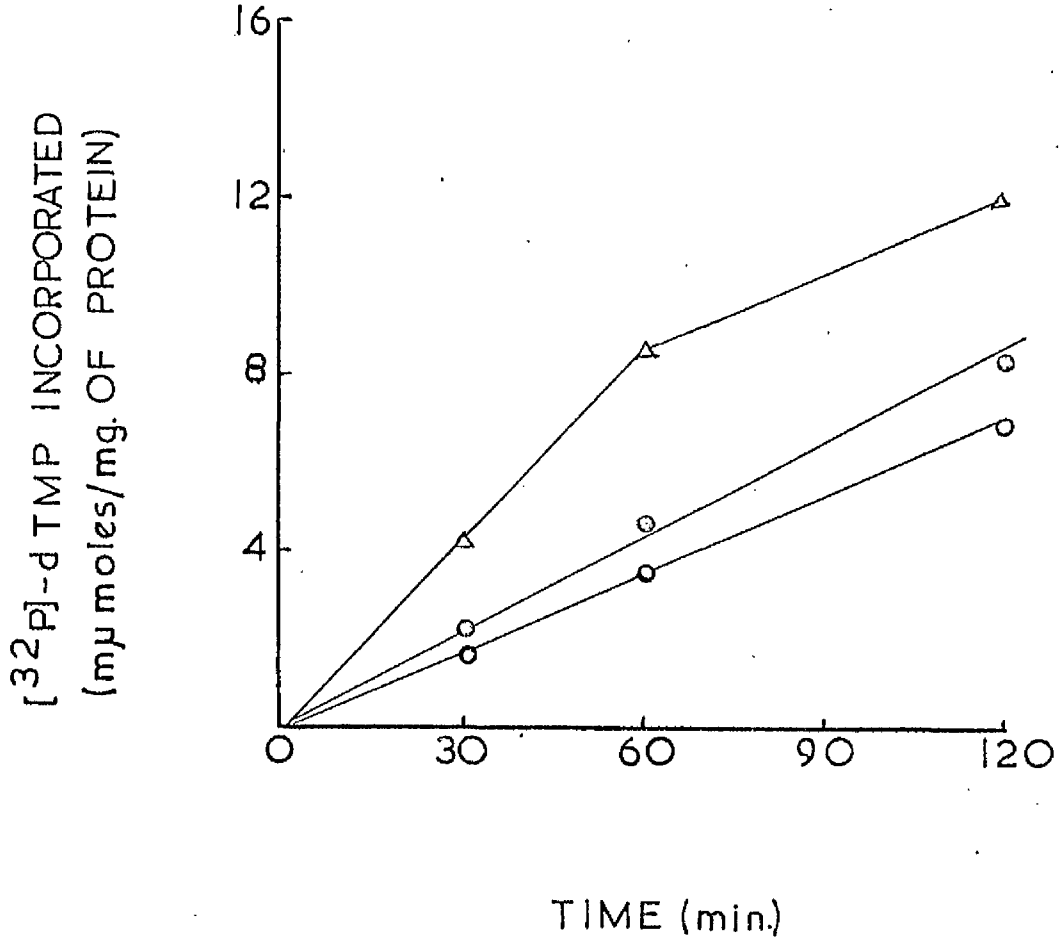

[32P]-d TMP INCORPORATED
(mμ moles/mg. OF PROTEIN)
16
12
8
4
0
30
60
90
120
TIME (min.)

FIGURE 26

4mM Mg^{2+}ions were also optimal for the calf thymus enzyme and that 50 µg. of heat-denatured DNA and 50 mumoles each of the four deoxyribonucleoside triphosphates sufficient to maintain activity over a 2 hr. incubation period. As has been shown for the Landschutz enzyme preparations, calf thymus DNA nucleotidyltransferase could be stored for longer periods in 2-mercaptoethanol at 0.005M and EDTA at 0.001M and had higher activities than its counterparts prepared in the absence of these compounds.

III Nature of the Product of DNA Nucleotidyltransferase Activity

In this section it is proposed to report only investigations into the nature of the product of Landschutz DNA nucleotidyltransferase since the product of the calf thymus enzyme has been well characterised by Bollum (1963a, b).

A) Precipitability of the Product

The (^{32}P)-labelled product was shown to be acid-precipitable, a property common to all high molecular weight polynucleotide material. Advantage was taken of this property in the assay method by precipitation of the product on paper discs. The product of the enzyme activity was also precipitated by the addition of two volumes of ethanol and was shown to be non-dialysable (see Experimental, section VIII), both factors indicating the macromolecular nature of the product molecule.

B) Ultracentrifugation Studies of the Product

The experiment, whose description follows, was set up to investigate the sedimentation properties of the product using the caesium chloride

density gradient technique (Meselson, Stahl & Vindgrad, 1957). The plan of the experiment was to run three caesium chloride gradients under the same centrifugation conditions, one containing native DNA, another heat-denatured DNA and the third (^{32}P)-labelled product DNA, and then compare their sedimentation patterns.

The reaction mixture (see legend Fig. 27) was incubated at 37° for 30 min. and the DNA was precipitated from it by the addition of two volumes of redistilled ethanol, washed several times with 70% (v/v) ethanol and then dissolved with stirring in a suitable volume of 1/10 SSC. After solution of the DNA, the product was made standard with respect to saline citrate and dialysed for several hours against 2l. SSC. Part of the preparation was then diluted with SSC to give an extinction of 1.0 at 260 mμ. 2 ml. of this solution (i.e., approximately 80 μg. of DNA) were centrifuged in a caesium chloride gradient as described in Experimental, section VIII. Native and heat-denatured calf thymus DNA preparations were also subjected to centrifugation in caesium chloride under the same conditions. In these cases however, 2 ml. portions of the respective preparations in SSC contained 0.5 extinction unit/ml. (i.e., approximately 40 μg. of DNA). Therefore, the product tube contained twice as much DNA as did the native and denatured DNA tubes. The gradients were collected, diluted and assayed for extinction at 260 mμ and radioactivity as described in Experimental section VIII.

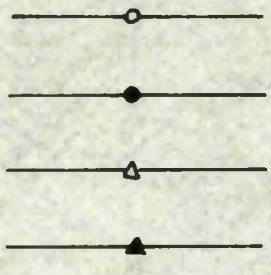
The results of this experiment are shown in graphical form in Fig. 27. As expected from preliminary calculations the native and denatured DNA

FIGURE 27

Caesium chloride density gradient patterns of native and heat-denatured calf thymus DNA, and of the product of a heat-denatured calf thymus DNA-primed reaction of a Landschutz ascites-tumour cell DNA nucleotidyltransferase fraction.

A 5 ml. reaction mixture was set up containing: 100 µmoles of tris-HCl buffer, pH 7.5; 300 µmoles of KCl; 1.6 µmoles of EDTA; 20 µmoles of $MgSO_4$; 25 µmoles of 2-mercaptoethanol; 1,000 µg. of heat-denatured DNA; 1,000 nmoles each of (α-^{32}P)-dATP, (α-^{32}P)-dGTP, (α-^{32}P)-dTTP and dCTP and 0.2 ml (1.5 mg. of protein) of a 20-45% ammonium sulphate fraction. Incubation was carried out at 37° for 30 min.

For other details, see text.



native DNA

heat-denatured DNA

product DNA

counts/min./ml.

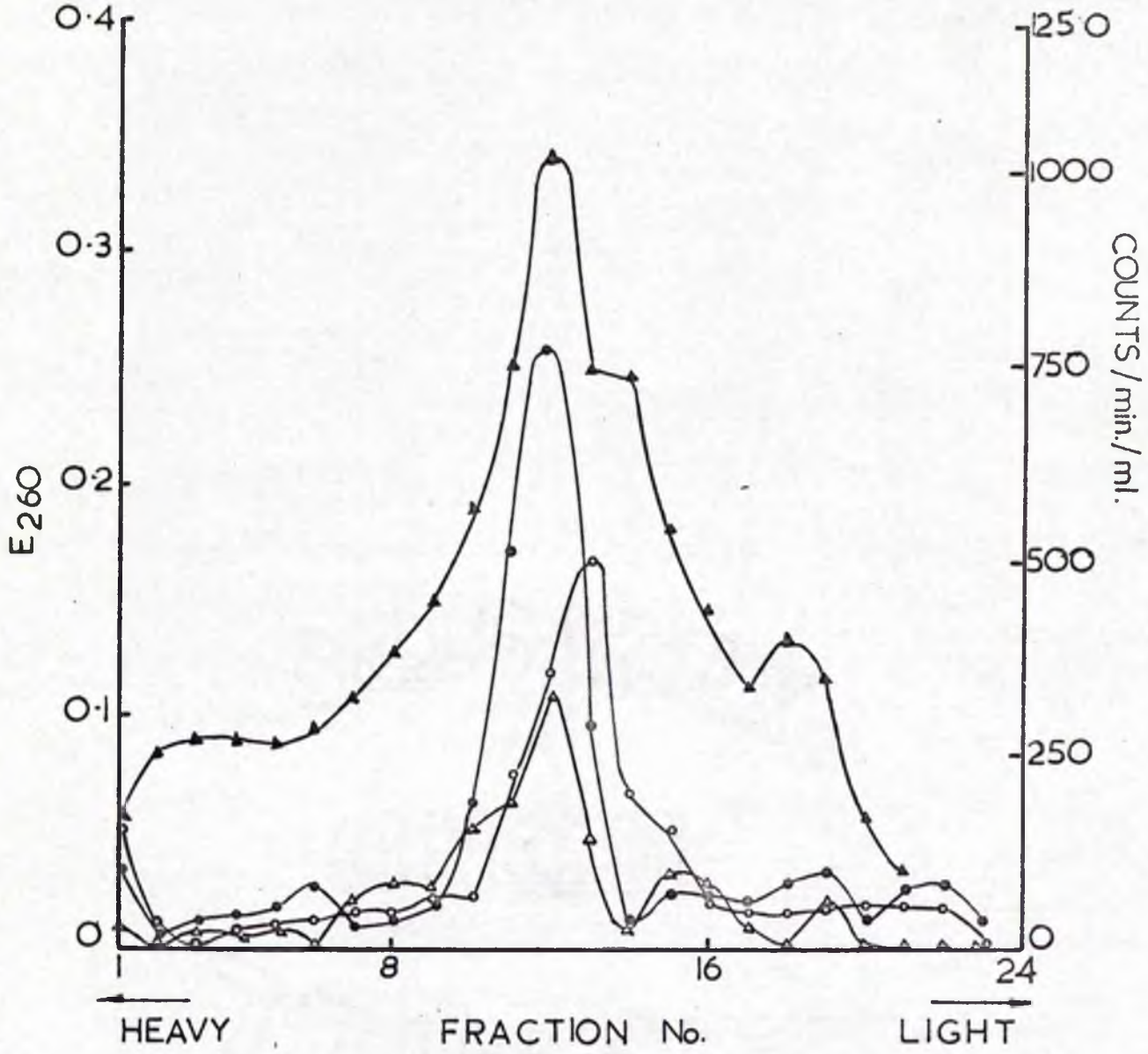

0·4
0·3
0·2
0·1
0
E260
1250
1000
750
500
250
0
COUNTS/min./ml.
1
8
16
24
HEAVY
FRACTION No.
LIGHT

FIGURE 27

preparations band in the middle of the gradient, the denatured form, because of its higher density, being slightly nearer the bottom of the tube. Both the product DNA and the radioactivity peak at the same point as does the denatured DNA but, it is important to note that the radioactivity curve also shows a shoulder at the density of native DNA. The product peak is much smaller than the native or denatured DNA peaks, although there was originally twice as much ultraviolet-light absorbing material applied to the product gradient. This can only be accounted for as partial loss of the product DNA macromolecular structure due to degradation by DNaseI during incubation. The relatively high background level of radioactivity in the test sample suggests that the gradient may contain a series of (^{32}P)-labelled polydeoxyribonucleotide molecules of varying lengths and conformation (arising from DNaseI action), some of which may be small enough to diffuse freely.

C) Differential Incorporation Studies

In an attempt to characterise the enzymic product further, (α-^{32}P)-labelled dATP and dGTP were obtained commercially in order to investigate, simultaneously, the relative incorporation of dAMP, dGMP and dTMP into acid-insoluble product. However, paper chromatography of the (α-^{32}P)-dATP and (α-^{32}P)-dGTP preparations (see Experimental, section IX), revealed that the dGTP contained a high proportion of dGDP (about 30%) and traces of another unidentified radioactive component. The dATP however appeared to be reasonably free of contaminants. Table 10 shows

TABLE 10

Relative incorporation of dAMP, dGMP and dTMP in product DNA by extracts of Landschuts ascites-tumour cells.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.10 µmole of EDTA; 1 µmole of $MgSO_4$; 1.25 µmoles of 2-mercaptoethanol; 50 µg. of heat-denatured DNA (from Landschutz ascites-tumour cells); 50 mµmoles each of dATP, dGTP, dCTP and dTTP and 0.05 ml (150 µg. of protein) of a pH 5 precipitate fraction, in a total volume of 0.25 ml. Incubation was carried out at 37° for 30 min.

The deoxyribonucleoside 5'-triphosphates had specific radioactivities of 7.9 , 6.7 and 1.3×10^6 counts/min./µmole respectively for dATP, dGTP and dTTP.

TABLE 10

(^{32}P)-Labelled Substrate	(^{32}P)-Deoxyribonucleotide Incorporated (mμmoles/mg. of protein)
dATP	5.10
dGTP	3.02
dTTP	4.73

the relative incorporation of dAMP, dGMP and dTMP in the DNA nucleotidyltransferase assay system, using a pH 5 precipitate enzyme fraction from Landschuts cells. Heat-denatured DNA from Landschuts cells was used as primer in the assay. This DNA has an adenine + thymine: guanine + cytosine ratio of 1.33 (H. M. Keir personal communication). Assuming that the incorporation of dCMP was equivalent to that of dGMP in the above experiment, the adenine + thymine: guanine + cytosine ratio for the radioactive bases incorporated into product DNA was 1.63. Allowing for the fact that the (α-^{32}P)-dGTP preparation used in the experiment contained a considerable amount of dGDP, which might inhibit (^{32}P)-dGMP incorporation thus increasing the ratio, it would seem valid to conclude that the ratio of adenine + thymine: guanine + cytosine incorporation catalysed by the enzyme approaches that previously determined by base analysis for the primer DNA. Therefore the nature of the product of DNA nucleotidyltransferase activity in preparations from Landschuts ascites-tumour cells used in this work appears to depend on the base ratios of the primer DNA.

IV Correlation of In Vivo and In Vitro Rates of DNA Synthesis by DNA Nucleotidyltransferase from Landschuts Ascites-Tumour Cells

For the purpose of comparing the ability of the enzyme fractions used in this work to synthesise DNA *in vitro* with the role of the enzyme within the cell during the S phase of the cell cycle, it was decided to compare the *in vivo* and *in vitro* rates of DNA synthesis.

A) In Vivo Rate of DNA Synthesis

DNA content/tumour cell = 1.42×10^{-6} µg. of phosphorus (Goldberg, Klein & Klein, 1950). Length of S phase = 10 hr. (Baserga & Lisco, 1963).

ion

Mean Generation Time 6 days after inoculation with 10 - 20 x 10^6 cells = approximately 40 hr. (Klein & Révész, 1953)

Ascitic fluid was withdrawn from 16 six-day-inoculated mice and the cells pooled in ice-cold 0.9% (w/v) NaCl.

Volume of saline cell suspension = 220 ml.

A small fraction of the suspension was diluted 1 in 50 and counted in the Coulter cell counter.

No. of cells in the 1 in 50 diluted sample = 0.53 x 10^6/ml.

Therefore total No. of cells in suspension = 0.53 x 10^6 x 50 x 220

= 5.83 x 10^9 cells.

Therefore the <u>in vivo</u> rate of DNA synthesis in these cells was calculated as follows, assuming that all the cells were viable:

Rate of DNA synthesis = 1.42 x 10^{-6} x 5.83 x 10^9 µg. of DNA phosphorus/10 hr.

= 26.7 µmoles deoxyribonucleotide incorporated/hr./total cell number.

However this assumes that all the cells are in the process of division, which will not be the case in an asynchronous population. If the mean generation time is approximately 40 hr. and the DNA synthetic phase is 10 hr. then approximately 10/40 or ¼ of the population will be in the process of DNA synthesis at any one time.

Therefore, the <u>in vivo</u> rate of DNA synthesis in the whole cell population should be of the order of:-

¼ x 26.7 = 6.7 µmoles deoxyribonucleotide incorporated into DNA/hr.

NB. This assumes a constant rate of DNA synthesis throughout the S phase.

B) In Vitro Rate of DNA Synthesis

The above population of cells was fractionated in the standard fashion to produce soluble extract, pH 5 precipitate and 20 - 45% ammonium sulphate fractions. Each fraction was assayed for protein content and DNA nucleotidyltransferase activity in order to estimate the *in vitro* rate of DNA synthesis. For the measurement of the rate of DNA synthesis the figure obtained for (^{32}P)-dTMP incorporation must be multiplied by 4 to allow for the incorporation of non-radioactive dAMP, dCMP and dGMP. The results of this fractionation are shown in Table 11, which also includes the total DNA synthesised/hr./fraction.

From the results given in Table 11 it would seem, superficially at least, that the rate of DNA synthesis in the *in vitro* system is at least as great if not greater than the *in vivo* rate of synthesis, irrespective of the fraction used in the *in vitro* system.

V The Terminal Enzyme

A) General Considerations

A *terminal* DNA nucleotidyltransferase which adds deoxyribonucleotides to the ends of primer DNA molecules has been identified in calf thymus preparations by several groups of workers (Kradow *et al.*, 1962; Keir & Smith, 1963; Bollum *et al.*, 1964) (see Introduction, section V C). In this work separation of the *replicative* and *terminal* forms of DNA nucleotidyltransferase in calf thymus has not been attempted since the primary aim of the project was to investigate the distribution of the *terminal* enzyme in other tissues, especially the Landschutz ascites-tumour

TABLE 11

In vitro rates of DNA synthesis in extracts of Landschutz ascites-tumour cells.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH.7.5; 15 µmoles of KCl; 0.10 µmole of EDTA; 1 µmole of $MgSO_4$; 1.25 µmoles of 2 mercaptoethanol; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and ($d\text{-}^{32}P$) -dTTP (11.2 x 10^6 counts/min./µmole); 0.05 or 0.01ml. of enzyme preparation, in a total volume of 0.25 ml. The protein content/assay was 102, 107 and 95 µg. respectively for soluble extract, pH5 precipitate and 20-45% ammonium sulphate fractions. Incubation was carried out at 37° for 60 min.

TABLE 11

Fraction	Protein Content (mg.)	(^{32}P)-dTMP Incorporated (mμmoles/mg. of protein/hr.)	Total Deoxyribonucleotide Incorporated (μmoles/fraction/hr.
Soluble Extract	514	4.43	9.12
pH 5 Precipitate	258	11.18	11.92
20 - 45% $(NH_4)_2SO_4$ Fraction	89	18.72	6.60

cell. The enzymic activities were differentiated by their method of assay, the replicative assay containing 50 mμmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP and the terminal assay containing 50 to 100 mμmoles of (α-^{32}P)-dTTP alone. Occasionally, for reasons given in Results, section VII, terminal activity was measured at 40mMK^+ ions and 10mM Mg^{2+} ions instead of 60mMK^+ ions and 4 mM Mg^{2+} ions which were routinely used for the assay of replicative DNA nucleotidyltransferase.

B) In the Calf Thymus Gland

Fig. 28 shows the relationship between the duration of incubation and (^{32}P)-dTMP incorporation for both the replicative and terminal DNA nucleotidyltransferases from a pH 5 precipitate fraction prepared from calf thymus. As might be expected the replicative activity is almost linear over 180 min. whereas the terminal activity reaches a maximum level after 120 min. presumably when all the available primer termini have been saturated with (^{32}P)-dTMP residues.

The effect of using DNA treated with pancreatic DNaseI to a limited extent (see Experimental, section VI B) and then heat-denatured, to prime the terminal DNA nucleotidyltransferase assay is shown in Fig. 29. This treatment, which involves the production of 3'-hydroxy-terminal groups in the primer molecule, appears to stimulate terminal activity a little more than twofold. Therefore, since there are more 3'-hydroxyl termini available in the primer DNA the attainment of a greater level of terminal addition by the enzyme is expected during a defined period of incubation.

FIGURE 28

Time courses of replicative and terminal DNA nucleotidyltransferase activities of extracts of calf thymus tissue.

The replicative assay reaction mixture contained: 5 μmoles of tris-HCl buffer, pH 7.5; 15 μmoles of KCl; 0.08 μmole of EDTA; 1 μmole of $MgCl_2$; 50 μg. of heat-denatured DNA; 75 mμmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (4.6×10^6 counts/min./μmole) and 0.05 ml. (138 μg. of protein) of a pH 5 precipitate fraction in a total volume of 0.25 ml.

The terminal assay reaction mixture contained: 5 μmoles of tris-HCl buffer pH, 7.5; 10 μmoles of KCl; 0.08 μmole of EDTA; 2.5 μmoles of $MgCl_2$; 50 μg. of heat-denatured DNA; 75 mμmoles of (α-^{32}P)-dTTP (4.6×10^6 counts/min./μmole) and 0.05 ml. (138 μg. of protein) of the same enzyme fraction in a total volume of 0.25 ml.

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

—●— replicative DNA nucleotidyltransferase activity

—○— terminal DNA nucleotidyltransferase activity

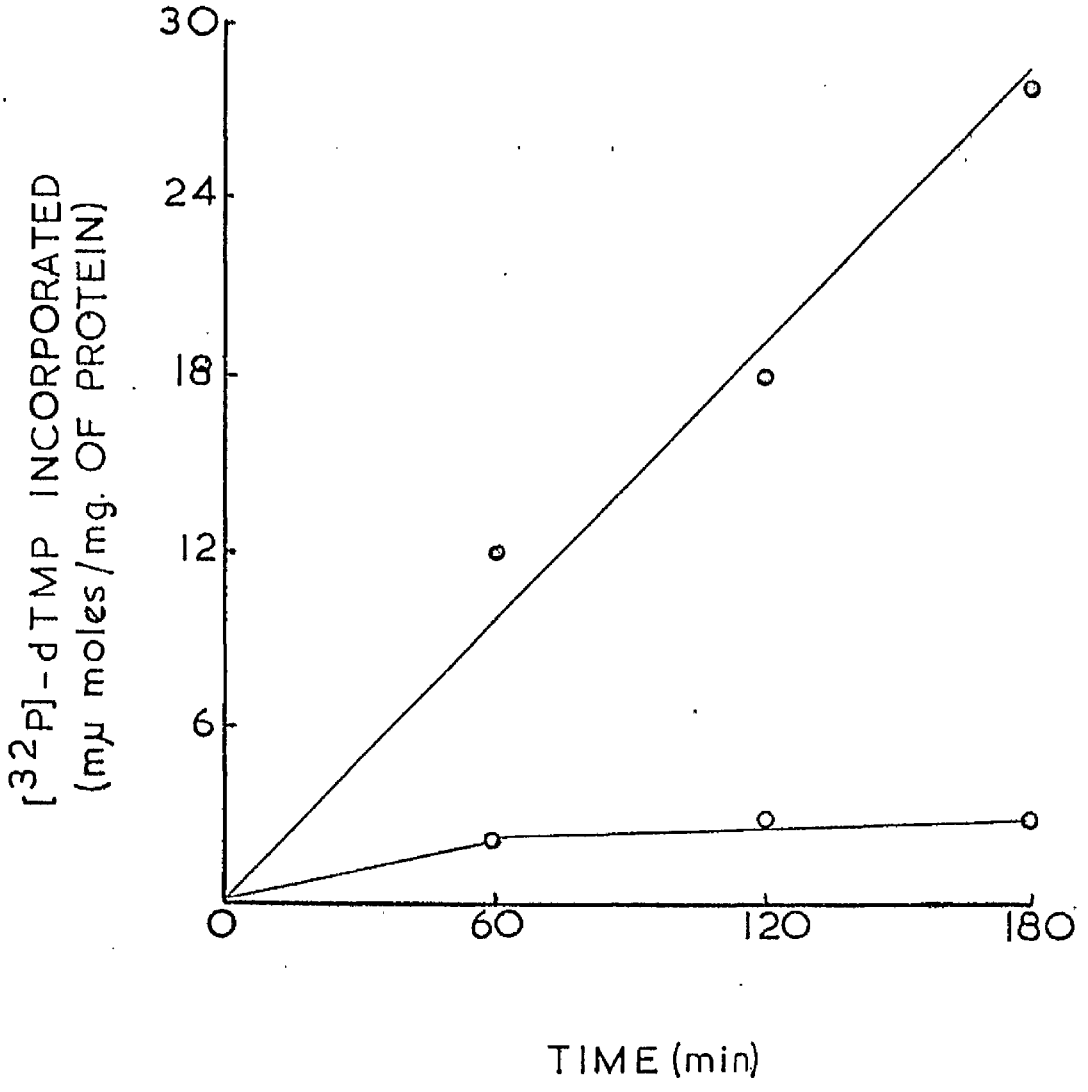

30
24
18
12
6
0
60
120
180
[32P]-dTMP INCORPORATED
(mμ moles/mg. OF PROTEIN)
TIME (min)

FIGURE 28

FIGURE 29

Stimulation of <u>terminal</u> DNA nucleotidyltransferase activity of extracts of calf thymus tissue by prior treatment of the primer with pancreatic DNaseI.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 10 µmoles of KCl; 0.08 µmole of EDTA; 2.5 µmoles of $MgCl_2$; 50 µg. of heat-denatured DNA or 50 µg. of DNase-treated, heat-denatured DNA; 75 mµmoles of (α-^{32}P)-dTTP (7.1×10^6 counts/min./µmole) and 0.05 ml. (188 µg. of protein) of a pH 5 precipitate fraction in a total volume of 0.25 ml. Incubation was carried out at 37° for the indicated time interval.

—o— heat-denatured primer

—●— DNase-treated, heat-denatured primer

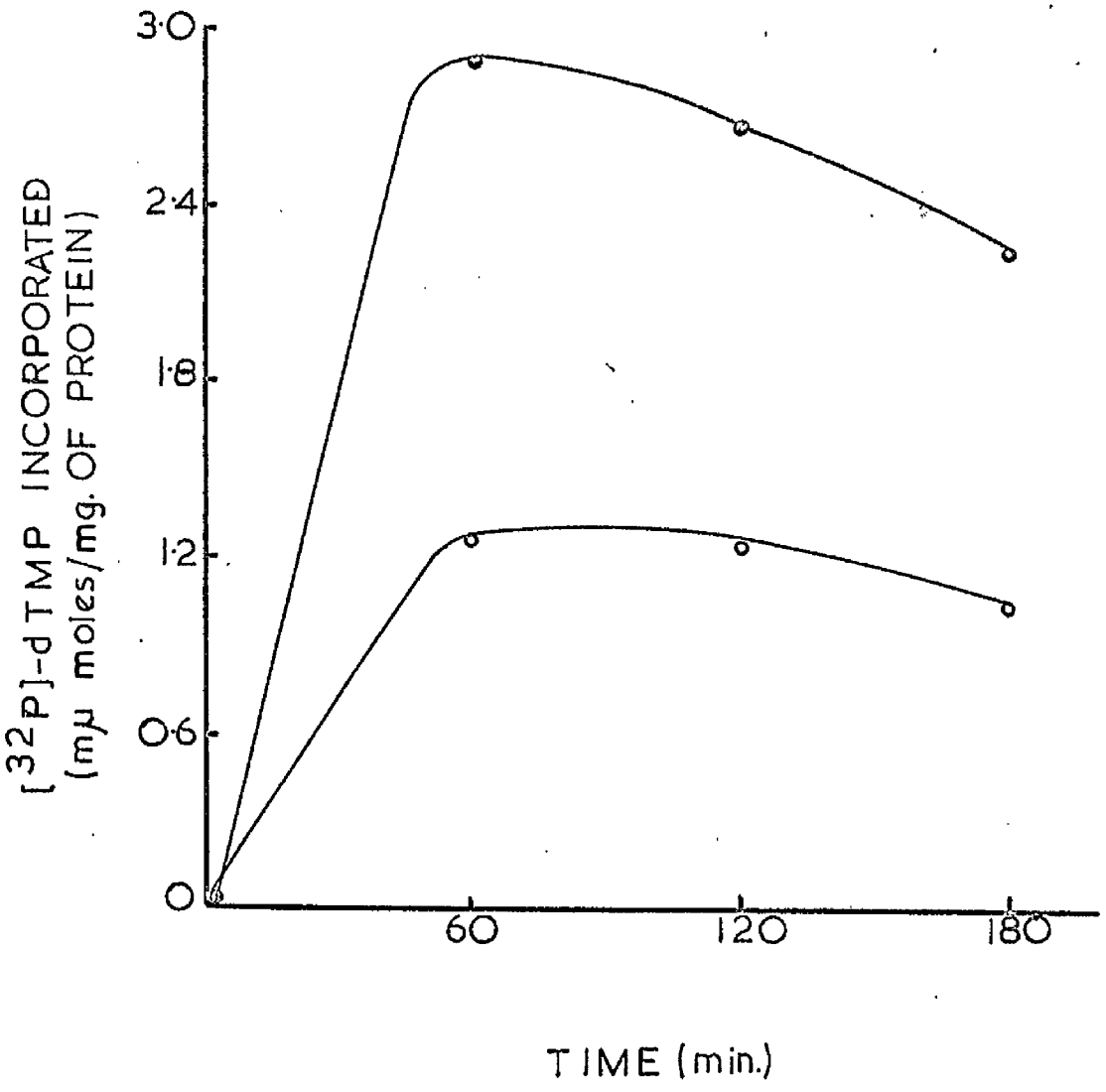

3·0
2·4
1·8
1·2
0·6
0
60
120
180
[32P]-dTMP INCORPORATED
(mμ moles/mg. OF PROTEIN)
TIME (min.)

FIGURE 29

It has also been shown in this laboratory, by snake venom phosphodiesterase digestion of the product of terminal DNA nucleotidyltransferase activity, that the incorporated (^{32}P)-dTMP units are located at terminal positions on the product DNA (H. M. Keir, J. Hay & J. B. Shepherd - collaborative experiments). The results of such an experiment are shown in Fig. 30, which demonstrates that the exonuclease (Adler et al., 1958) renders acid-soluble the product of terminal DNA nucleotidyltransferase activity much more readily than it does the product of replicative activity. This suggests that the terminal assay conditions allow only addition to the ends of the primer whereas during replicative assays formation of new complementary strands of DNA is apparently taking place.

A highly active calf thymus enzyme preparation kindly supplied by Dr. M. G. Burdon was assayed under both replicative and terminal conditions for DNA nucleotidyltransferase activity in turn with (α-^{32}P)-dATP, (α-^{32}P)-dGTP and (α-^{32}P)-dTTP (see Results section III C). This fraction corresponded to fraction D of the purification procedure of Bollum (1960). The relative incorporation of the three deoxyribonucleotides for both replicative and terminal conditions are shown in Table 12. The replicative figures are roughly equivalent suggesting true synthesis of complementary DNA. The terminal figures, however, although showing the greatest activity towards dATP appear to contradict the work of Bollum et al. (1964) which claims that the terminal enzyme in calf thymus is highly specific for dATP.

Nevertheless, there would seem to be little doubt that the assay method

FIGURE 30

Action of 5'-phosphodiesterase on the products synthesized by the replicative and terminal DNA nucleotidyltransferase from calf thymus tissue.

2 mg. of DNA product were isolated from each of two reaction mixtures (2.5 ml.) representing terminal and replicative DNA nucleotidyltransferase as described in Fig. 28. Incubation time for the terminal enzyme was 90 min., and for the replicative enzyme, 120 min. The product was precipitated from the synthetic reaction mixture by 2.5 ml. of N-$HClO_4$ and was freed from acid-soluble radioactivity by four cycles of solution in 0.1N-NaOH and reprecipitation in N-$HClO_4$. The product was finally dissolved in 1 ml. of 0.1N-NaOH and adjusted to pH 8.5 with HCl. A reaction mixture was set up (5 ml.) containing 200 µmoles of tris-HCl buffer pH 8.5, 50 µmoles of $MgCl_2$ and 1.7 units of 5'-phosphodiesterase, and was incubated at 37°. 0.5 ml. samples were withdrawn at intervals, added to 0.15 ml. of calf thymus DNA solution (260 µg. of DNA) and 0.5 ml. of N-$HClO_4$ was added; precipitated material was removed by centrifuging. 0.2 ml. portions of the supernatant fluid were plated for measurement of radioactivity, and the extinction of the remainder of the fluid was measured at 260 mµ.

—o— terminal DNA nucleotidyltransferase product

—•— replicative DNA nucleotidyltransferase product

—△— E_{260}

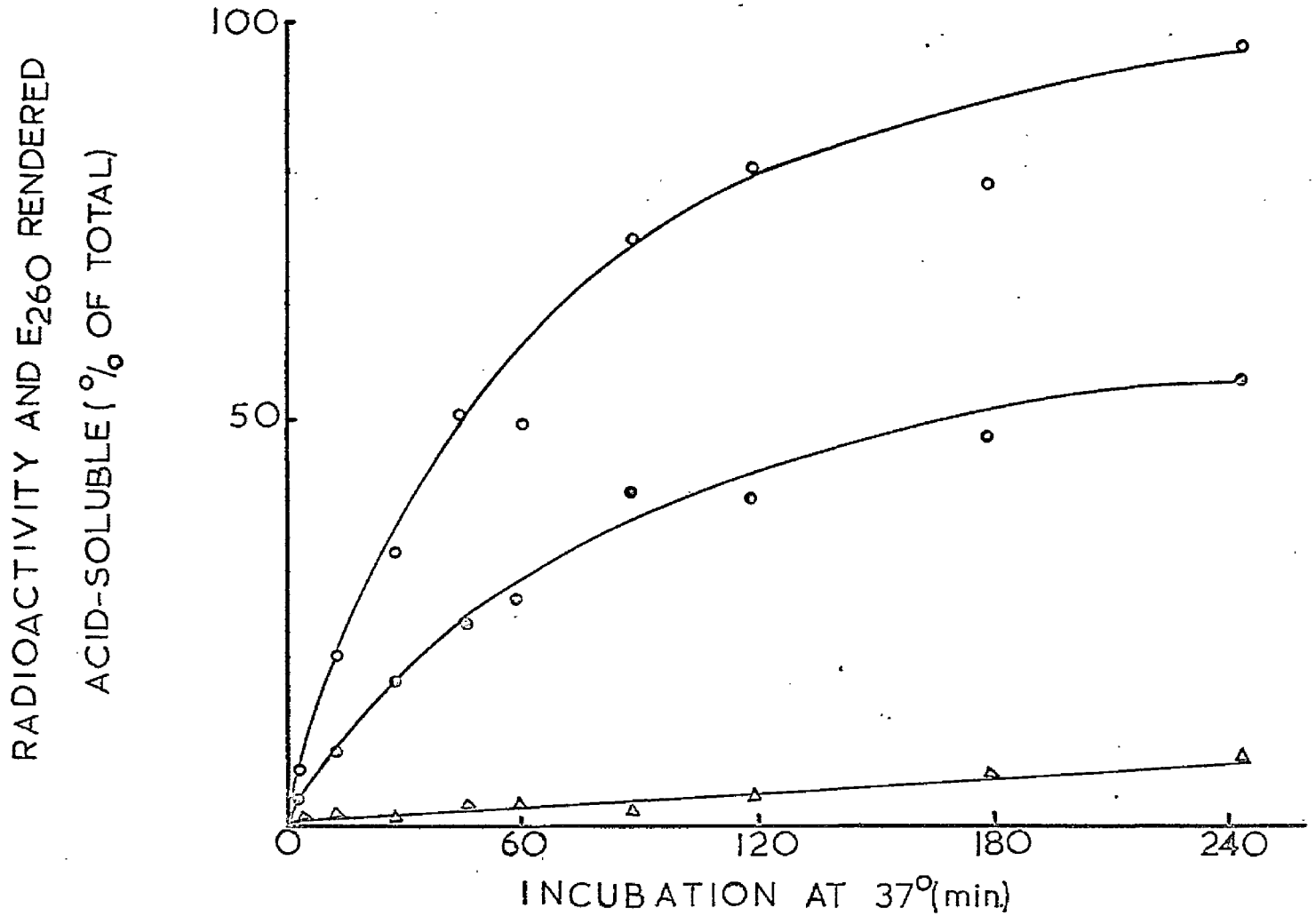

RADIOACTIVITY AND E_{260} RENDERED ACID-SOLUBLE (% OF TOTAL)
100
50
0
60
120
180
240
INCUBATION AT 37°(min)

FIGURE 30

TABLE 12

Relative incorporation of dAMP, dGMP and dTMP into product DNA under replicative and terminal assay conditions by a highly active DNA nucleotidyltransferase preparation from calf thymus tissue.

The replicative reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.10 µmole of EDTA; 1 µmole of $MgSO_4$; 1.25 µmoles of 2-mercaptoethanol; 50 µg. of heat-denatured DNA (from Landschutz ascites-tumour cells); 50 mµmoles each of dATP, dGTP, dCTP and dTTP and 0.02 ml. (20 µg. of protein) of enzyme, in a total volume of 0.25 ml.

Terminal reaction mixtures were the same as their replicative counterparts except that only one of the (α-^{32}P)-labelled deoxyribonucleoside 5'-triphosphates, dATP, dGTP or dTTP was present at a concentration of 100 mµmoles/assay.

The (^{32}P)-labelled deoxyribonucleoside 5'-triphosphates had specific radioactivities of 7.9, 6.7 and 1.3×10^6 counts/min./µmole respectively, for dATP, dGTP and dTTP. Incubation was carried out at 37° for 30 min.

TABLE 12

(^{32}P)-Labelled Substrate	(^{32}P)-Deoxyribonucleotide Incorporated (mμmoles/mg. of protein)	
	Replicative	Terminal
dATP	28.51	8.67
dGTP	34.35	4.34
dTTP	32.13	5.70

employed in this work for the measurement of terminal DNA nucleotidyltransferase activity does in fact measure the terminal addition enzymic activity reported in calf thymus by Krakow et al., (1962) and Keir & Smith (1963).

C) In Landschuts Ascites-Tumour Cells

Terminal DNA nucleotidyltransferase activity was investigated in pH 5 precipitate fractions prepared from Landschutz cells using the criteria established in section B) for the measurement of terminal activity. Actinomycin D has been shown to be a much more potent inhibitor of replicative DNA nucleotidyltransferase, activity than of terminal DNA nucleotidyltransferase activity obtained from both the cytoplasmic and nuclear fractions of non-aqueous calf thymus preparations (H. M. Keir, J. Hay & J. B. Shepherd - collaborative experiments). This is, perhaps, not unexpected, since the binding of the antibiotic at guanine residues (Reich, Goldberg & Rabinowitz, 1962; Goldberg, Rabinowitz & Reich, 1962) along the DNA-primer molecules would certainly be expected to interfere seriously with incorporation of the replicative type, whereas terminal incorporation would be expected to be less susceptible to inhibition since direct interference would arise only in primer molecules bearing deoxyguanylyl residues at or near 3'-hydroxy-terminal positions. The terminal and replicative DNA nucleotidyltransferase activities of pH 5 precipitate fractions from Landschutz cells in the presence and absence of Actinomycin D are shown in Fig. 31. 25 µg. of primer DNA and 5 µg. of Actinomycin D were used in each assay, since these were

FIGURE 31

Replicative and terminal DNA nucleotidyltransferase activities of extracts of Landschuts ascites-tumour cells in the presence and absence of Actinomycin D.

The replicative assay mixtures contained: 10 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.08 µmole of EDTA; 1 µmole of $MgCl_2$; 25 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (10.8×10^6 counts/min./µmole) and 0.05 ml. (150 µg. of protein) of a pH 5 precipitate fraction in a total volume of 0.25 ml.

The terminal assay mixtures contained: 10 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.08 µmole of EDTA; 1 µmole of $MgCl_2$; 1 µmole of 2-mercaptoethanol; 25 µg. of heat-denatured DNA; 50 mµmoles of (α-^{32}P)-dTTP (10.8×10^6 counts/min./µmole) and 0.05 ml. (150 µg. of protein) of a pH 5 precipitate fraction in a total volume of 0.25 ml. Incubation was carried out at 37° for the indicated time interval.

The incorporation scale for the two terminal activity plots is given on the right of the figure. (N.B. this scale is tenfold smaller than that for the replicative assay)

—○— replicative activity

—●— replicative activity in the presence of 5 µg. of Actinomycin D

—△— terminal activity

—▲— terminal activity in the presence of 5 µg. of Actinomycin D

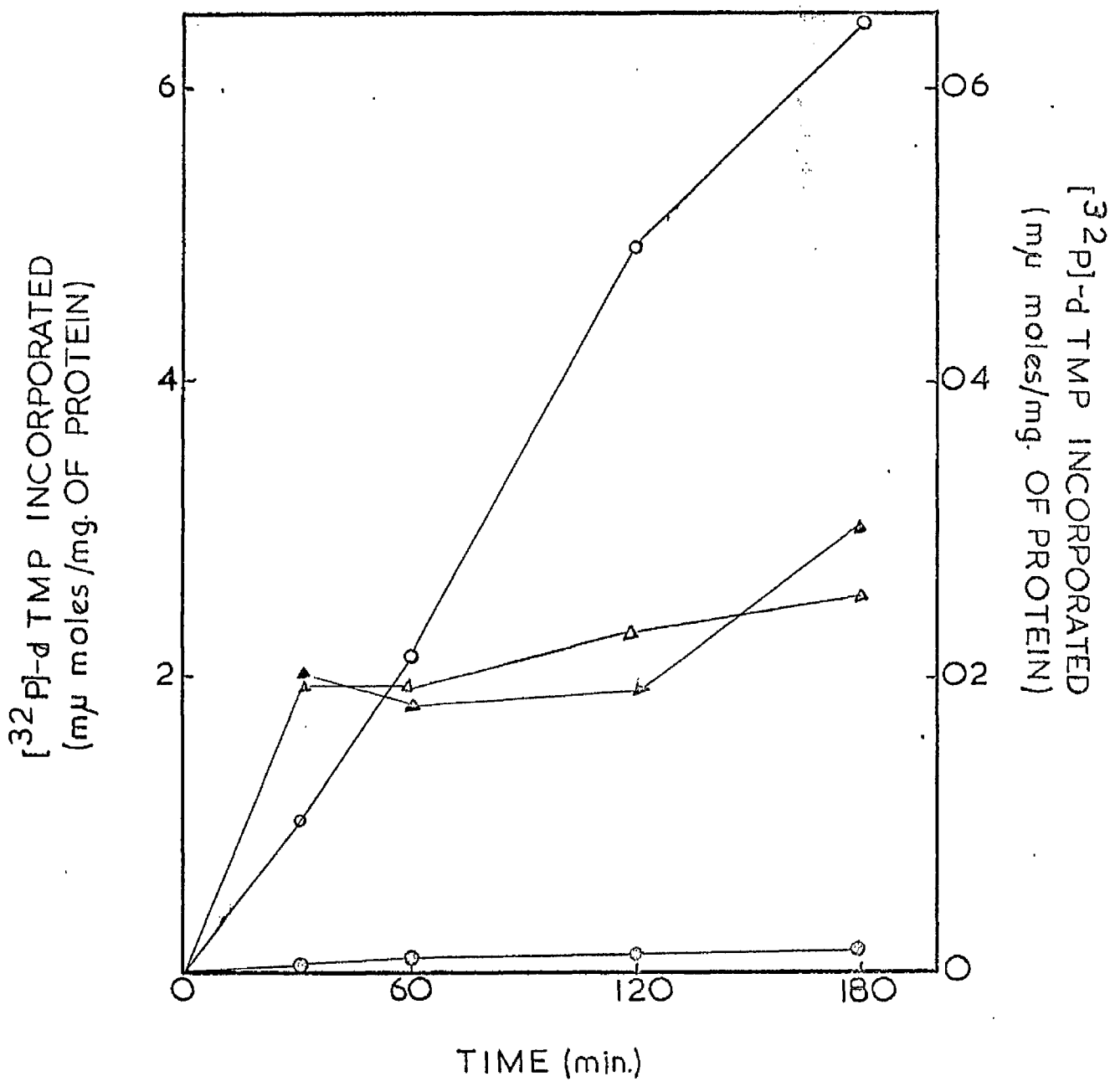

[32P]-d TMP INCORPORATED
(mμ moles/mg. OF PROTEIN)
6
4
2
0
60
120
180
TIME (min.)
06
04
02
0
[32P]-d TMP INCORPORATED
(mμ moles/mg. OF PROTEIN)

FIGURE 31

conditions suitable for demonstration of inhibition of replicative DNA nucleotidyltransferase from calf thymus tissue. The results obtained corroborate those obtained with the calf thymus enzyme since the replicative activity is almost abolished by Actinomycin D, while the terminal activity remains unchanged. However, the relative amount of terminal activity in Landschutz cells appears to be much less than that found in thymus tissue (see section D).

D) In Other Tissues

Terminal DNA nucleotidyltransferase activity was also investigated in certain tissues of the rabbit (appendix, liver, spleen and thymus) and of the rat (spleen and liver). pH 5 precipitate fractions of all of these tissues together with pH 5 precipitate fractions from Landschutz cells and from non-aqueous nuclear and non-aqueous cytoplasmic fractions (prepared by the method of Kay et al., 1956) from calf thymus were assayed for replicative and terminal DNA nucleotidyltransferase activity. The results of these assays are recorded in Table 13 which shows that the calf thymus gland is the tissue which has the highest proportion of terminal DNA nucleotidyltransferase relative to the replicative activity of the tissue, while Landschutz ascites-tumour has little or no terminal DNA nucleotidyltransferase activity when compared to its replicative DNA nucleotidyltransferase activity and to the terminal activities of other tissues.

VI Inhibition Studies on DNA Nucleotidyltransferase from Landschutz Ascites-Tumour Cells

A) Thiol Group Inhibitors

DNA nucleotidyltransferase activity in pH 5 precipitate fractions

TABLE 13

Replicative and terminal DNA nucleotidyltransferase activities of various mammalian tissues.

The replicative reaction mixture contained: 10 μmoles of tris-HCl buffer, pH 7.5; 15 μmoles of KCl; 0.08 μmole of EDTA; 1 μmole of $MgCl_2$; 35 μg. of heat-denatured DNA; 50 mμmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (10^7 counts/min./μmole) and 100 - 150 μg. of protein from the indicated pH 5 precipitate fraction, in a total volume of 0.25 ml.

The terminal reaction mixture contained: 10 μmoles of tris-HCl buffer, pH 7.5; 10 μmoles of KCl; 0.08 μmole of EDTA; 2.5 μmoles of $MgCl_2$; 35 μg. of heat-denatured DNA; 50 mμmoles of (α-^{32}P)-dTTP (10^7 counts/min./μmole) and 100 - 150 μg. of protein from the indicated pH 5 precipitate fraction, in a total volume of 0.25 ml.

Incubation time at 37° was 2.5 hr. with the mouse and rabbit preparations, 2 hr. with the calf thymus and rat spleen preparations and 1.5 hr. with the rat liver preparation.

TABLE 13

Tissue	(^{32}P)-dTMP Incorporated (mμmoles/mg. of protein)	
	Replicative	Terminal
Rabbit		
Appendix	10.31	1.32
Liver	0.34	0.38
Spleen	2.64	0.63
Thymus	17.40	2.04
Rat		
Spleen	6.27	1.62
Liver	0.68	0.61
Mouse		
Landschutz Ascites-Tumour Cells	9.34	0.17
Calf		
Thymus Non-Aqueous Nuclei	15.5	9.90
Thymus Non-Aqueous Cytoplasm	6.9	6.40

was inhibited by several compounds known to react with thiol groups, and in this way to modify the action of enzymes which contain thiol groups. Figs. 32 & 33 show the effect of increasing amounts of sarkomycin (2-methylene-3-oxocyclopentane carboxylic acid), iodoacetamide and p-hydroxymercuribenzoate on the activity of DNA nucleotidyltransferase. The complete reaction mixtures were allowed to stand for 30 min. at 0° prior to incubation proper for 60 min. at 37°, in order to allow reaction of the enzyme with the inhibitor. It is recognised that complete equilibration may not have been achieved but prior incubation at 25° for 20 min. or at 37° for 15 min. in the absence of deoxyribonucleoside triphosphates did not alter the pattern of results significantly. Prolonged prior incubation was not desirable because of the relative lability of the enzyme.

The effect of increasing amounts of 2-mercaptoethanol and GSH on the activity of DNA nucleotidyltransferase is shown in Fig. 34. Neither 2-mercaptoethanol nor glutathione stimulates DNA nucleotidyltransferase activity under standard assay conditions, but both compounds begin to inhibit enzymic activity above 20 mM, GSH being the more potent in this respect.

Inhibition by p-hydroxymercuribenzoate could be prevented if 2-mercaptoethanol at 30 mM was added to the assay mixture immediately prior to the 37° incubation period. Inhibition by sarkomycin could only be partially prevented by this procedure, while inhibition by iodoacetamide was in no way reversed. Indeed when these concentrations of 2-

FIGURE 32

Inhibition of DNA nucleotidyltransferase activity of extracts of Landschutz ascites-tumour cells by sarkomycin.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.1 µmole of EDTA; 1.2 µmoles of $MgSO_4$; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (0.8×10^6 counts/min./µmole); 0.02 ml. (156 µg. of protein) of a pH 5 precipitate fraction and the indicated amount of sarkomycin in a total volume 0.25 ml. The complete reaction mixture was allowed to stand for 30 min. at 0° before incubation for 60 min. at 37°.

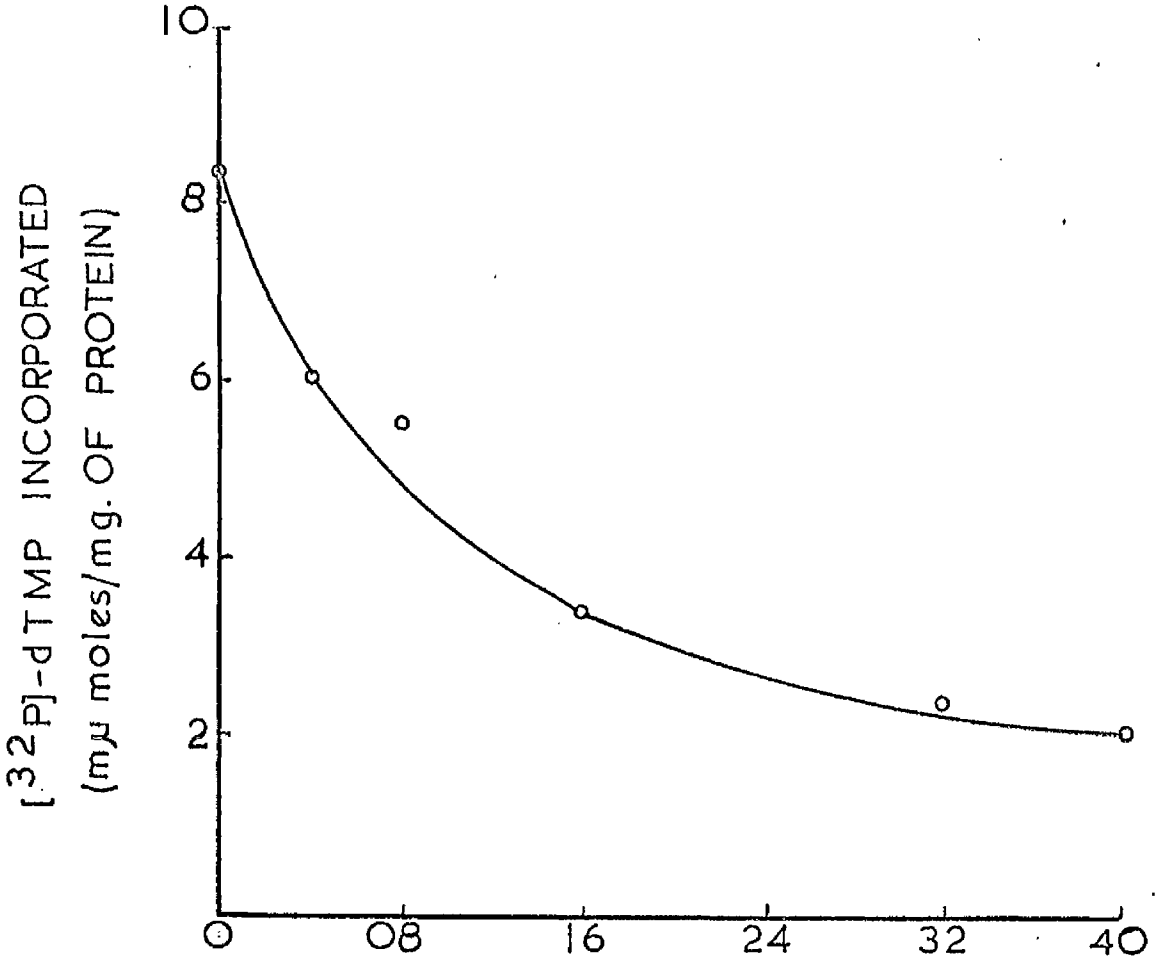
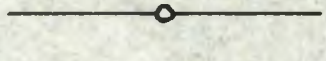

[32P]-dTMP INCORPORATED
(mμ moles/mg. OF PROTEIN)
10
8
6
4
2
0
08
16
24
32
40
SARKOMYCIN (mM)

FIGURE 32

FIGURE 33

Inhibition of DNA nucleotidyltransferase activity of extracts of Landschutz ascites-tumour cells by iodoacetamide and p-hydroxymercuribenzoate.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.1 µmole of EDTA; 1.2 µmoles of $MgSO_4$; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dCTP, dGTP and (α-^{32}P)-dTTP (1 x 10^6 counts/min./µmole); 0.05 ml. (110 µg. of protein) of a pH 5 precipitate fraction and the indicated amount of iodoacetamide or p-hydroxymercuribenzoate in a total volume of 0.25 ml. The complete reaction mixture was allowed to stand for 30 min. at 0° before incubation at 37° for 60 min.



iodoacetamide

p-hydroxymercuribenzoate

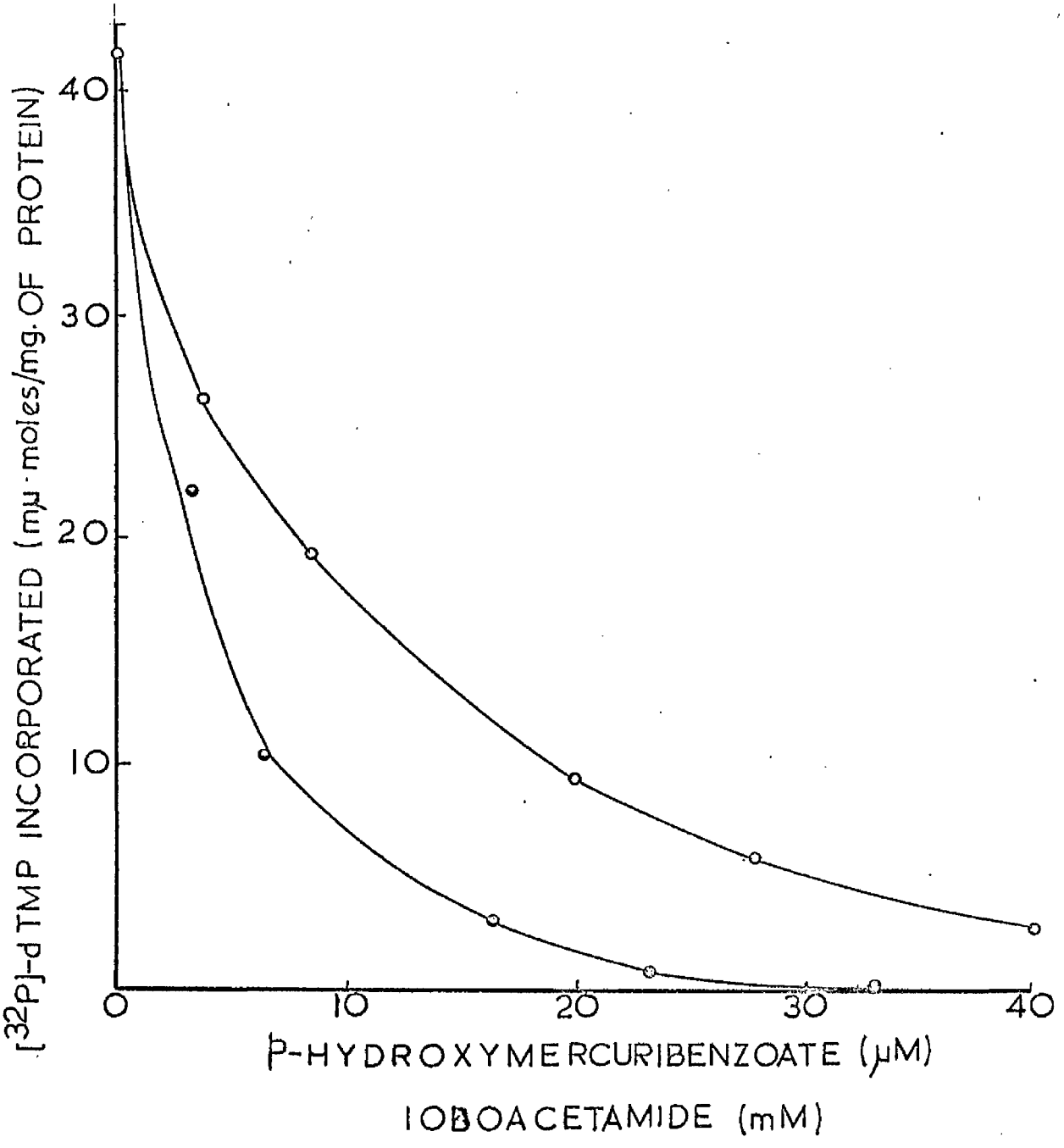
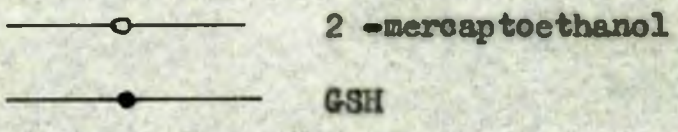

[32P]-d TMP INCORPORATED (mμ·moles/mg. OF PROTEIN)
40
30
20
10
0
10
20
30
40
P-HYDROXYMERCURIBENZOATE (μM)
IOBOACETAMIDE (mM)

FIGURE 33

FIGURE 34

Effects of GSH or 2-mercaptoethanol on DNA nucleotidyltransferase activity of extracts of Landschutz ascites-tumour cells.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.10 µmole of EDTA; 1.2 µmoles of $MgSO_4$; 50 µg. of heat-denatured DNA; 50 mµmoles each of dGTP, dCTP, dTTP and (8-^{14}C)-dATP (6.2×10^6 counts/min./µmole); 0.05 ml. (110 µg. of protein) of a pH 5 precipitate fraction and the indicated amount of 2-mercaptoethanol and GSH. Incubation was carried out at 37° for 60 min.



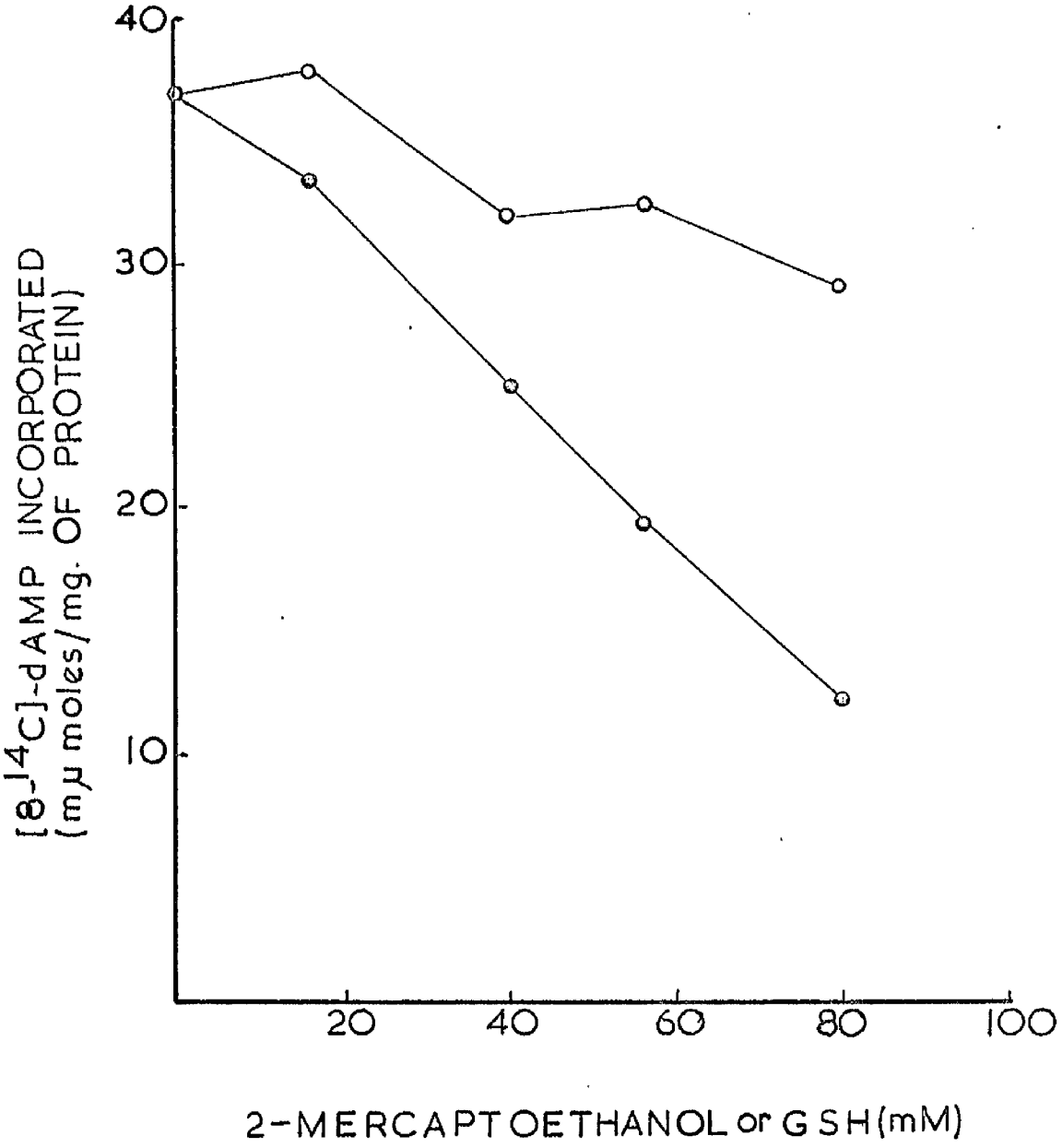

40
30
20
10
[8-14C]-dAMP INCORPORATED
(mμ moles/mg. OF PROTEIN)
20
40
60
80
100
2-MERCAPTOETHANOL or GSH(mM)

FIGURE 34

mercaptoethanol and GSH were added to iodoacetamide or iodoacetate inhibited assays the residual incorporation of (^{32}P)-dTMP was virtually abolished; evidence suggests that the DNA nucleotidyltransferase, along with most of the other proteins in the enzyme preparation, was precipitated and rendered inactive, when 2-mercaptoethanol or GSH plus iodoacetate or iodoacetamide were present in the assay mixture. These results of inhibition and release of inhibition are summarised in Table 14.

Similar experiments investigating the inhibition of the terminal addition reaction by iodoacetamide and p-hydroxymercuribenzoate were performed (see Table 15). These compounds inhibited the terminal addition reaction in a manner essentially similar to their inhibition of the replicative reaction; however, levels of inhibition imposed on the replicative reaction required 2.5-fold higher concentrations of these inhibitors to be achieved in the terminal addition reaction. The phenomenon of 100% inhibition exerted by iodoacetamide plus GSH was observed also in the terminal addition assay.

The effects on the DNA nucleotidyltransferase reaction of varying the concentration of DNA-primer were determined at constant concentrations of inhibitors. The results for iodoacetamide, p-hydroxymercuribenzoate and sarkomycin are presented in Fig. 35 as double reciprocal plots (Lineweaver & Burk , 1934). The results for each inhibitor gave a linear plot intersecting at the base line with the corresponding control plot, which suggests that the inhibition exerted in each case conforms to non-

TABLE 14

Inhibition of DNA nucleotidyltransferase activity of extracts of Landschutz ascites-tumour cells by p-hydroxymercuribenzoate, iodoacetamide and sarkomycin.

Enzymic activities are expressed as percentages of the activity obtained under standard assay conditions which were: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.10 µmole of EDTA; 1.2 µmole of $MgSO_4$; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (0.8×10^6 counts/min./µmole;) 110 µg. of protein from a pH 5 precipitate fraction and the indicated concentrations of inhibitor and reversal agent, in a total volume of 0.25 ml.

Reaction mixtures were kept at 0° for 30 min. with inhibitor and enzyme present. GSH or 2-mercaptoethanol was then added as indicated, and incubation carried out at 37° for 60 min. The standard assay representing 100% activity gave an incorporation of 38.4 mµmoles of (^{32}P)-dTMP/mg. of protein/hr.

TABLE 14.

Additions to standard assay	^{32}P)-dTMP incorporated (% of standard assay)
Nil	100
Iodoacetamide at 8mM	50
Iodoacetamide at 20mM	24
Iodoacetamide at 8mM, GSH at 20mM	0
Iodoacetamide at 20mM, GSH at 20mM	0
Iodoacetamide at 8mM, 2-mercaptoethanol at 30mM	2
Iodoacetamide at 20mM, 2-mercaptoethanol at 30mM	0
p-hydroxymercuribenzoate at 6.6 µM	27
p-hydroxymercuribenzoate at 16.6 µM	8
p-hydroxymercuribenzoate at 6.6 µM, GSH at 20mM	92
p-hydroxymercuribenzoate at 16.6 µM, GSH at 20mM	98
p-hydroxymercuribenzoate at 6.6 µM, 2-mercaptoethanol at 30mM	100
p-hydroxymercuribenzoate at 16.6 µM, 2-mercaptoethanol at 30mM	108
Sarkomycin at 12mM	19
Sarkomycin at 12mM, GSH at 20mM	59
Sarkomycin at 12mM, 2-mercaptoethanol at 30mM	56

TABLE 15

Inhibition of the terminal addition reaction of extracts of Landschutz ascites-tumour cells by iodoacetamide and p-hydroxymercuribenzoate.

Enzymic activities are expressed as percentages of the activity obtained under standard assay conditions which were: 5 µmoles of tris-HCl buffer pH 7.5; 15 µmoles of KCl; 0.10 µmole of EDTA; 1.2 µmoles of $MgSO_4$; 50 µg. of heat-denatured DNA; 80 mµmoles of (α-^{32}P)-dTTP (10^6 counts/min./µmole) and 100 µg. of protein from a pH 5 precipitate fraction, in a total volume of 0.25 ml.

The complete reaction mixtures were kept at 0° for 30 min. in the presence of iodoacetamide or p-hydroxymercuribenzoate before assaying. The appropriate assays received G-SH immediately before incubation at 37° for 60 min.

TABLE 15

Additions to Standard Assay	(^{32}P)-dTMP incorporated (% of standard assay)
Nil	100
Iodoacetamide at 20mM	46
p-hydroxymercuribenzoate at 16.6 μM	25
GSH at 20 mM	97
Iodoacetamide at 20mM plus GSH at 20mM	0
p-hydroxymercuribenzoate at 16.6 μM plus GSH at 20mM	95

FIGURE 35

Inhibition of DNA nucleotidyltransferase activity of extracts of Landschutz ascites-tumour cells by iodoacetamide, p-hydroxymercuribenzoate and sarkomycin.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.1 µmole of EDTA; 1.2 µmoles of $MgSO_4$; 50 µg. of heat-denatured DNA; 50 mµmoles each of dGTP, dCTP, dTTP and (8-^{14}C)-dATP (6.2×10^6 counts/min./µmole); 0.05 ml. (110 µg. of protein) of a pH 5 precipitate fraction and the indicated concentrations of iodoacetamide, p-hydroxymercuribenzoate or sarkomycin in a total volume of 0.25 ml. The complete reaction mixture was allowed to stand at 0° for 30 min. before incubation at 37° for 60 min. The results are expressed as double reciprocal plots (1/v versus 1/s)

v = mµmoles of (8-^{14}C)dAMP incorporated/mg. of protein/hr.

s = µg. of DNA/assay

A = sarkomycin at 12 mM

B = p-hydroxymercuribenzoate at 6.6 µM

C = iodoacetamide at 8mM (—●—) and at 20 mM (—△—)

—○— uninhibited standard assay

—●— —△— inhibitor present

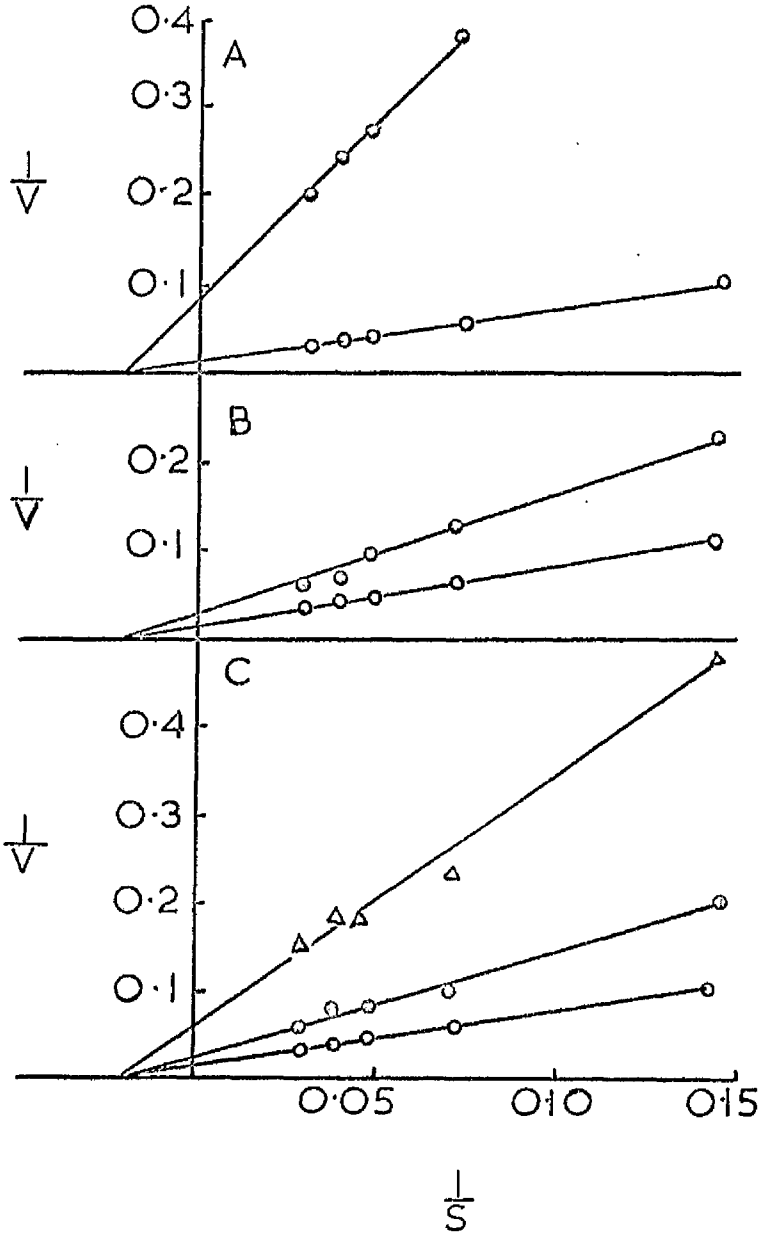


FIGURE 35

competitive kinetics, the values of K_i being 6.1×10^{-3}M, 2.5×10^{-3}M and 7.1×10^{-6}M for iodoacetamide, sarkomycin and p-hydroxymercuribenzoate respectively.

B) Inhibition by Actinomycin D

Studies on the inhibition of DNA nucleotidyltransferase by Actinomycin D were performed in collaboration with Dr. H. M. Keir and Dr. H. Omura.

Fig. 36 shows inhibition of the enzyme activity by increasing amounts of Actinomycin D. The inhibitory effect exerted by a constant amount of Actinomycin D in the presence of varying amounts of primer-DNA is presented in Fig. 37 as a double reciprocal plot (Lineweaver & Burk , 1934). These plots of 1/v against 1/s result in lines which have an upward curve in both the inhibited and non-inhibited cases. This deviation from the standard Lineweaver-Burk relationship will be examined in more detail later (see Results section VII). However, the 1/v against 1/s plots intersect on the vertical axis indicating that Actinomycin D inhibits the enzyme in a competitive manner. Actinomycin D inhibition of DNA-primed RNA synthesis has been shown to be due to its ability to bind to guanine residues of the DNA primer (Reich et al., 1962; Goldberg et al., 1962). Kirk (1960) showed that Actinomycin D inhibited DNA nucleotidyltransferase from E. coli, and it has been shown that the antibiotic inhibits highly purified DNA and RNA nucleotidyltransferases from E. coli in a manner which obeys competitive kinetics with respect to DNA-primer. Therefore, it is not unexpected that Actinomycin D should

FIGURE 36

Inhibition of DNA nucleotidyltransferase activity of extracts of Landschutz ascites-tumour cells by Actinomycin D.

The reaction mixture contained: 10 µmoles of tris-HCl buffer, pH 7.5; 9 µmoles of KCl; 0.08 µmole of EDTA; 1 µmole of $MgCl_2$; 0.5 µmole of potassium phosphate buffer, pH 7.5; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (1.6×10^6 counts/min./µmole); 0.05 ml. (80 µg. of protein) of a pH 5 precipitate fraction and the indicated amounts of Actinomycin D in a total volume of 0.25 ml. Incubation was carried out at 37° for 120 min.

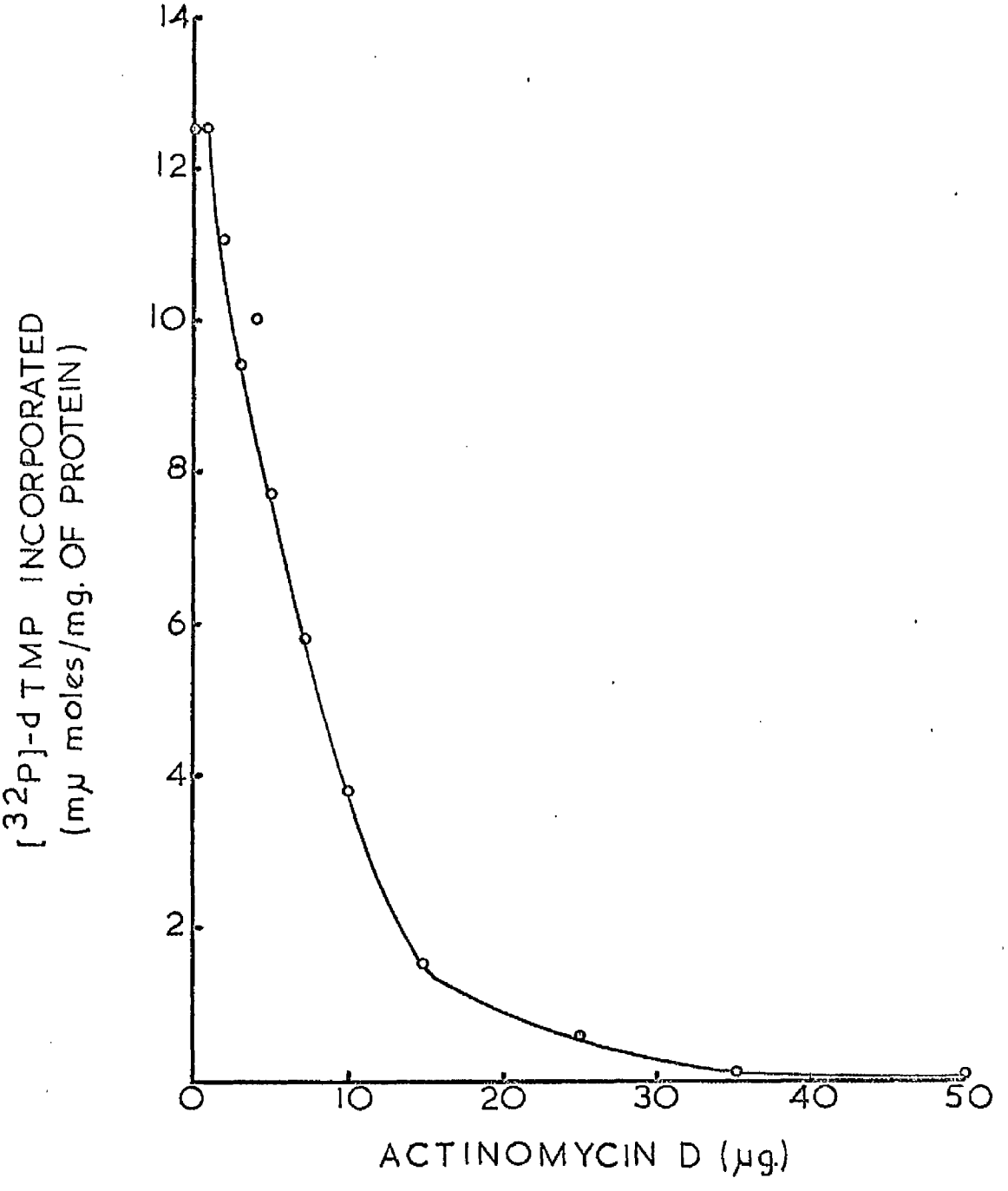

[32P]-d TMP INCORPORATED
(mμ moles/mg. OF PROTEIN)
14
12
10
8
6
4
2
0
10
20
30
40
50
ACTINOMYCIN D (μg.)

FIGURE 36

FIGURE 37

Competitive inhibition of DNA nucleotidyltransferase activity of extracts of Landschuts ascites-tumour cells by Actinomycin D.

The reaction mixture contained: 10 µmoles of tris-HCl buffer pH 7.5; 9 µmoles of KCl; 0.08 µmole of EDTA; 1 µmole of $MgCl_2$; 0.5 µmole of potassium phosphate buffer pH 7.5; heat denatured DNA as indicated (µg.); 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (16 x 10^6 counts/min./µmole) and 0.05 ml. of a pH 5 precipitate fraction (209 µg. of protein) in a total volume of 0.25 ml. Incubation was carried out at 37° for 120 min.

The results are expressed as double reciprocal plots (Lineweaver & Burk, 1934).

——o—— (no addition) control

——•—— 2 µg. of Actinomycin D added.

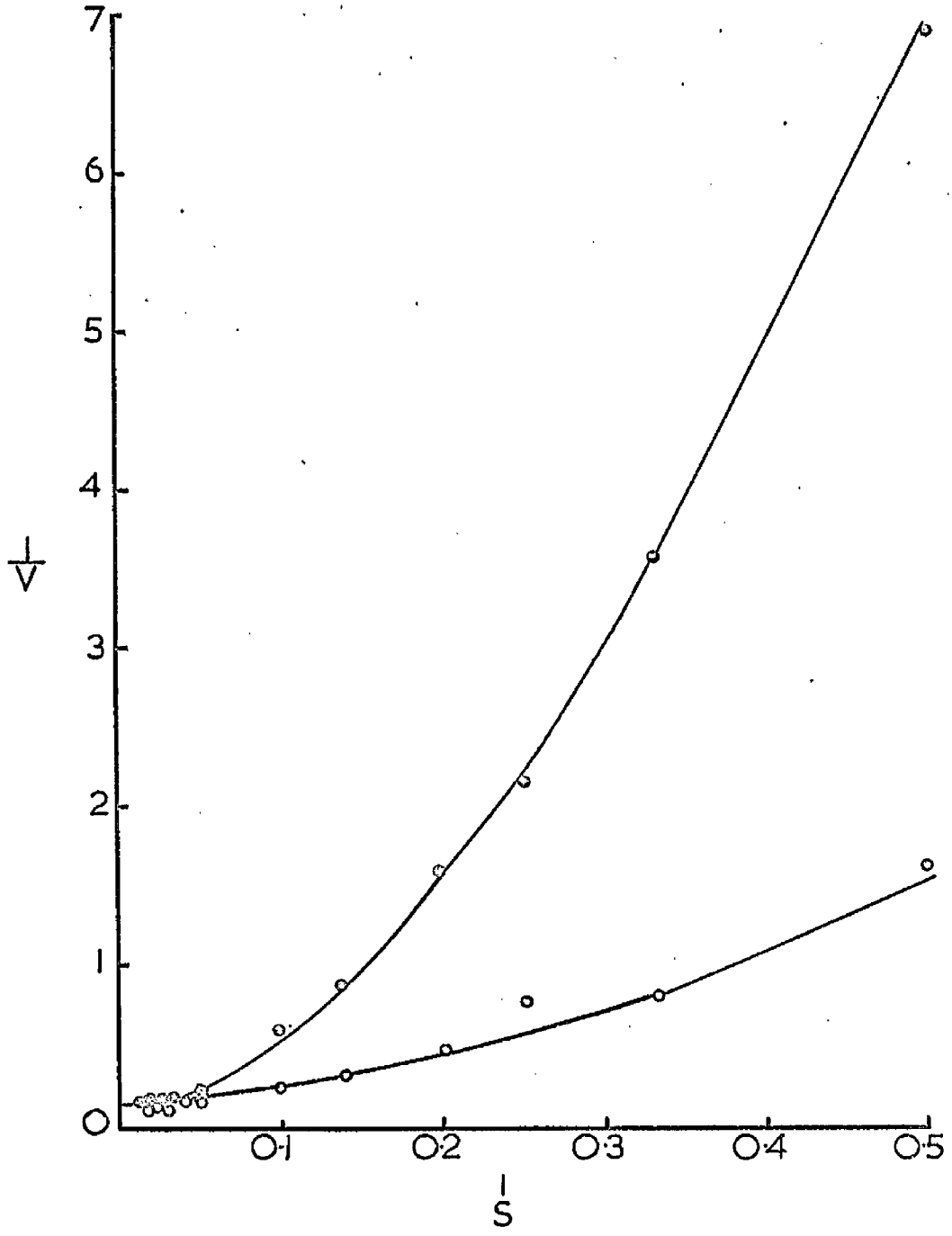


FIGURE 37

behave in a similar manner towards a mammalian DNA nucleotidyltransferase.

VII A Possible Approach to Characterisation of the Molecular Conformation of DNA Nucleotidyltransferase

A) General Considerations

During this work attempts to purify DNA nucleotidyltransferase preparations from Landschutz ascites-tumour cells and calf thymus tissue were repeatedly frustrated by inactivation of the enzyme during fractionation, in spite of the fact that the utmost care was taken to prevent such a result. In addition, it was found that different enzyme fractions prepared in exactly the same manner could vary enormously in total activity and in their optimal ionic requirements. This apparent extreme fragility and wide variability in properties of the enzyme preparations led to the concept that, *in vivo*, the enzyme is possibly a highly organised, well-defined structure, perhaps a "DNA-synthesising particle", but, that in the present *in vitro* studies a substantial proportion of the enzyme molecules being investigated readily undergo some sort of molecular modification or fragmentation. It was with a view to testing this concept that experiments of the type described in this section were undertaken.

B) Studies on the Heat-Stability of the Enzyme

Assuming the validity of the enzyme fragmentation concept, one would expect that the greater the degree of fractionation or purification of a particular enzyme preparation the greater would be the extent of fragmentation or degradation. A technique designed to exploit and

investigate this idea was that of prior heating of the enzyme (preincubation) followed by incubation proper at 37° (see Experimental, section IV A, 1). The results of preincubation of soluble extract, pH 5 precipitate, 20 - 45% ammonium sulphate fraction and nuclear extract at 45° over a period of 25 min. are shown in Fig. 38. The activity of all four fractions is stimulated over the first 2 min. period of preincubation. However, the soluble extract fraction is stimulated by 41% of the initial activity and the nuclear extract by 18%, while the pH 5 precipitate and the 20 - 45% ammonium sulphate fractions show stimulation of the order of only 4% and 2% respectively. The extreme lability of the enzyme is demonstrated by the facts that prior incubation at 50° under the same conditions completely abolishes the activity of all four fractions within 2 min. and prior incubation at 45° in the absence of DNA results in complete inactivation of the enzyme within a similar time interval.

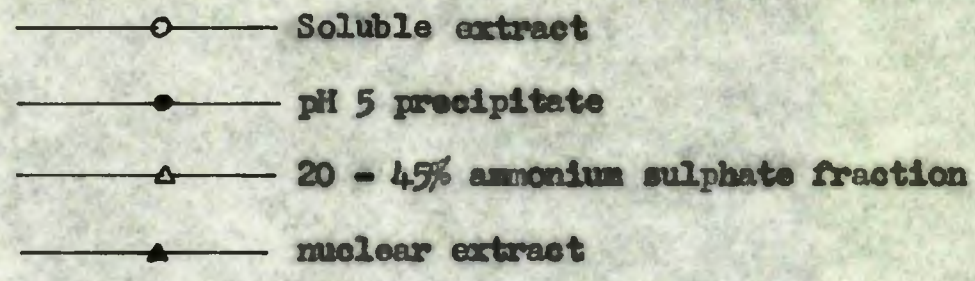
C) Variation in Ionic Requirements

During investigations designed to establish the optimal ionic conditions for DNA nucleotidyltransferase activity in both Landschutz ascites-tumour cell and calf thymus fractions, it was noted on occasion that the optimal requirements for K^+, Na^+ and Mg^{2+} ions differed significantly from those described previously in section II A of the Results. At first this was interpreted as the expression of different ionic optima by the replicative and terminal DNA nucleotidyltransferases and led to the assay, on occasion, of the terminal enzyme at 10mM $MgCl_2$

FIGURE 38

Heat-stability of DNA nucleotidyltransferase activity of extracts of Landschuts ascites-tumour cells.

The assay mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.15 µmole of EDTA; 1.2 µmoles of $MgSO_4$; 1.25 µmoles of 2-mercaptoethanol; 150 µg. of heat-denatured DNA; 75 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (1.9×10^6 counts/min./µmole) and 0.05 ml. of the appropriate enzyme fraction in a total volume of 0.25 ml. Prior incubation was carried out for the indicated time with enzyme and 75 µg. of heat-denatured DNA, pH 7.0 before the addition of the remainder of the assay mixture containing a further 75 µg. of heat-denatured DNA plus the other constituents mentioned above. Incubation then proceeded at 37° for 60 min. Soluble extract, nuclear extract, pH 5 precipitate and 20 - 45% ammonium sulphate fractions contained respectively 112, 135, 99 and 161 µg. of protein/assay.



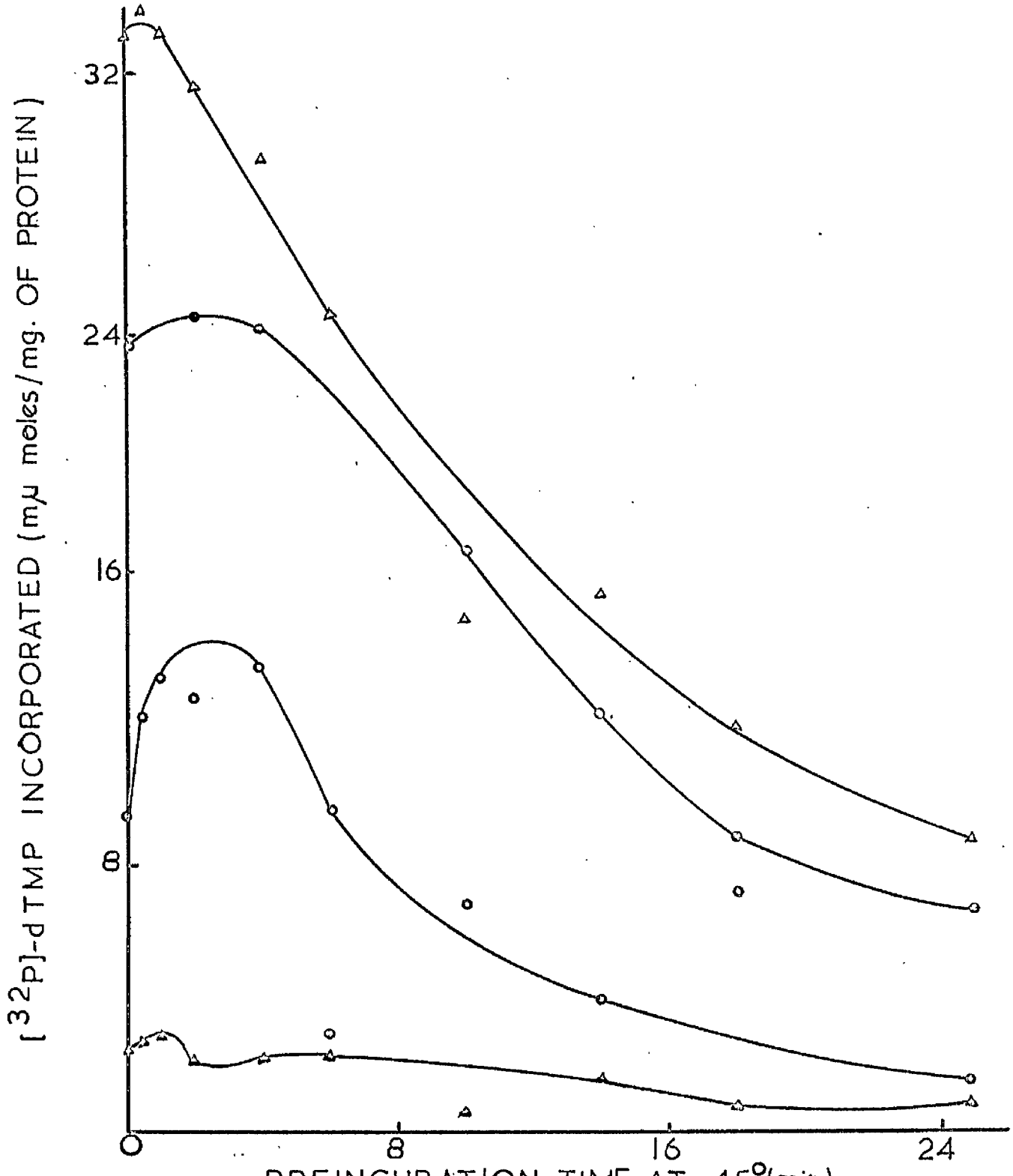

[32P]-d TMP INCORPORATED (mμ moles/mg. OF PROTEIN)
32
24
16
8
0
8
16
24
PREINCUBATION TIME AT 45°(min.)

FIGURE 38

and 40mMKCl. However, as more information became available, it seemed reasonable to suggest, among other possibilities, that these preparations contained DNA nucleotidyltransferase molecules differing in structure from those molecules which displayed standard ionic requirements. An example of this phenomenon is shown in Fig. 39 which consists of Mg^{2+} ion concentration curves for two such anomalous 20 - 45% ammonium sulphate fractions, one from calf thymus and the other from Landschutz ascites-tumour cells. The form of Fig. 39 differs very much from the relatively sharp optimum at 4mM Mg^{2+} ions usually obtained for the Landschutz enzyme (see Fig. 21) and the calf thymus enzyme (See Results, section II B). This phenomenon of irregular response to ionic conditions was also observed on several occasions with respect to K^{+} and Na^{+} ions. In order to investigate this problem further, the ionic characteristics of preparations from both calf thymus and Landschutz tissues were examined simultaneously. This approach had the advantage that it brought a third variable, namely the difference in source of the enzyme fractions, into play in addition to the Mg^{2+} ion variability and the physical separateness of the two enzyme preparations. It was hoped in this way to show that there can be variabilities in the optimal ionic requirements of different DNA nucleotidyltransferase preparations depending on such factors as method of extraction, duration of extraction and source of enzyme. The results of two such co-ordinated experiments are shown in Figs. 40 & 41. Fig. 40 represents the ratio of the DNA nucleotidyltransferase activity of a calf thymus 20 - 45% ammonium sulphate fraction to that

FIGURE 39

An example of an anomalous relationship between Mg^{2+} ion concentration and the DNA nucleotidyltransferase activities of 20-45% ammoniumsulphate fractions prepared from calf thymus tissue and Landschutz ascites-tumour cells.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH7.5; 15 µmoles of KCl; 0.08 µmole of EDTA; the indicated amount of $MgSO_4$; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (7.2×10^6 counts/min/µmole) and 0.01 ml. of calf thymus enzyme fraction (114 µg. of protein) or 0.02 ml. of Landschutz enzyme fraction (94 µg. of protein) in a total volume of 0.25 ml.

Incubation was carried out at 37° for 45 min.

——o—— calf thymus preparation

——●—— Landschutz ascites - tumour preparation.

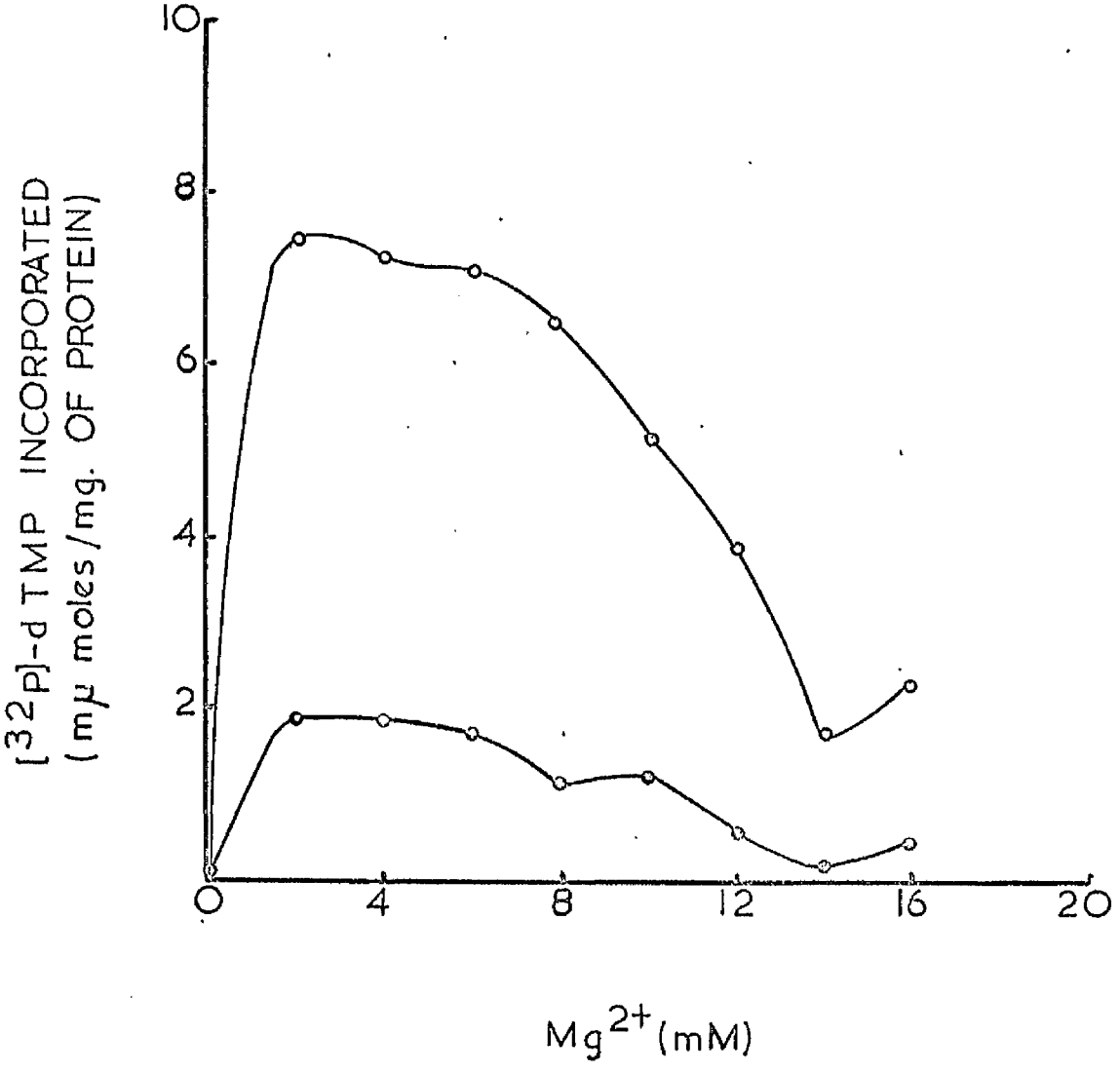

[32P]-d TMP INCORPORATED
(mμ moles/mg. OF PROTEIN)
10
8
6
4
2
0
4
8
12
16
20
Mg2+ (mM)

FIGURE 39

FIGURE 40

DNA nucleotidyltransferase activity of extracts of calf thymus tissue and Landschutz ascites-tumour cells as functions of Mg^{2+} ion concentration.

The fractions were obtained by identical extraction and purification procedures (see Results, section I), and the results are expressed as the ratio of thymus activity to Landschutz activity.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.08 umole of EDTA; the indicated amount of $MgSO_4$; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (8.8×10^6 counts/min./µmole) and 0.01 ml. (171 µg. of protein) of a calf thymus ammonium sulphate fraction or 0.02 ml. (142 µg. of protein) of a Landschutz ammonium sulphate fraction in a total volume of 0.25 ml. Incubation was carried out at 37° for 90 min.

Optimal enzymic activity at 4 mM $MgSO_4$ was:

1) calf thymus; 21.8 mµmoles of (32p)-dTMP incorporated/mg. of protein/90 min.

2) Landschutz; 11.5 mµmoles of (32p)-dTMP incorporated/mg. of protein/90 min.

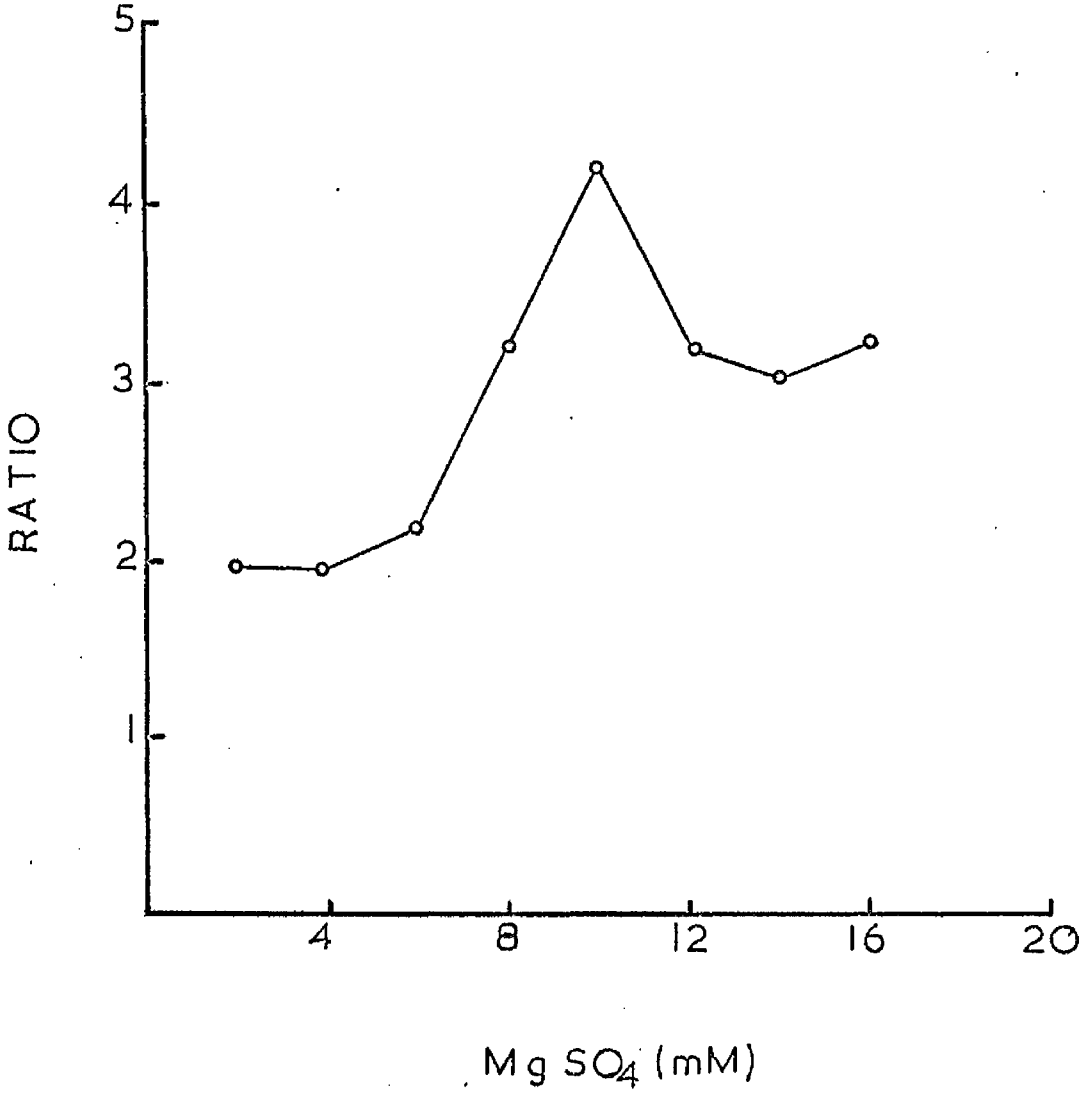


FIGURE 40

FIGURE 41

DNA nucleotidyltransferase activities of extracts of calf thymus tissue and Landschuts ascites-tumour cells as functions of K^+ and Na^+ ion concentrations.

The results are expressed as percent stimulation or percent inhibition of the enzymic activity in the absence of Na^+ and K^+ ions. The latter activity was 5.75 and 2.99 mμmoles of (^{32}P) -dTMP incorporated/ mg. of protein/45 min. respectively for the calf thymus and the Landschuts enzymes.

The reaction mixture contained: 5 μmoles of tris-HCl buffer, pH 7.5; the indicated amount of KCl or NaCl; 0.08 μmole of EDTA; 1 μmole of $MgCl_2$; 50 μg. of heat-denatured DNA; 50 mμmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (15.7 x 10^6 counts/min./μmole) and 0.01 ml. (114 μg. of protein) of a calf thymus 20-45% ammonium sulphate fraction or 0.02 ml. (94 μg. of protein) of a Landschuts 20-45% ammonium sulphate fraction in a total volume of 0.25 ml. Incubation was carried out at 37° for 45 min.

△, ○, calf thymus preparation

▲, ●, Landschuts preparation

– – – – – – K^+ ion concentration

——————— Na^+ ion concentration.

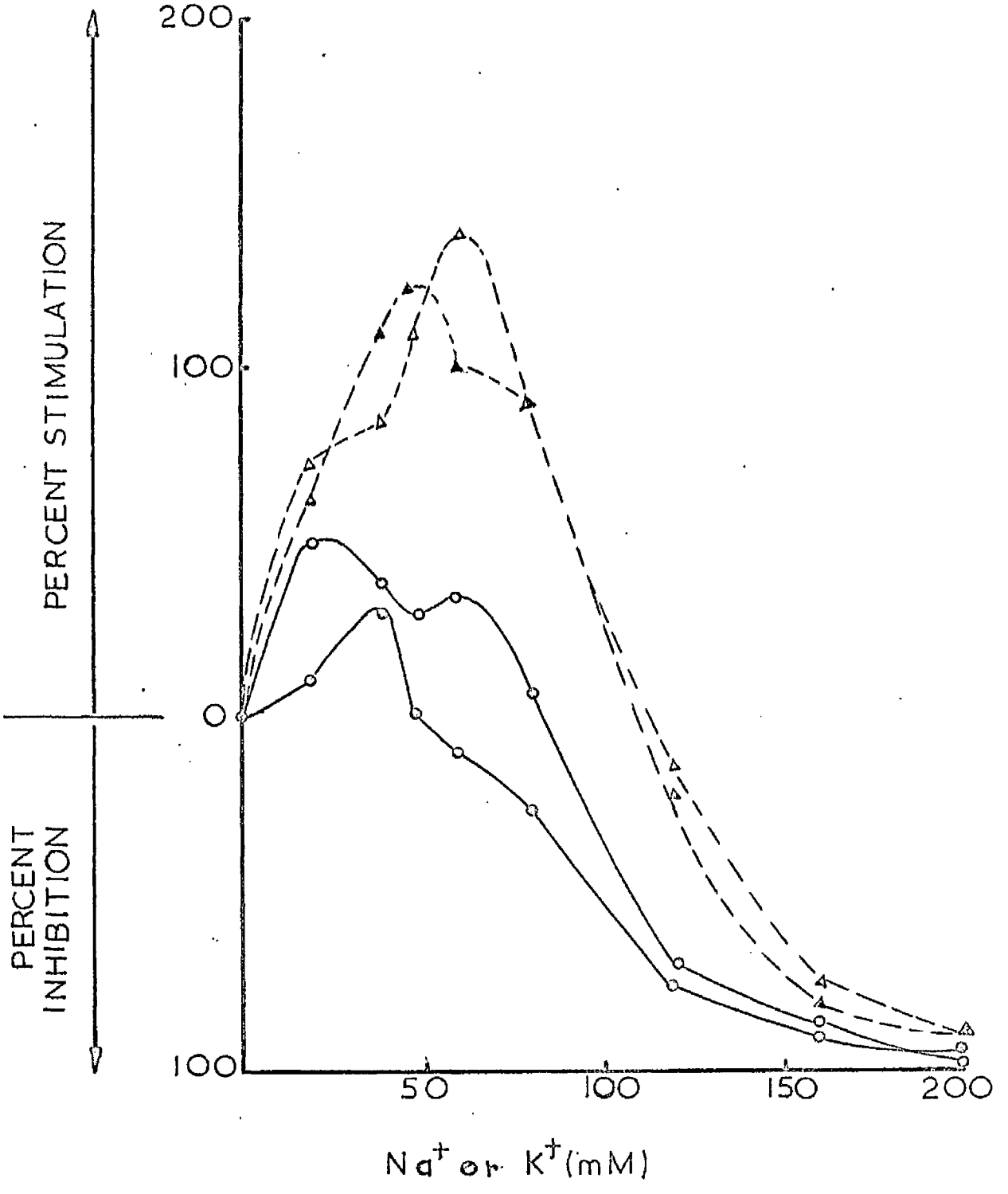

200
100
0
100
PERCENT STIMULATION
PERCENT INHIBITION
50
100
150
200
Na+ or K+ (mM)

FIGURE 41

of a similar fraction from Landschutz cells at various Mg^{2+} ion concentrations. Fig. 41 is the result of a similarly designed experiment investigating simultaneously the Na^+ and K^+ ion requirements of 20 - 45% ammonium sulphate fractions from calf thymus tissue and Landschutz cells. To permit a better correlation of the various activities the results are expressed as percent stimulation of the enzymic activity in the absence of Na^+ or K^+ ions.

These two sets of results together with those shown in Figs. 21 & 39 indicate that there can be variability of ionic requirements among enzyme fractions prepared at different times or from different sources, which may be due to differences in the macromolecular integrity of the DNA nucleotidyltransferase molecules in these preparations.

D) Kinetic Studies

Preliminary kinetic studies involving the effect of DNA concentration on the rate of the reaction for enzyme preparations from Landschutz cells were undertaken. Fig. 42 shows a typical double reciprocal plot ($1/v$ against $1/s$) plot over the range, 0 - 15 µg. of DNA phosphorus/assay for a pH 5 precipitate fraction. The sigmoid curve is representative of many results obtained with soluble extract pH 5 precipitate and 20 - 45% ammonium sulphate fractions. The reciprocal of the square of the substrate concentration was then plotted against the reciprocal of the velocity (i.e., $1/v$ against $1/s^2$) for the range 0.9 - 15 µg. of DNA phosphorus/assay (Fig. 43). When points at the higher substrate concentrations are presented as $1/v$ against $1/s^2$,

FIGURE 42

Double reciprocal plots (1/v against 1/s) for DNA nucleotidyltransferase activity of extracts of Landschutz ascites-tumour cells.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.08 µmole of EDTA; 1 µmole of $MgSO_4$; 1.25 µmole of 2-mercaptoethanol; 0-0.15 µg. of DNA phosphorus (heat-denatured) as indicated; 50 mµmoles each of dATP, dGTP, dCTP and (α-^{32}P)-dTTP (9.6×10^6 counts/min./µmole) and 0.05 ml. (149 µg. of protein) of a pH 5 precipitate fraction in a total volume of 0.25 ml. Incubation was carried out at 37° for 60 min.

v = enzyme velocity is expressed as mµmoles (^{32}P)-dTMP incorporated/mg. of protein/hr.

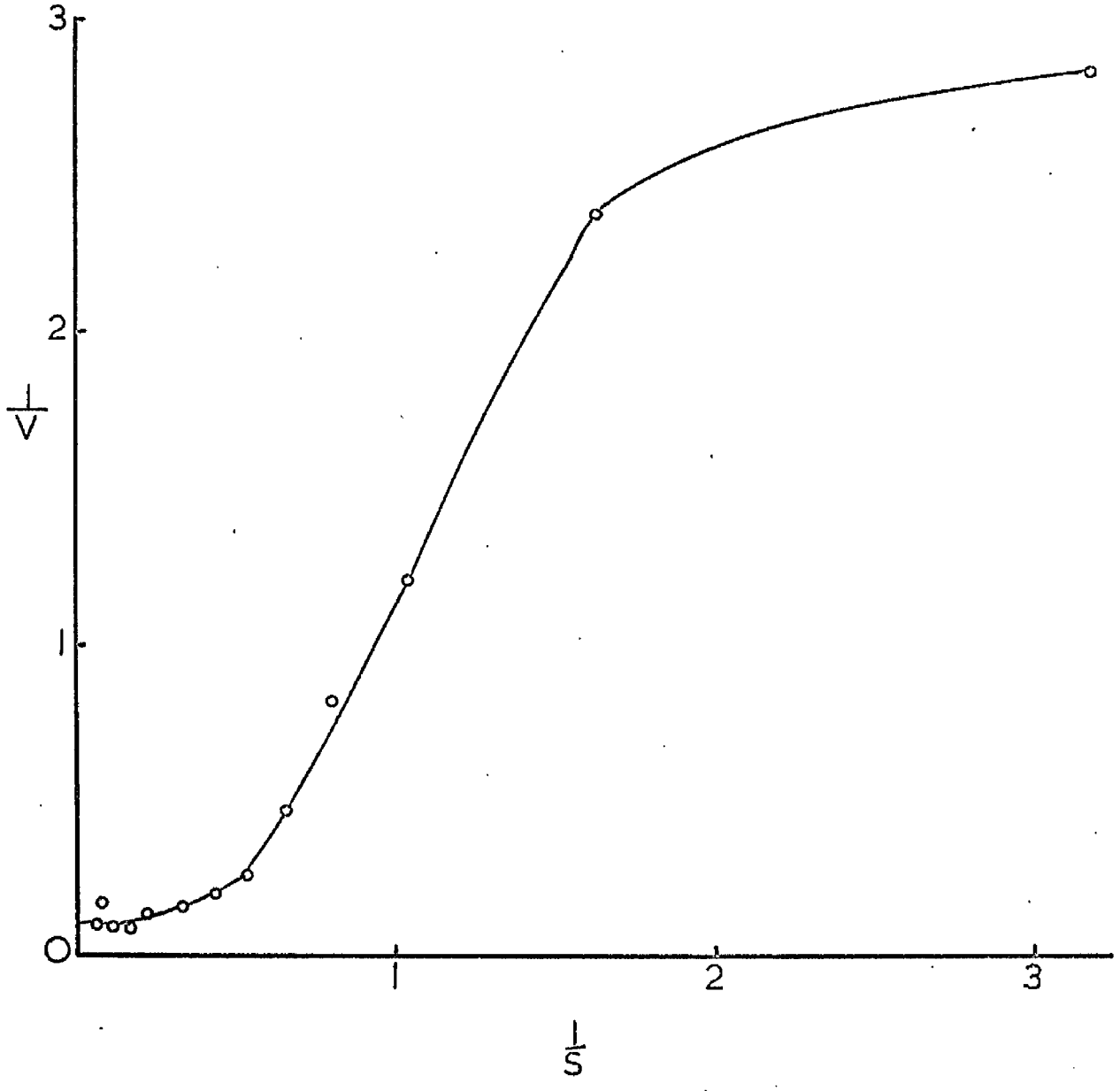


FIGURE 42

FIGURE 43

Double Reciprocal plots ($1/v$ against $1/s^2$) for DNA nucleotidyltransferase activity of extracts of Landschuts ascites-tumour cells.

This figure is composed of the information in Fig. 42 presented in another manner (see text).

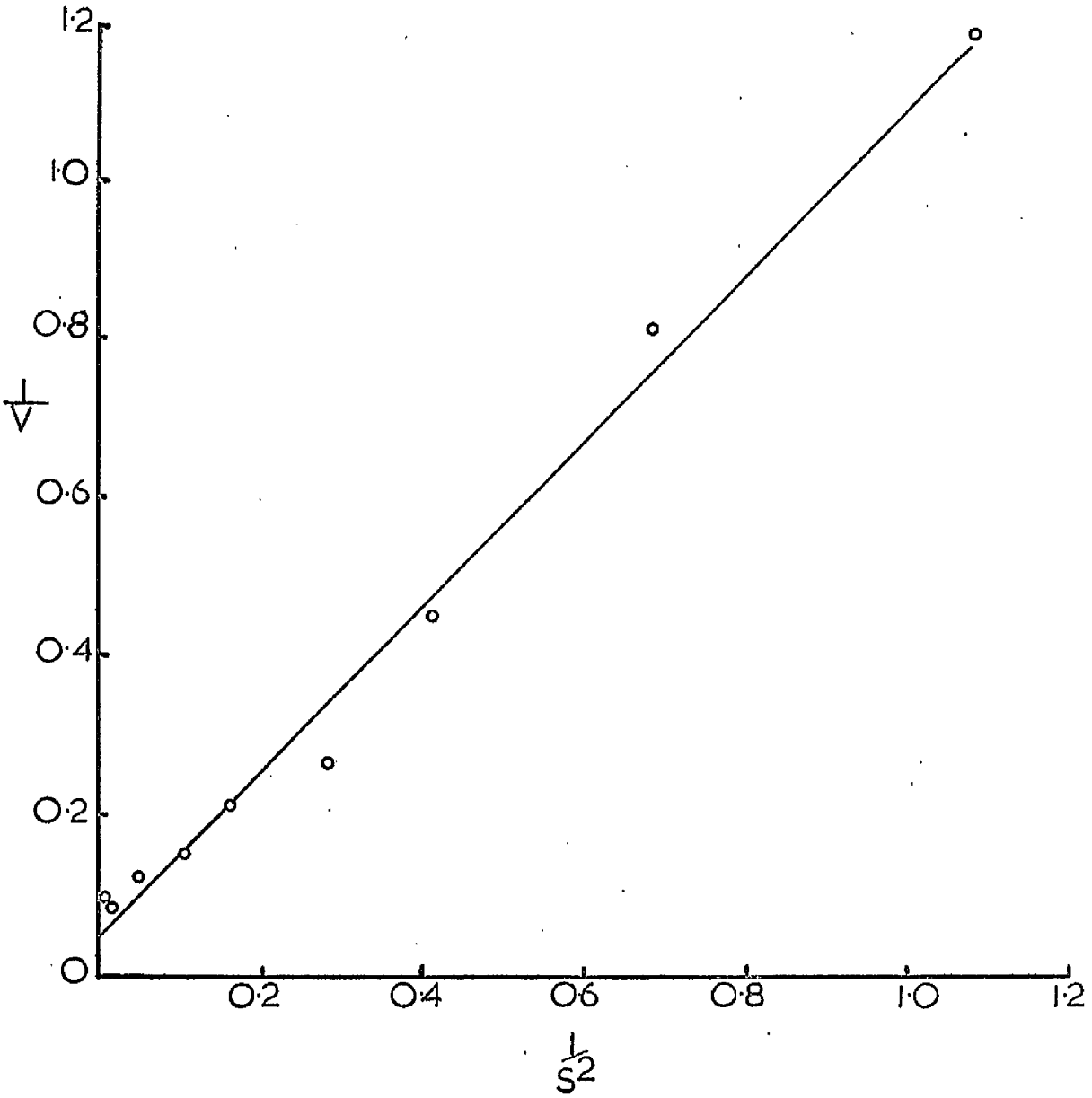


FIGURE 43

the plot is apparently linear but it deviates from linearity when the lower substrate concentrations are presented in a similar manner (see Figs. 42 & 43). This might possibly be explained by assuming that at higher DNA concentrations each molecule of enzyme binds two molecules of single-stranded DNA, but only one at low DNA concentrations (Webb, 1963; Frieden, 1964). In this context it is interesting that the rate of DNA nucleotidyltransferase activity would seem to be greater for a given quantity of DNA at the lower DNA concentrations. Also Fig. 37, which consists of 1/v against 1/s curves for control and Actinomycin D inhibited assays, shows non-linear relationships for the inhibited and non-inhibited enzyme. However, when 1/v is plotted against $1/s^2$ for the results in Fig. 37 a relationship which approaches linearity is obtained (Fig. 44). This kinetic evidence suggests that at higher concentrations of substrate the enzyme may bind simultaneously two molecules of DNA (Reiner, 1959; Frieden, 1964; Webb, 1963).

E) Enzyme Inactivation

It is apparent from the rapid loss of enzymic activity on storage at 0° in the absence of EDTA and 2-mercaptoethanol that DNA nucleotidyltransferase from Landschuts ascites-tumour cells is an enzyme which is very easily inactivated and consequently must be handled with great care. Before 2-mercaptoethanol and EDTA were routinely included in all enzyme fractionation and storage operations, the enzyme very rapidly lost activity even when the utmost caution was observed during manipulation. During this "pre-mercaptoethanol phase", many other enzyme

FIGURE 44

Double reciprocal plots ($1/v$ against $1/s^2$) for control and Actinomycin D inhibited DNA nucleotidyltransferase activities of Landschutz ascites-tumour cells.

This figure is composed of the information in Fig. 37 presented in another manner (see text).

—o— (no addition) control

—●— 2 µg. of Actinomycin D added

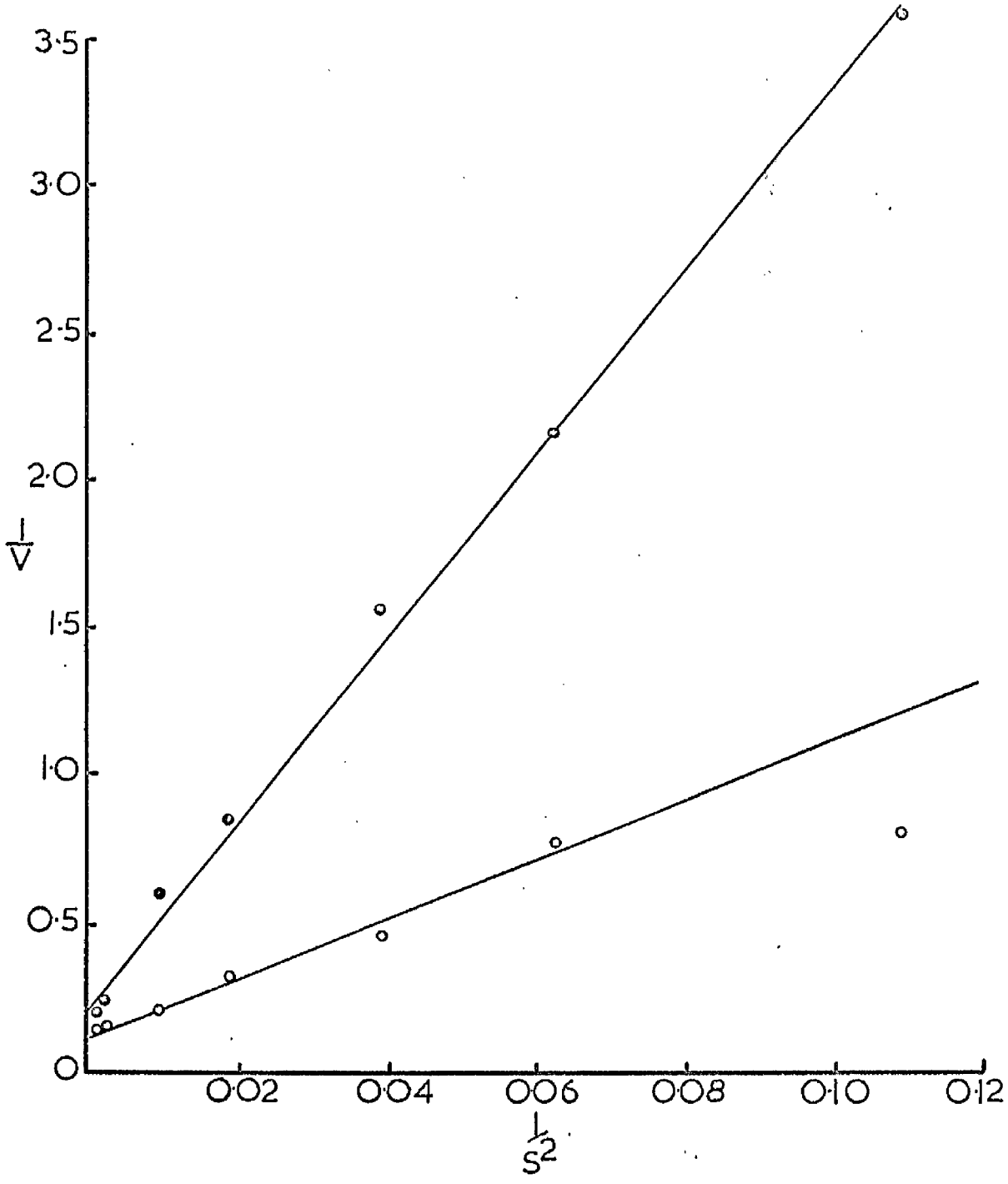


FIGURE 44

fractionation techniques were investigated with a view to purifying the enzyme, both from Landschuts cells and calf thymus tissue. These included acetone fractionation and column chromatography by ion-exchange (DEAE-cellulose, DEAE-Sephadex and CM-Sephadex) under conditions which allowed the adsorption of the enzyme on to the column. The protein recovered during these procedures was completely lacking in DNA nucleotidyltransferase activity. Indeed, gel filtration was the only technique which achieved any measure of success during this period. Whether these techniques will meet with greater success when they are employed in the presence of 2-mercaptoethanol remains to be seen, but their earlier failure only serves to emphasise the fragility of the DNA nucleotidyltransferase molecule from both sources. It is indeed significant that later and successful experiments involving chromatography on hydroxylapatite have incorporated 2-mercaptoethanol and EDTA.

During enzyme fractionation, dialysis was routinely employed as the method for the removal of unwanted ions which might inhibit DNA nucleotidyltransferase activity e.g., $(NH_4)^+$ ions after ammonium sulphate fractionation, or excess K^+ ions in the pH 5 precipitate or soluble extract fractions. Loss of activity was often observed after dialysis and an example of this is shown in Fig. 45 for both calf thymus and Landschutz enzymes. Thus prolonged periods of dialysis have been shown to inactivate DNA nucleotidyltransferase from both sources. Whenever possible dialysis was restricted to periods of 2 - 3 hr. Since there

FIGURE 45

The effect of dialysis on the DNA nucleotidyltransferase activity of extracts of calf thymus tissue and Landschutz ascites-tumour cells.

The reaction mixture contained: 5 µmoles of tris-HCl buffer, pH 7.5; 15 µmoles of KCl; 0.08 µmole of EDTA; 50 µg. of heat-denatured DNA; 50 mµmoles each of dATP, dCTP, dGTP and (α-^{32}P)-dTTP (2.4×10^6 counts/min./µmole) and 0.02 ml. of the indicated 20 - 45% ammonium sulphate fraction in a total volume of 0.25 ml. Incubation was carried out at 37° for 60 min.

Dialysis was performed on a 1 ml. portion of enzyme in a 7l. volume of 0.001M-potassium phosphate buffer, pH 7.5. The protein content of each fraction was assayed before and after each dialysis, and was in the range 140 - 180 µg./assay for all enzyme assays.

———●——— calf thymus preparation

———○——— Landschutz preparation

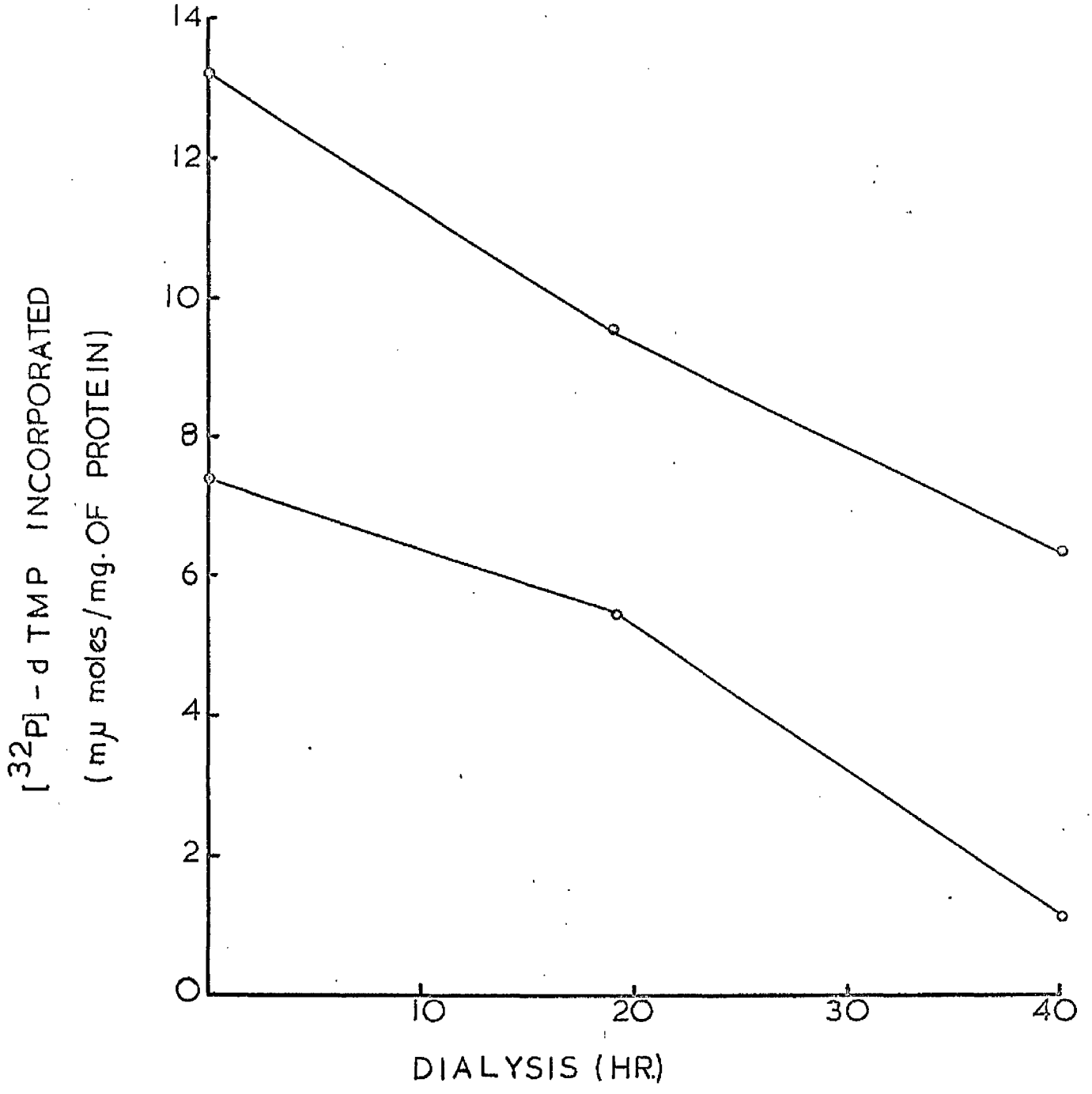

14
12
10
8
6
4
2
0
10
20
30
40
DIALYSIS (HR.)
[32P] - d TMP INCORPORATED
(mμ moles/mg. OF PROTEIN)

FIGURE 45

is little or no change in the environment of the enzyme during the experiment described in Fig. 45 it would seem fair to conclude that the inactivation observed is simply an acceleration of the decay process encountered during storage at 0° (see Fig. 25).

The results reported in this section serve to emphasise the extreme fragility of the enzymes which are engaged in DNA biosynthesis within mammalian cells.

DISCUSSION

DISCUSSION

I Studies on Mammalian DNA Nucleotidyltransferases

A Landschutz Ascites-Tumour Cell Enzyme Fractionation

1) Comparison with Previous Work on Landschutz DNA Nucleotidyltransferase

The results shown in Table I for the soluble extract and pH 5 precipitate fractions are essentially the same as those of Keir (1962) who used the same fractionation procedures. However, the purification is not as great as that previously obtained, because the inclusion of 2-mercaptoethanol and EDTA during extraction has almost doubled the activity of the soluble extract, which results in a fall in the overall purification (see Discussion, section I B 6 & 7).

The ammonium sulphate step also achieves a significant purification, but its greatest merit lies in the fact that the 20 - 45% fraction is much more readily soluble than the pH 5 precipitate fraction. Thus, it is possible to prepare concentrated solutions (up to 30 mg. of protein/ml.) of the 20 - 45% ammonium sulphate fraction, which are satisfactory for application of the enzyme to columns for further fractionation, when it is necessary to add as small a volume as possible. Deviation from linearity with respect to incubation time for the ammonium sulphate fraction (see Fig. 15) after 60 - 90 min. is unexpected, when compared to the linearity of the activities of the soluble extract and pH 5 precipitate fractions, as it seems unlikely that it will be caused by such factors as exhaustion of substrate or excessive nuclease activity.

It may be that the ammonium sulphate step in some way makes the enzyme more susceptible to inactivation so that on incubation at 37° for long periods it is more readily denatured than the other fractions (see Discussion, section I H).

The hydroxylapatite step is an important advance in the fractionation process and promises to make large amounts of nuclease-free DNA nucleotidyltransferase readily available, since column chromatography on a large scale under gravity flow (i.e., without artificially applied pressure) has been shown to be efficient for the separation of the two enzymes in E. coli DNA nucleotidyltransferase preparations. (H.V. Aposhian personal communication).

2) Evaluation of Nuclease Activity

2.75% of the DNA-primer is rendered acid-soluble during a 60 min. incubation period by an average DNA nucleotidyltransferase preparation, while DNA amounting to 7.4% of the primer is synthesised. For reasons given in the Results, section I B 3, it may be that even less of the primer-DNA is lost in the acid-soluble fraction in the DNA nucleotidyltransferase assay. Therefore, from the point of view of investigating at least some of the properties of the enzyme, the present system is fairly satisfactory. However, for definitive experiments investigating the role of primer and the nature of the product in the reaction it will be necessary to use DNA nucleotidyltransferase preparations free from nuclease contamination, for, although only a small fraction of the primer becomes acid-soluble, there are almost certainly many nuclease breaks in the remaining acid-precipitable primer and product. A good example of this

problem was encountered in the course of caesium chloride density gradient analyses of the product of the reaction (see Results, section III B). The information which could have been gained from the same experiment using a nuclease-free enzyme would have been of much greater value than that obtained in this work, and therefore, efforts to rid large amounts of the enzyme of nuclease contamination must be continued. This could clearly be achieved by hydroxylapatite chromatography (see Fig. 12), but additionally, it would be advisable to check that the DNA nucleotidyltransferase peak is completely free of nuclease, using an assay which is more sensitive than the spectrophotometric one employed in this work, e.g., retention of transforming activity of bacterial DNA or of infectivity of viral DNA would offer an excellent criterion of absence of nuclease (Nester, Schafer & Lederberg, 1963; Weil, 1961).

3) Evaluation of Phosphatase Activity

The deoxyribonucleoside triphosphatase activity of the various Landschutz enzyme preparations is negligible under the conditions of the DNA nucleotidyltransferase assay. This is perhaps not surprising, since, for cellular economy, it would be unsuitable to have within the cell enzymes which destroy optimally the precursors of DNA, under the conditions most favourable for DNA synthesis. However, these phosphatases may be chromosomally bound and not released into the DNA nucleotidyltransferase fractions used here (Siebert, 1963). Therefore, problems which would have arisen in this work, if there had been appreciable triphosphatase activity can be discounted. However, Gold & Helleiner (1964) using (^{32}P)-labelled

substrates have found that their L cell DNA nucleotidyltransferase preparations contain appreciable triphosphatase activity. Therefore, it is possible that different cell systems may give different degrees of contamination with triphosphatase.

4) Comparison with Other Mammalian Systems

DNA nucleotidyltransferases from three other mammalian tissues, calf thymus (Bollum, 1960), L cells (Gold & Helleiner, 1964) and Walker carcinoma (Furlong, 1965) have been purified in a manner very similar to that described here for the purification of the Landschutz enzyme.

With the calf thymus enzyme a purification of some 50-fold has been achieved in a preparation which has undergone acid precipitation, ammonium sulphate fractionation, DEAE-cellulose chromatography and refractionation with ammonium sulphate. The activity of this fraction was 38 mμmoles of (^{32}P)-deoxyribonucleoside 5'-monophosphate incorporated/mg. of protein/hr. The L cell system has been purified 15-fold in exactly the same manner except that the acid precipitation step was omitted, resulting in a fraction with an activity of the order of 23 mμmoles of (^{32}P)-deoxyribonucleoside 5'-monophosphate incorporated/mg. of protein/hr. The Walker carcinoma enzyme has been purified by a method closely similar to that described here to give a fraction with an activity of 6-7 mμmoles of (^{32}P)-deoxyribonucleoside-5'-monophosphate incorporated/mg. of protein/hr., resulting in a purification of approximately 20-fold. The higher purification figures, when compared to those obtained in this work for the Landschutz enzyme, can be accounted for by the fact that all three enzymes have a very low activity in the fraction which corresponds with the Landschutz soluble extract, the activities being respectively 0.7, 1.6 and

0.3 mμmoles of (^{32}P)-deoxyribonucleoside 5'-monophosphate incorporated/mg. of protein/hr., whereas with the Landschutz enzyme an activity of at least 4 - 5 mμmoles has been achieved routinely in the soluble extracts (see Discussion, section I B 6 & 7).

Therefore, it would seem either that the extraction conditions employed in this work were superior or that the enzyme from Landschutz cells is more soluble or more active than the others. In other respects the purification procedures for all four DNA nucleotidyltransferases and for the DNA nucleotidyltransferase from regenerating rat liver (Mantsavinos, 1964) are very similar, suggesting very probably that DNA nucleotidyltransferases from these sources are basically alike.

5) Comparison with Bacterial Systems

Investigation of DNA nucleotidyltransferases from bacterial sources has been pursued most extensively by Kornberg and his colleagues with the E. coli and B. subtilis systems (see Introduction, section V A 3).

Using techniques very similar to those used for the fractionation of several mammalian DNA nucleotidyltransferases (see section 4 above) a purification of DNA nucleotidyltransferase from E. coli of approximately 5,000-fold has been achieved. The B. subtilis enzyme, using the same procedure of soluble extraction, followed by phase separation in Dextran 500 and polyethylene glycol to remove nucleic acid, ammonium sulphate fractionation, DEAE-cellulose chromatography, phosphocellulose chromatography and finally hydroxylapatite chromatography, has been purified 400-fold. These bacterial enzymes are apparently much more stable

than their mammalian counterparts, and can therefore withstand more rigorous treatment. This appears to be a general rule, when comparing bacterial and mammalian enzymes, and in this respect DNA nucleotidyltransferase is no exception.

These two bacterial DNA nucleotidyltransferases appear to be very similar in their purification patterns and properties except for the fact that it has been impossible, so far, to obtain from E. coli an enzyme preparation, which is completely free of nuclease (Richardson, Schildkraut, Aposhian & Kornberg, 1964a), whereas this has been achieved in preparations of the B. subtilis enzyme (Okazaki & Kornberg, 1964). It has been shown that the nuclease activity (exonuclease II), which accompanies the E. coli DNA nucleotidyltransferase throughout the purification procedure, acts by sequentially releasing deoxyribonucleoside 5'-monophosphates from the 3'-hydroxy-termini of the primer molecules (Lehman & Richardson, 1964). Whether this final (hydroxylapatite) fraction of E. coli DNA nucleotidyltransferase consists of one protein with two enzyme activities or of two separable enzymes is a question which has not yet been settled. Certain observations, however, suggest that the two enzyme activities of the fraction may be due to the presence of two proteins. These include the facts that there are different pH optima for the two enzyme activities, 7.4 for DNA nucleotidyltransferase and 9.2 for exonuclease II, that while 3'-phosphoryl nucleotides are not attacked by the nuclease they do not inhibit it as they do the DNA nucleotidyltransferase, and that the preparation has not been crystallised.

Therefore, when comparing the purification of the Landschutz enzyme with those obtained for bacterial DNA nucleotidyltransferases one readily appreciates the greater advances, which have been made in the enzymology of the bacterial cell. Nevertheless, the similarity of the fractionation procedures suggests that mammalian and bacterial DNA nucleotidyltransferases probably have a similar structure. In fact, from the likeness of the respective hydroxylapatite chromatographic patterns it might be contended that the nuclease-DNA nucleotidyltransferase complement of Landschutz cells has much in common with those of E. coli and B. subtilis cells. However, such conclusions await a more comprehensive purification and characterisation of both the DNA nucleotidyltransferase and nuclease activities of the Landschutz ascites-tumour cell.

6) Location of DNA Nucleotidyltransferase Within the Cell

Current knowledge of the location of DNA nucleotidyltransferase within the mammalian cell has been reviewed (see Introduction, section V D). This problem was not extensively investigated during this work, but it seems appropriate to mention, at this point, impressions formed as a result of observations on nuclear extract preparations and on fractions isolated by a modification of the non-aqueous extraction procedure of Kay et al., (1956) (see Experimental, section II D). The information given in Fig. 38 shows that the soluble extract is approximately 6 times as active as the nuclear extract, which is typical of the distribution of DNA nucleotidyltransferase activity between the 105,000 g supernatant and sediment fractions. Also prior treatment of the cells with non-aqueous solvents did not yield

fractions with appreciably higher activity, as might have been expected if the non-aqueous treatment had removed lipid-soluble components from the nuclear membrane and other intracellular structures, thereby facilitating the release of more DNA nucleotidyltransferase from these various structures during the subsequent aqueous extraction procedure. These results serve to substantiate previous evidence from this laboratory, which suggests that the DNA nucleotidyltransferase of Landschutz cells may be predominantly located in the initially soluble material of the cell (Smellie et al., 1959; Smellie & Eason, 1960). However, a definitive study of the intracellular location of the enzyme in Landschutz cells has not yet been undertaken owing to the relative resistance of the cells to disruption by the traditional methods.

A further complication arises from the fact that a large proportion of the cells, from which enzyme fractions were prepared, may not have been actually engaged in DNA synthesis at the time of removal from the mice, for it is known that most of the cells present in the tumour fluid at the 8th and 9th days after inoculation are necrotic (Siegler & Koprowska, 1962). During this work a practice was made of collecting ascites-tumour fluid 5 - 7 days after inoculation, but even at these earlier times variations in the activity of the extracted DNA nucleotidyltransferase have been observed suggesting that a variable proportion of the cells contain active enzyme at the time of enzyme preparation. Thus, these observations do not contradict the more objective work of Keir et al., (1962), Smith & Keir (1963), Mazia & Hinegardner (1963), Main & Cole (1964), Littlefield et al., (1963) Keir & Gold (1963), and Gold & Helleiner (1964) with various mammalian

tissues, which has led to the conclusion that the enzyme may be bound in particulate form, within the nucleus during DNA synthesis and is distributed, in soluble form, throughout the cell at other times (see Introduction, section V D).

B Optimal Assay Conditions

1) Protein Concentration and Incubation Time

Fig. 16 shows that DNA nucleotidyltransferase activity was linear for protein concentrations up to 120 µg./assay. Deviation from linearity in the range 120 - 200 µg. was minimal; indeed, in several soluble extract and pH 5 precipitate fractions linearity up to 240 µg. of protein/assay has been observed. Loss of the linear relationship at high protein concentrations was possibly due to interference of a steric nature with the polymerisation reaction or chelation of the Mg^{2+} ion requirement by the other proteins present in the enzyme preparation. This explanation would seem more probable than the postulates that substrate exhaustion and product destruction are the responsible factors, since it has been shown that there is very little, if any, triphosphatase activity in the assay (see Results, section I B 4) and that the DNaseI activity of the enzyme preparation also falls off with increasing protein concentration (see Fig. 14).

Fig. 15 shows that the enzyme activity was linear over a 120 min. incubation period at 37° for soluble extract and pH 5 precipitate fractions. However, 20 - 45% ammonium sulphate fractions tended to lose activity after 60 - 90 min. of incubation at 37°. Beyond the 120 min. period loss of proportionality with incubation time was very variable and tended to

depend very largely on the individual enzyme preparation. On occasion linearity beyond 180 min. was observed. The most probable reason for loss of activity on prolonged incubation would seem to be heat inactivation at 37°. Fig. 38 shows that all enzyme fractions have lost more than 50% of their initial activity after prior incubation at 45° for 25 min. Prior incubation at 50° has been shown to abolish almost all enzyme activity within a few minutes, while prior incubation at 40° for 25 min. has been shown to inhibit a soluble extract fraction by more than 20% Therefore, heat-inactivation of the enzyme would seem to account for loss of activity on prolonged incubation more reasonably than exhaustion of substrate or destruction of product. However, where an actual fall of activity was observed on prolonged incubation (see Fig. 29) it seems probable that this was due to destruction of product by nuclease after heat-activation of DNA nucleotidyltransferase. The earlier inactivation of the 20 - 45% ammonium sulphate fraction suggests that it may be more labile than the soluble extract or pH 5 precipitate fractions (see Discussion, sections I A 1 & I H).

2) Primer Requirement

DNA nucleotidyltransferase has been shown to have an absolute requirement for primer DNA (see Fig. 19); 50 µg. of heat-denatured DNA appears to be sufficient to support maximal activity over an incubation period of 60 min. (see Figs. 19 & 20). Table 5 presents data which indicates that heat-denatured DNA primes the reaction to a greater extent than native DNA for soluble extract, pH 5 precipitate and 20-45% ammonium sulphate fraction

This presents a conceptual problem since, these results suggest that denatured DNA is a more suitable substrate than native DNA for DNA nucleotidyltransferase. However, substantial regions of single-stranded DNA have not been detected in the chromosomes of mammalian systems and one wonders how these two facts can be reconciled. This question will be discussed more fully in the Discussion, section I H. Highly polymerised RNA from two sources has been shown to be unable to prime the reaction (see Table 5). This fact agrees with the experience encountered in all other in vitro DNA synthesising systems.

3) Bivalent Cation Requirement

DNA nucleotidyltransferase has been shown to have an absolute requirement for a bivalent cation (see Fig. 21). Mg^{2+} ions are by far the most efficient for stimulation of enzymic activity. Mn^{2+} ions will support synthesis but only to about one fifth of the activity at optimal concentrations of Mg^{2+} ions. Ca^{2+} ions on the other hand fail entirely to support synthesis. The role played by Mg^{2+} ions in promoting the activity of DNA nucleotidyltransferase is probably an ionic binding effect by which it maintains the enzyme primer complex in the configuration required for efficient polymerisation of deoxyribonucleoside 5'-triphosphates (Lowenstein, 1958; Dixon & Webb, 1960).

Zn^{2+} ions have been found to inhibit DNA nucleotidyltransferase in the presence of both Mg^{2+} ions and EDTA. This fact is important because Lieberman & Ove (1962) have shown that there is a requirement for Zn^{2+} ions for the development of the ability by certain cells in tissue culture to enter DNA synthesis. The increase in DNA nucleotidyltransferase activity,

which appeared in cells engaged in DNA synthesis, was prevented by having EDTA in the culture medium throughout the experiment; the effect was abolished by Zn^{2+} ions (Lieberman et al., 1963). The nature of the Zn^{2+} ion requirement, which disappears after the initial phases of preparation for cell division have been completed, is unknown. However, it seems that in the Landschuts in vitro system a simple cofactor function of free Zn^{2+} ions in the reaction can be ruled out.

4) pH Optimum: Univalent Cation and EDTA Requirements

DNA nucleotidyltransferase exhibited a pH optimum in the region pH 7.2 - 7.4 (see Fig. 18), which represents the upper range of the pH environment of most mammalian cells. The intracellular pH of the Landschuts cell in vivo may, however, be slightly on the acid side as the metabolic processes of the cells within the peritoneum are almost totally anaerobic.

DNA nucleotidyltransferase activity did not show an absolute requirement for K^+ or Na^+ ions, but it was stimulated by both of them, the former being by far the more effective (see Fig. 22). The optimal concentration for stimulation by each ion was in the region of 50 - 60mM, K^+ and Na^+ ions, stimulating activity by 120% and 25% respectively. Therefore, it is clear that K^+ ions have a greater capacity than Na^+ ions to stimulate DNA nucleotidyltransferase. The biological significance of this finding, which agrees with that of Walwick & Main (1962), who used rat thymus DNA nucleotidyltransferase, will remain obscure until the nature, site of action and location within the cell of DNA

nucleotidyltransferase become more fully established, but it presumably is closely related to the size of the hydrated ion and its participation along with Mg^{2+} ions in catalysis (Lowenstein, 1960). Finally, it must be emphasised in this content that the enzyme was never assayed in conditions completely free from a univalent cation because of the presence of tris-HCl buffer in the assay system.

The EDTA requirement of DNA nucleotidyltransferase as shown in Fig. 23 was absolute. However, in many cases there was substantial activity in the absence of EDTA, and this was optimally stimulated by the addition of EDTA to the assay mixture at concentrations in the range 0.32 - 0.48mM. These observations together with those concerning the preparation and storage of the enzyme in the presence of 2-mercaptoethanol and EDTA (see Table 7) indicated that the EDTA effect in the assay was due to its chelation of inhibitory cations like Ca^{2+} and Zn^{2+} which might be present as impurities in chemicals or in the water supply used in the assay system. The enhancement of storage properties of enzyme fractions prepared in a medium containing EDTA would appear to be due to these same chelation properties which would prevent slow inactivation of the enzyme by trace amounts of inhibitory cations. In support of this theory of the mechanism of EDTA activation is the evidence of Williams (1959), which shows that cations such as Zn^{2+} and Ca^{2+}, which inhibit DNA nucleotidyltransferase, are complexed in preference to Mg^{2+} ions by EDTA. Therefore, it would appear that those preparations, which displayed an absolute requirement for EDTA, did so because they or their assay mixtures

contained abnormally high quantities of inhibitory bivalent cations.

5) Deoxyribonucleoside Triphosphate Requirement

The relationship between deoxyribonucleoside 5'-triphosphate concentration and enzyme activity is presented in Fig. 24, from which it would appear that 50 mμmoles each of dATP, dGTP, dCTP and dTTP/assay were sufficient for maximal DNA nucleotidyltransferase activity during a 60 min. incubation period, the other components of the reaction mixture being at optimal concentrations.

Table 6 indicates that the reaction required all four deoxyribonucleoside 5'-triphosphates for optimal activity. Figures quoted in the Results, section II A 7, for cases in which each of the four deoxyribonucleoside 5'-triphosphates was in turn replaced by the corresponding deoxyribonucleoside 5'-monophosphate substantiate the requirement for four deoxyribonucleoside 5'-triphosphates. When the four deoxyribonucleoside 5'-monophosphates or (α-^{32}P)-dTTP alone were present in the assay mixture no significant incorporation could be detected. However, figures for the four deoxyribonucleoside 5'-diphosphates, and especially those for (α-^{32}P)-dTTP plus the three complementary deoxyribonucleoside 5'-monophosphates and those for (α-^{32}P)-dTTP plus the three complementary 5'-diphosphates seem to be rather high for an enzyme, which is thought to require all four deoxyribonucleoside 5'-triphosphates for DNA synthesis. This can be most readily explained by the presence of an enzyme or enzymes in the 20 - 45% ammonium sulphate fractions, which catalyse transphosphorylation reactions. Thus, significant amounts of the four deoxyribon-

ucleoside 5'-triphosphates would be formed and DNA synthesis catalysed by DNA nucleotidyltransferase would follow. This theory is supported by two further observations from Table 6:-

i) Where (α-^{32}P)-dTTP was present, this unexpected incorporation was greatest. This might be expected, since transphosphorylation is more likely to take place when nucleoside triphosphates, which have a high energy content, are present (Strominger, Heppel & Maxwell, 1959; Heppel, Strominger & Maxwell, 1959).

ii) Where (α-^{32}P)-dTTP was present together with the three complementary deoxyribonucleoside 5'-diphosphates incorporation was higher than when it was present together with the three deoxyribonucleoside 5'-monophosphates. Since, in the first case a lesser degree of transphosphorylation would have to take place before a sufficient amount of the four deoxyribonucleoside 5'-triphosphates became available for DNA synthesis, this result might also be expected.

Therefore, it may be concluded from the data presently available that DNA nucleotidyltransferase from Landschutz ascites-tumour cells is specific in its requirement for the four complementary deoxyribonucleoside 5'-triphosphates and that the enzyme preparations used in this work appeared to be contaminated with an enzyme or enzymes, which were capable of catalysing transphosphorylation reactions among the four deoxyribonucleotides at all levels of phosphorylation.

6) <u>2-mercaptoethanol Requirement</u>

From observations made during this work it was apparent (see Fig. 34) that 2-mercaptoethanol was neither an absolute requirement nor a stimulant

for DNA nucleotidyltransferase activity. Nevertheless, it became clear that thiol groups played an important role in the maintenance of enzymic activity (see Figs. 32 & 33), and it was established that 2-mercaptoethanol greatly enhanced the activity and storage properties of fractions prepared in its presence (see Table 7). Therefore, 2-mercaptoethanol at 0.005M was included in the assay medium to help maintain the integrity of the enzyme molecules during incubation rather than to stimulate enzyme activity (see Discussion, section I H).

7) Comparison with Other Mammalian Systems

When the characteristics of DNA nucleotidyltransferase from Landschutz ascites-tumour cells are compared with DNA nucleotidyltransferases from other mammalian tissues with a view to detecting some significant difference between the DNA-synthesising machinery of the malignant cell and that of the normal cell, it is found, on the contrary, that the Landschutz enzyme resembles DNA nucleotidyltransferases from other mammalian sources in most respects.

All the mammalian DNA nucleotidyltransferase systems so far investigated have demonstrated an absolute requirement for primer-DNA. These include DNA nucleotidyltransferase from calf thymus (Bollum, 1960), rat thymus (Walwick & Main, 1962), regenerating rat liver (Bollum, 1958a; Mantsavinos, 1964), L cells (Gold & Helleiner, 1964) and Walker carcinoma (Furlong, 1965). In all of these systems except the partially purified preparation from regenerating rat liver (Mantsavinos, 1964), it has been shown that denatured DNA primes to a much greater extent than native DNA

(Bollum, 1959, 1960; Walwick & Main, 1962; Skidmore, Main & Cole, 1963; Gold & Helleiner, 1964; Furlong, 1965). However, other workers have found that the soluble extract from regenerating rat liver is primed more efficiently by denatured DNA (De Recondo et al., 1964). Bollum (1963a) states categorically that native, double-stranded DNA is inactive in priming the calf thymus enzyme, whereas in this work it has been shown that a similar enzyme fraction from calf thymus is primed one tenth as effectively by native DNA compared with denatured DNA (see Table 5). Most other DNA nucleotidyltransferases from mammalian sources have been found to be primed to a limited extent by native DNA. Bollum (1962) has shown that a 3'-hydroxy-terminal trinucleotide is the smallest molecule which can serve as primer for the calf thymus DNA nucleotidyltransferase. (The nature and role of the primer will be discussed in more detail in the Discussion, sections I D & I H).

A bivalent cation seems to be required for the activity of all the DNA nucleotidyltransferases mentioned above. As is generally the case, (see Discussion, section I H) for the Landschutz enzyme it would appear that all the DNA nucleotidyltransferases listed above display optimal activity in the region 4 - 8mM Mg^{2+} ions. Zn^{2+} ions have been shown to be essential for synthesis of DNA by some mammalian cells (Lieberman & Ove, 1962; Fujioka & Lieberman, 1964). The possibility that this may be due to a direct cofactor relationship with DNA nucleotidyltransferase has been considered (see Discussion, section I B 3).

The pH optimum of DNA nucleotidyltransferase from Landschutz cells

is very similar to those of the mammalian DNA nucleotidyltransferases so far characterised, failing to show a divergence from the general pattern, in spite of the unusual environment of the cells within the peritoneum of the mouse.

Stimulation of enzymic activity by a univalent cation has not been investigated for many DNA nucleotidyltransferases, but the results with respect to K^+ and Na^+ ions (see Fig. 22) for the Landschutz enzyme are very similar to those obtained by Walwick & Main (1962) for the rat thymus enzyme. It has also been shown in this work that the calf thymus enzyme is stimulated by K^+ and Na^+ ions in a manner closely similar to that observed for the Landschutz enzyme (see Results, sections II A 5 & VII C and Fig. 41). Bollum (1960) failed to detect stimulation of calf thymus DNA nucleotidyltransferase on the addition of several univalent cations but as pointed out by Walwick & Main (1962), during these experiments the reaction mixture contained potassium phosphate buffer, pH 7.0 at 40 mM (60 mM K^+ ions at pH 7.0), which would conceal true levels of stimulation and inhibition by the cations tested.

EDTA has not been routinely included in the assay systems used by other laboratories engaged in the investigation of DNA synthesis in mammalian tissues. As discussed earlier (see Discussion, section I B 4) EDTA is thought to stimulate by removal of inhibitory cations, and is not necessary for enzyme activity. Therefore, it may be that in the Landschutz system there is more contamination by such ions and that the high activity in the soluble extract fraction is due to the chelation of these cations

at an early stage, while they are not removed from the enzyme preparation until later stages (pH 5 precipitation or ammonium sulphate fractionation) in other fractionation procedures.

2-mercaptoethanol has been shown to enhance the activity of most mammalian DNA nucleotidyltransferases, and since results of this work (see Results, section VI A and Discussion, sections I B 6 & I G 1) indicate that DNA nucleotidyltransferase from Landschutz cells contains thiol groups, it would seem valid to conclude that all mammalian DNA nucleotidyltransferases probably contain thiol groups readily susceptible to oxidation.

All the DNA nucleotidyltransferases mentioned above show optimal activity in the presence of all four deoxyribonucleoside 5'-triphosphates; if any one of them is omitted from the reaction mixture incorporation is greatly reduced. Therefore, the DNA nucleotidyltransferase from Landschuts ascites-tumour cells, which shows similar deoxyribonucleoside 5'-triphosphate requirements can be classified along with the DNA nucleotidyltransferases of calf thymus tissue, regenerating rat liver, L cells and Walker carcinoma, as being capable of replicative DNA nucleotidyltransferase activity (see Discussion, section I C).

The nuclear DNA nucleotidyltransferase from an invertebrate system (sea urchin embryo - Mazia & Hinegardner, 1963) appears to be very like the Landschuts enzyme in almost all respects, but there are two important exceptions, for the former system appears to display a preference for Mn^{2+} ions rather than for Mg^{2+} ions, and for native DNA rather than for

heat-denatured DNA. Whether these are features of invertebrate systems or whether they are due to the fact that the enzyme activity was measured in whole nuclei is a question which must await further experimentation before an answer will be forthcoming.

8) Comparison with Bacterial Systems

The highly purified bacterial DNA nucleotidyltransferase preparations from E. coli and B. subtilis (Richardson et al., 1964a; Okazaki & Kornberg, 1964) appear to have properties very similar to those described for Landschutz and other mammalian cell DNA nucleotidyltransferases in the preceding sections.

The bacterial enzymes express an absolute requirement for a bivalent cation, Mg^{2+} ions being optimal at 4 - 8mM and Mn^{2+} ions at 0.8mM. Optimal pH ranges from 7.4 to 8.5 depending on the buffer employed. The evaluation of the optimal pH is obviously complicated by the possible stimulatory effects of univalent cations, which have not been systematically investigated for bacterial enzymes. DNA-primer is essential for the activity of the bacterial enzymes, (with the important exception of the unprimed synthesis of poly d(A-T) and poly (dG) : (dC)- see Introduction, section II A); native and heat-denatured DNA are almost equivalent in their priming capacities over short incubation periods. Stimulation of DNA nucleotidyltransferase activity by EDTA has not been investigated for bacterial enzyme preparations, but 2-mercaptoethanol has been routinely included in the DNA nucleotidyltransferase assays during these investigations.
For optimal activity all four of the complementary deoxyribonucleoside triphosphates are required, incorporation being greatly reduced

on the omission of one of them, or on the replacement of one of them by an analogue which does not conform to the hydrogen-bonding pattern of the original base.

The DNA nucleotidyltransferase newly synthesised within the bacterial cell on the infection of E. coli with bacteriophage T_2 has been shown in vitro to resemble the Landschutz enzyme inasmuch as it is primed ten times more effectively by denatured DNA than by native DNA and is more susceptible to p-chloromercuribenzoate inhibition (Aposhian & Kornberg, 1962). It has also been shown that DNA from vegetative T_2 bacteriophage must be heat-denatured to act as an effective primer of T_2 DNA nucleotidyltransferase (Masamune, Hori & Takagi, 1964).

These observations suggest that the Landschutz enzyme has much in common with the DNA nucleotidyltransferases from E. coli and B. subtilis with respect to structure and mode of action. At first it seems strange that there are not greater divergencies among the properties of enzymes whose origins are so disparate, but, on reflection, it might be expected that enzymes which perform the same fundamental, biological function, should have essentially similar properties regardless of their sources. The small differences, which do become apparent, may be attributable to the probability that bacterial enzymes are less delicate entities and withstand the various manipulations of isolation and purification (see Discussion, section I H).

C) Calf Thymus DNA Nucleotidyltransferase

As mentioned in the Results, section II B the investigation of calf

thymus DNA nucleotidyltransferase in this work was undertaken largely for comparative purposes. The information given in Tables 5, 8, & 9 indicate that the enzyme activity of the fractions used in this work corresponds to that described by Bollum (1960) for DNA nucleotidyltransferase from calf thymus. The figures in Table 9 for the cases in which one of the four deoxyribonucleoside 5'-triphosphates was replaced by the corresponding monophosphate in the assay mixture, suggest that in these cases there is partial replication of DNA. This might well be expected if there are stretches of the primer, which lack the complement of the base which is present as the 5'-monophosphate, or if the enzyme preparation retains the ability to catalyse transphosphorylation reactions (see Discussion, section I B 5).

One important result obtained from the ancillary work with the calf thymus enzyme was a partial separation of DNaseI and DNA nucleotidyltransferase by gel filtration on Sephadex G-200 (see Fig. 17). The fact that the DNA nucleotidyltransferase peak was retarded to the region 1.75 - 1.90 void volumes indicates that the molecular weight of the enzyme is certainly below 200,000 and possibly more than 100,000 (see Bollum, 1963a).

D Nature of the Product

Since it was established in this laboratory that soluble extracts prepared from Ehrlich ascites-tumour cells catalysed the random incorporation of thymidine, dCMP and dTMP into central regions of newly synthesised polydeoxyribonucleotide chains, and that net synthesis of DNA was achieved during this process, (Smellie et al., 1960) further

investigation of this problem with Landschuts cells has been concentrated on the purification of DNA nucleotidyltransferase to remove nuclease from the enzyme preparation. Consequently, the nature of the product has not been comprehensively investigated. Indeed, the worth of such experiments until an enzyme preparation completely free of nuclease has become available is a matter of some debate (see Discussion, section I A 2).

Nevertheless several points concerning the nature of the product of the activity of DNA nucleotidyltransferase from Landschuts ascites-tumour cells ought to be considered at this point.

Firstly the precipitability of the product in 5% (w/v) trichloroacetic acid and from 67% (v/v) ethanol, and the non-dialysable nature of the product, establish its macromolecular nature.

Secondly, the caesium chloride density gradient centrifugation of the product (see Fig. 27) shows that the peak of radioactivity occurs in molecules which band at the same density as native and denatured primer molecules. Since considerably more DNA (about twice the amount of ultraviolet-light absorbing material) was applied to the "product" gradient than to either of the "marker" gradients, it might have been expected that the ultraviolet-light absorbing peak of the product coincident with the radioactive peak, would have been greater than the native and denatured DNA "marker" peaks. Fig. 27 shows how essential it is, in product studies of this nature, to obtain nuclease-free preparations. Since only about 1.3% of the primer was rendered acid-soluble according to the standard DNaseI assay, one might have expected the degradation to have been almost

negligible, but, clearly, the product has been degraded sufficiently to produce a spectrum of pieces of DNA, such that the sedimentation band has a greater spread and there is a background distribution throughout the gradient. Nevertheless, taken into account with the data of Smellie et al., (1960) and the precipitable, non dialysable properties of the product, this ultracentrifugal evidence confirms that the product of Landsohuts DNA nucleotidyltransferase activity is high molecular weight newly-synthesised polydeoxyribonucleotide material.

The requirement for all four deoxyribonucleoside 5'-triphosphates for optimal activity, and the substantial activities in conditions favourable for the formation of the four deoxyribonucleoside 5'-triphosphates (see Table 6 and Duscussion, section I B 5), have implied throughout this work that the enzyme was capable of catalysing the synthesis of DNA according to the pattern of the primer or template DNA in a manner dictated by the Watson-Crick hydrogen-bonding rules (see Introduction, section V A 3). The results presented in Table 10 measuring the incorporation of (^{32}P)-dAMP, - dGMP, and - dTMP suggest that, assuming dCMP incorporation to be the same as that for dGMP, the pattern of nucleotide incorporation into product DNA is determined by the base composition of the primer. Although on some counts, this experiment may be open to question (see Results, section III C), from the results presented in Tables 6 & 10 and the evidence of Smellie et al.,(1960) it seems justifiable to conclude that the nature of the product DNA is dictated by the template or primer DNA. Previously the term "template"

has been avoided where possible in relation to the Landschutz enzyme, but, hereafter, it shall be contended that the various Landschutz DNA nucleotidyltransferase fractions employed in this work were capable of catalysing the complementary synthesis (or complementation - see Discussion, section I E 1) of a copy of the template DNA, and that they are identical with or derived from the enzyme, which, *in vivo*, catalyses the replication or duplication of cellular DNA during the S phase prior to cell division (see Discussion, section I H).

It would be pointless to draw any further conclusions from the nature of the product of Landschutz DNA nucleotidyltransferase action until further purification is achieved. However, in the DNA nucleotidyltransferase systems which have been most highly purified, those of *E. coli* in the bacterial sphere and of calf thymus in the mammalian sphere, great difficulty has been encountered in correlating the product of *in vitro* DNA synthesis with DNA as it occurs within the cell. Bollum (1963a, b) has shown that the calf thymus enzyme catalyses complementary synthesis on the single-stranded template to produce a double-stranded product, after which synthesis is terminated. On separation of primer from product molecules, which have been complemented to form double-stranded DNA, it has been found that the product of calf thymus DNA nucleotidyltransferase action will not undergo strand separation in a manner analogous to natural DNA (see Introduction, section II A). It was proposed that there are two factors *in vivo* which are lacking in the *in vitro* system. The function of the first is to convert the interphase chromosome to "priming" DNA (presumably

with some single-stranded regions), which serves as template for DNA nucleotidyltransferase during the S phase; the role of the second factor is to convert the product of DNA nucleotidyltransferase action from the initial, anomalous, enzymic product to natural DNA. Like the product of the calf thymus enzyme that of the DNA nucleotidyltransferase from E. coli has been shown to be much more difficult to denature than the natural E. coli double-stranded DNA used as primer in the system. Also, electron micrographs of the product DNA show that there is a 10 to 20-fold synthesis of primer and that the product consists of branched structures (Schildkraut, Richardson & Kornberg, 1964; Inman, Schildkraut & Kornberg, 1965). On the other hand the repair of a primer partially degraded by E. coli exonuclease III, which renders the DNA molecule single-stranded (Richardson, Lehman & Kornberg, 1964b), proceeds more rapidly than the reaction primed by native DNA, and the initial product is covalently linked to the primer and resembles natural DNA, even retaining genetic activity (Richardson, Inman & Kornberg, 1964c). The fact that the calf thymus enzyme (Bollum, 1960) is primed only by denatured DNA is consistent with the termination of synthesis after one cycle of replication since the double-stranded product would not be expected to prime the reaction. However, the E. coli system might be expected to perform multiple synthesis as the enzyme is primed by native DNA. Therefore, the unusual properties of both products suggest that there may well be some factor within the cell, absent from the in vitro system, which can convert the DNA nucleotidyltransferase product into natural DNA.

However, the evidence with the partially degraded primer in the E. coli system indicates that the anomaly in the product may be due to complications in the structure and orientation of the template. Nevertheless, this does not apply to the mammalian system and these anomalies in the product DNA may well be the consequence of the disruption of the natural environment in which DNA replication takes place. Therefore, these anomalies may possibly be attributed to modification of the DNA nucleotidyltransferase molecule during extraction or to the loss or destruction of factors which control the degree and manner of replication (see, Discussion section I H)

E Quantitative Assessment of Synthesis

1) Relationship Between Synthesis and Degradation

From the Results section I B 3 it has been shown that DNA equivalent to 7.4% of the primer can be synthesised by preparations of Landschutz DNA nucleotidyltransferase used in this work, and during the same 60 min. incubation period a maximum of 2.75% of the primer DNA is rendered acid-soluble. Since it has been demonstrated that (^{32}P)-incorporation measures the synthesis of long chain polydeoxyribonucleotide material, the fate of the components of the assay mixture can be described in the following manner. The primer DNA is hydrolysed by endonucleolytic activity producing large acid-precipitable polynucleotides and also smaller acid-soluble oligo-and mononucleotides. (Exonucleolytic activity may contribute to this process). The DNA nucleotidyltransferase molecules in the enzyme preparation appear to catalyse the synthesis of the polynucleotide chain

complementary to the single-stranded primer molecules and to the polynucleotide products of endonucleolytic action. Since the heat-denatured template of the *in vitro* system contains both complementary chains of the original DNA molecule, during complementation by DNA nucleotidyltransferase DNA with base ratios dictated by the primer is synthesised.

At this point it seems appropriate to define more fully the terms *complementation* and *replication* in the context of DNA synthesis. *Complementation* is the synthesis of the DNA strand complementary to a single-stranded template to give a double-helical product. *Replication* is the synthesis of an exact copy of a double-stranded template to give two identical double helices. Consequently *replication* resembles much more closely than complementation the mechanism of DNA duplication *in vivo*. Also, it is apparent that *in vitro* DNA synthesising systems which utilise only denatured DNA as primer are capable of *complementation* but not of *replication* of template DNA, whereas a system like that from *E. coli*, which is primed by both native and denatured DNA is superficially capable of performing both functions. In the long term, of course, *complementation in vitro* is equivalent to replication because heat-denaturation followed by complementary synthesis of both single-strands results in the formation of two identical double helices (see Discussion, sections I H & II A).

Stimulation of enzymic activity by the use of an *in vitro* template partially treated with DNase is well documented for both mammalian and

bacterial systems (Keir, 1962; Richardson et al., 1963a; Richardson et al., 1963b; Richardson et al., 1964a; Okazaki & Kornberg, 1964) This stimulation appears to be due to the production of 3'-hydroxy-terminal groups resulting from the nucleolytic activity (Bollum, 1962). The products of DNaseII action (which bear 3'-phosphoryl terminal groups), inhibit DNA nucleotidyltransferase from both mammalian and bacterial sources. Therefore, it seems that the stimulation of DNA nucleotidyl-transferase activity by DNase-treated primers results from the provision of more 3'-hydroxy-termini in the primer, which in turn act as new sites for the initiation of DNA synthesis (see Fig. 8). Nuclease may have a function to play in the initiation or control of DNA synthesis, but it seems more likely that this phenomenon is simply an artifact of the *in vitro* system. However, the persistence of nuclease activity in the most highly purified fraction of *E. coli* DNA nucleotidyltransferase (Richardson et al., 1964a) and the proposed conversion factors of Bollum (1963a, b) for the production of "primer" DNA and "natural" product DNA, influence one to keep an open mind on this question.

2) In Vivo and In Vitro Rates of Synthesis

From the Results, section IV and Table 11 it appears that the rate of DNA synthesis in the Landschutz *in vitro* system is commensurate with the *in vivo* DNA synthetic rate of ascites-tumour cells. However, this calculation is subject to certain restrictions. Although these restrictions preclude an accurate evaluation of the respective rates of DNA synthesis, they do not invalidate the general conclusion that, subject to the various

situations which might arise within the cell during replication, the DNA-synthesising capacity of the *in vitro* system is sufficient for it to perform the replication of the DNA complement of ascites-tumour cells *in vivo*.

The first restriction is that the *in vitro* system measures DNA nucleotidyltransferase from all cells, whether they are dead or alive, whether they are in the S phase or otherwise. Therefore, for accurate comparison of the *in vitro* and *in vivo* rates of DNA synthesis an estimation of the proportion of the enzyme actively engaged in DNA synthesis would have to be determined. This leads on to the second possible restriction, which concerns the problem of location of the DNA nucleotidyltransferase within the mammalian cell and the possible migration of enzyme from nucleus to cytoplasm and *vice versa* during the cell cycle (see Introduction, section V D and Discussion, sections I A 6 & II B). The great doubt which enshrouds this problem makes it impossible to calculate figures to compensate for errors due to variable location and activity of the enzyme. Finally, the probability that, *in vivo*, the true template of DNA nucleotidyltransferase is native DNA (see Discussion, section I H) further complicates the matter of comparison of the DNA synthetic rates.

Nevertheless the value of this type of calculation lies not in its accuracy but rather in its general implication that the enzyme fractions studied in this work have the quantitative capacity to perform their function *in vivo*.

F Terminal DNA Nucleotidyltransferase

1) Properties of the Terminal Addition Reaction

From the data presented in Figs. 28, 29 & 30 for calf thymus preparations, there is little doubt that the incorporation measured under the conditions of terminal assay was that of addition of (^{32}P)-dTMP to the ends of primer molecules corresponding to the action of the terminal DNA nucleotidyltransferase, which has been separated from the replicative DNA nucleotidyltransferase in calf thymus tissue by Krakow et al., (1962) and by Keir & Smith (1963), (see Introduction, section V C).

The time course of calf thymus terminal DNA nucleotidyltransferase (see Figs. 28 & 29) indicates that maximum activity is attained after a 60 min. incubation period. This implies that the addition of (^{32}P)-dTMP to the ends of primer molecules is limited to a certain level. This may be due to lower affinity of terminal DNA nucleotidyltransferase for long stretches of poly-dTMP than for DNA because, if this is the case, as the terminal homopolymer of dTMP lengthens, a point will be reached when the enzyme must bind to a portion of polynucleotide composed largely or perhaps entirely of dTMP residues, and under such circumstances the enzyme-substrate complex may no longer associate in a satisfactory manner and synthesis will be terminated.

In experiments with (^{32}P)-dATP,-dGTP and-dTTP under terminal DNA nucleotidyltransferase assay conditions it was shown (see Table 12) that there is a slight preference, but certainly not a complete specificity, for the incorporation of dAMP by a highly purified fraction of calf thymus

DNA nucleotidyltransferase (see Results, section V B). This appears to contradict the results of Bollum et al., (1964), who found, in calf thymus extracts, a terminal DNA nucleotidyltransferase, which was specific for dATP. However, these workers used calf thymus fractions, which had been further purified either by hydroxylapatite chromatography or by gel filtration on Sephadex G-100, and they may have separated by these means a factor responsible for the specific terminal addition of dAMP residues.

Fig. 31 shows that there is very little terminal addition activity in extracts from Landschuts ascites-tumour cells. Since no evidence has been obtained that this terminal addition activity was physically separable or distinct from the replicative DNA nucleotidyltransferase, the alternative remained that the reaction was catalysed by replicative DNA nucleotidyltransferase. In deference to this possibility this activity in Landschuts preparations will be termed the terminal addition reaction.

It has been shown that Actinomycin D inhibits the action of replicative DNA nucleotidyltransferase to a much greater extent than it inhibits terminal DNA nucleotidyltransferase activity in calf thymus tissue (see Results, section V B). Similar results are presented in Fig. 31 for the terminal and replicative activities of Landschuts enzyme preparations. It might be expected that terminal addition would would be less affected by the presence of Actinomycin D, since interruptions along the strands of the DNA-primer would not interfere with terminal addition. As this appears to be the case it must be concluded that the Actinomycin D-primer complex (see Results, section VI B & Discussion

section I G 2) does not prevent the binding of the terminal DNA nucleotidyltransferase to the extremities of the primer molecules.

Table 15 describes the inhibition of the terminal addition reaction in DNA nucleotidyltransferase preparations from Landschuts ascites-tumour cells by the thiol group inhibitors, iodoacetamide and p-hydroxymercuribenzoate. The terminal addition reaction is much less sensitive to these inhibitors than the replicative reaction (see Table 14). It would seem from these observations that certain susceptible groups which are essential for normal functioning of replicative DNA nucleotidyltransferase, may not be so intimately involved in the enzyme catalysis during the terminal addition reaction. It must be emphasised that this interpretation assumes that both types of reaction are catalysed by the same enzyme.

At earlier stages in this work variation in the response of replicative DNA nucleotidyltransferase preparations to concentrations of Mg^{2+} and K^{+} ions (see Results, section VII C) suggested that the terminal enzyme might be responsible for irregularities of activity at 10mM Mg^{2+} ions and 40 mM K^{+} ions. Thus terminal DNA nucleotidyltransferase activity was for a period measured at these concentrations. However, subsequent observations have led to a different interpretation of these findings (see Discussion, section I H), and latterly terminal DNA nucleotidyltransferase was measured under conditions standard for the replicative enzyme except that (α-^{32}P)-dTTP was the only deoxyribonucleoside 5'-triphosphate present in the assay mixture.

2) Distribution of the Terminal Enzyme

The results presented in Table 13 show that rabbit appendix and thymus are more efficient with respect to the terminal incorporation reaction than rabbit liver or spleen. The enzyme preparation from Landschutz ascites-tumour cells had a particularly low level of terminal addition activity, while those from calf thymus, non-aquous nuclei and calf thymus non-aqueous cytoplasm had by far the highest levels relative to their replicative DNA nucleotidyltransferase activities. Rat spleen had greater terminal addition activity than rat liver or rabbit spleen, but it had also greater replicative DNA nucleotidyltransferase activity than had rabbit spleen, in proportion to its excess of terminal activity. Further experimentation is of course required to determine whether the terminal addition activity of these tissues can be ascribed to a separable terminal DNA nucleotidyltransferase.

3) Possible Role of the Terminal Enzyme

After the work of Krakow et al., (1962) and Keir & Smith (1963) on the terminal DNA nucleotidyltransferase from calf thymus, the immediate reaction was to assign a function to the new enzyme. This was one of the reasons for undertaking the survey of the DNA nucleotidyltransferases of several mammalian tissues (see Tables 4 & 13). The results failed to demonstrate terminal DNA nucleotidyltransferase activity of the order of that observed in calf thymus in any of these normal tissues and in the malignant tissue, the Landschutz ascites-tumour cell.

One possible conclusion is that the existence of terminal DNA

nucleotidyltransferase is peculiar to thymus tissue, (among other figures in Table 13 rabbit thymus has the highest terminal DNA nucleotidyltransferase activity). The modern view of the role of the thymus in immunity suggests that thymus tissue might reasonably be expected to play a part in the synthesis of antibody by providing immunologically-competent cells (Miller, 1962, 1963a, b). Therefore, terminal DNA nucleotidyltransferase from thymus may be involved in the synthesis of modified DNA molecules, which have a specialised role in the direction of antibody synthesis, and it would be interesting to investigate the levels of terminal addition activity in such tissues as spleen, lymph node and appendix after antigenic stimulation of, say, rabbits.

Another possibility is that the terminal enzyme may represent detachment of some enzymically active protein from the parent replicative DNA nucleotidyltransferase during synthesis or extraction of the latter (see Discussion, section I H).

G) Enzyme Inhibition

1) Thiol Group Inhibitors

a) Inhibition

The results presented in Figs. 32 & 33 and in Table 14 indicate that all three of the traditional thiol group inhibitors investigated, (iodoacetamide, iodoacetate and p-hydroxymercuribenzoate) and also sarkomycin, are capable of inhibiting the activity of DNA nucleotidyltransferase from Landschuts ascites-tumour cells. These experiments were carried out on enzyme preparations lacking 2-mercaptoethanol, but

if this compound was present much higher concentrations of inhibitors were required to effect the same inhibition.

The modes of action of iodoacetamide, iodoacetate and p-hydroxymercuribenzoate as inhibitors of thiol group enzymes are well-documented (Boyer, 1959). Sarkomycin is produced by Streptomyces erythrochromogenes and displays antitumour and weak antibacterial activity (Umezawa et al., 1954). Sung & Quastel (1963) have described its inhibitory action on the incorporation of (^{3}H)-thymidine into DNA by cell free extracts of Ehrlich ascites-tumour cells.

The actions of iodoacetamide, iodoacetate, p-hydroxymercuribenzoate and sarkomycin in this experimental system do not reveal the precise mechanism of inhibition, but it seems reasonable to conclude that thiol group(s) in the DNA nucleotidyltransferase molecule are at least the primary site(s) of action of these inhibitors. Iodoacetamide, iodoacetate and sarkomycin probably act in this system by alkylation of the thiol group(s) in the enzyme, while p-hydroxymercuribenzoate probably inhibits by mercaptide formation with the same group(s). The failure of 2-mercaptoethanol and GSH to reverse inhibition by iodoacetate and iodoacetamide, while they are able to reverse inhibition by p-hydroxy-mercuribenzoate are observations to be expected from knowledge of their alkylating or mercaptide forming potentials (Boyer, 1959). With sarkomycin it is probable that electron displacement induced by the 3-oxo-group gives rise to a positive tendency on the 2-methylene carbon, thus promoting reactivity towards thiol groups.

The double reciprocal plots shown in Fig. 35 indicate that these compounds inhibit in a non-competitive fashion as would be expected from the mechanism by which they exert their inhibition.

The inhibition by these compounds of the terminal addition reaction in Landschutz cells (see Table 15) has been discussed (see Discussion, section I F 1). These inhibitors may have important uses in the elucidation of the structure and mechanism of action of more purified preparations of DNA nucleotidyltransferase.

b) Protection

The effects of 2-mercaptoethanol and GSH on DNA nucleotidyltransferase activity of extracts of Landschutz ascites-tumour cells are shown in Fig. 34. Very high concentrations of either compound are required to produce apparent inhibition. GSH has a greater tendency to inhibit and this is due probably to chelation of the Mg^{2+} ions in the assay mixture. Similar effects were observed in the same DNA nucleotidyltransferase assay system when cysteine was included in the reaction medium (Keir & Smith, 1963).

The ability of these compounds to protect against the inhibition of thiol group inhibitors (see section a above) is an example of a property which has been well known for many years. The reason for the inclusion of 2-mercaptoethanol and EDTA in all buffers employed in preparation and storage of DNA nucleotidyltransferase fractions is discussed in sections I B 4, 6 & 7 of the Discussion.

2) Action of Actinomycin D

The evidence presented in Figs. 31, 36 & 37 demonstrates that DNA

nucleotidyltransferase from Landschutz ascites-tumour cells is strongly inhibited by Actinomycin D. Double reciprocal plots of reaction velocity against substrate concentration (see Fig. 37) indicate that the inhibition obeys competitive kinetics. Actinomycin D is known to inhibit DNA-primed RNA synthesis because of its ability to bind guanine residues in the primer (see Results, section VI B). In the experiment described in Fig. 36, 50 µg. of DNA-primer was used, i.e., 153 mµmoles of total deoxyribonucleotide or 32 mµmoles of guanine deoxyribonucleotide as indicated by base analysis (see Results, section III C). Since the lowest amount of Actinomycin D giving 100% inhibition was about 45 µg. (approximately 36 mµmoles), it would appear that complete inhibition was effected when the molar ratio of Actinomycin D to guanine in the primer approaches unity. These results suggest that Actinomycin D inhibition of DNA nucleotidyltransferase from Landschutz ascites-tumour cells occurs by the binding of the antibiotic to guanine residues on the primer. Similar observations have been reported for DNA nucleotidyltransferase from E. coli (Kirk, 1960; Hurwitz, Furth, Malamy & Alexander, 1962; Elliot, 1963). Therefore, it would seem that in this respect at least the bacterial and mammalian DNA nucleotidyltransferases are similar in their requirements for formation of enzyme-substrate complex and for template replication.

Other evidence (see Discussion, section I F 1 and Fig. 31) indicates that the terminal addition reaction is much less sensitive than the replicative reaction to inhibition by Actinomycin D. This suggests that

Actinomycin D may well be a useful tool for distinguishing the two forms of the enzyme. Finally, the binding of Actinomycin D to the primer-DNA may modify conditions within the assay mixture so that possible features of the structure of the enzyme can be inferred from studies of Actinomycin D inhibition (see Results, section VII D and Discussion section I H).

H) Some Thoughts on the Conformation of DNA Nucleotidyltransferase

The data presented in section VII of the Results were obtained in order to acquire information on the possible conformation of DNA nucleotidyltransferase from Landschutz cells and other mammalian tissues. As mentioned in the introductory remarks to section VII of the Results, a conceptual image has been formed during this work that DNA nucleotidyltransferase *in vivo* might be a highly organised, well defined structure which, *in vitro*, may be modified to variable extents by such factors as extraction and purification methods. In this section it is proposed to describe the possible nature of DNA nucleotidyltransferase molecules which, within the cell, are capable of carrying out complete replication of the chromosome in preparation for cell division, and to compare this with the "structurally-altered" DNA nucleotidyltransferase molecules which possibly exist in all *in vitro* systems. Having established the concept of variation between the DNA nucleotidyltransferase molecules of *in vivo* systems and those of *in vitro* systems the results of experiments designed to substantiate this theory will be considered (see Results, section VII).

The first observation relevant to this concept was that DNA nucleotidyltransferase preparations from Landschutz cells and calf thymus tissue were very readily inactivated by the mildest of fractionation

procedures (see Results, section VII E). The discovery that the enzyme from Landschutz cells contained thiol groups which appeared to be active in preserving the integrity of the molecule suggested that these groups may well play a part in determining the tertiary structure of the enzyme. The variable responses (see Fig. 39) of different enzyme preparations from calf thymus tissue and Landschutz cells to ionic conditions of the in vitro assay lent support to the idea that the enzyme fractions in the in vitro systems were composed of molecules differing considerably from each other and differing from the enzyme as it occurs in vivo. The existence in calf thymus gland of a terminal DNA nucleotidyltransferase, which catalyses the addition of deoxyribonucleoside 5'-triphosphates to the ends of polydeoxyribonucleotides, at first implied that this may be an enzyme with a specialised function, which was found only in that tissue. However, detection of similar, though proportionally much less, activity in other tissues later suggested that this might be simply a breakdown product of the replicative DNA nucleotidyltransferase. Inhibition, by thiol group inhibitors, of the relatively small fraction of terminal addition activity found in Landschutz ascites-tumour cells confirmed that this activity was enzymic, and was consistent with the concept that the terminal addition reaction might be due to a breakdown product of the replicative DNA nucleotidyltransferase molecule (N.B. this reasoning assumes that the two types of enzymic activity are due to two separable proteins - see Discussion, section I F 1).

Thus, the idea gradually formed that DNA nucleotidyltransferase

extracts from calf thymus and Landschutz cells used in this work contained a variable proportion of collapsed enzyme molecules which retained residual activity. The bulk of the evidence from mammalian DNA nucleotidyltransferase *in vitro* systems indicates that they are primed to a much greater degree by denatured DNA than by native DNA (see Discussion, sections I B 2 & I D). However, this seems to be an anomaly since, so far it has been found impossible to demonstrate the existence of substantial areas of single-stranded DNA along the chromosome *in vivo*. Therefore, it seems probable that DNA nucleotidyltransferase *in vivo* must replicate native DNA (see Discussion, section II A). The appropriate conclusion to be drawn from these observations is that the DNA nucleotidyltransferase molecules in *in vitro* preparations must be in some way modified so that they have lost the ability to accept native DNA as template, but have retained the ability to execute complementary synthesis along single-stranded DNA. The fact that bacterial DNA nucleotidyltransferases appear to be primed by native DNA may simply be a reflection of their greater stabilities during extraction and purification. However, *in vitro* DNA-synthesising systems from both bacterial and mammalian sources are apparently unable to synthesise a product which has the same physical properties as DNA extracted from the cell of origin of the DNA nucleotidyltransferase (see Discussion, section I D). This may, in part, be due to modification of the template and loss of factors controlling synthesis (see Discussion, section II B) in the *in vitro* system, but it may also be due to modification of the enzyme during its removal

from the cell and subsequent purification - a factor which, clearly, has not been taken into consideration in the past.

Having considered these observations the following pictures of DNA nucleotidyltransferase within the cell (intact form) and of modified DNA nucleotidyltransferase in cell extracts (altered form) were drawn up purely as working hypotheses for the purpose of investigation of the conformation of DNA nucleotidyltransferase.

It is proposed that the intact form of DNA nucleotidyltransferase might be a dimeric molecule, which has the capability of simultaneous complementation of both strands of DNA (see Discussion, section II A). This molecule might consist of two identical or mirror image subunits held together by intermolecular bonds. This would explain its ability to replicate native DNA. On mechanical stress these subunits would tend to dissociate, and individually they would retain only the ability to utilise single-stranded DNA. As it appears that at the site of DNA replication (see Discussion, section II A) a nucleus of single-stranded DNA is necessary (perhaps only a few nucleotides long) it might also be expected that the intact form of the enzyme would be required to initiate synthesis on a wholly double-stranded primer.

Furthermore this concept can be extended to account for the terminal DNA nucleotidyltransferase from calf thymus tissue. The two subunits postulated above may themselves be composed of four subunits, each of which is specific for binding one of the four deoxyribonucleoside 5'-triphosphates prior to polymerisation according to the base sequence of

the template DNA: Therefore, in a manner analogous to that proposed for the separation of the first two subunits (template subunits) these four (base subunits) may be, in turn, split by more extensive fractionation into single fragments capable of performing the function of terminal addition. The fractionation procedure of Bollum et al., (1964), for example, may be selective in the purification of a fragment specific for the polymerisation of dAMP residues. Further support for this proposal is obtained from the fact that the terminal enzyme from calf thymus, demonstrated by these workers, was eluted well after the replicative DNA nucleotidyltransferase from a Sephadex G-100 column, indicating that it had a much lower molecular weight and that it might well be a subunit of the parent replicative enzyme.

It must be emphasised that all the proposed forms of DNA nucleotidyltransferase, intact and altered (subunits), may be present in cell free enzyme preparations, their relative proportions perhaps depending upon the degree of mechanical stress to which the extracts has been subjected. The fact that the ability of native DNA to prime synthesis by these fractions decreases as enzyme fractionation proceeds (see Table 5) supports this concept. Finally, it should be borne in mind that these models of the DNA nucleotidyltransferase molecule represent a purely hypothetical attempt to rationalise the results of many workers in the field of DNA biosynthesis.

It is now appropriate to discuss the results of some experiments performed in an attempt to substantiate this theory.

Studies on the heat stability of DNA nucleotidyltransferase preparations

preparations from Landschuts ascites-tumour cells indicate that prior incubation of the enzyme at 45° initially stimulates the activity of all four enzyme fractions investigated (see Fig. 38 and Results section VII B). As purification proceeds the degree of stimulation due to prior incubation tends to fall off. These results may be interpreted in the following manner. Prior thermal activation of the enzyme molecule might be expected to modify its tertiary structure. Such modification might initially favour access of substrate to the active site of the enzyme causing the stimulation of activity observed in Fig. 38. This might be correlated with the conversion of DNA nucleotidyltransferase from the intact to an altered form. The fact that this effect diminishes as purification proceeds supports this concept as the more purified preparations may already be largely in an altered form of some kind. The fall-off in activity on prior incubation for periods longer than 3-4 min. can be attributed to progressive heat-inactivation and eventual total denaturation. These characteristics were exhibited by almost all Landschutz DNA nucleotidyltransferase preparations investigated, and it may be concluded that they demonstrate the initial activation of a highly organised, three-dimensional structure followed by its progressive denaturation.

More detailed studies on the ionic responses of DNA nucleotidyl-transferase were performed in order to investigate their possible interpretation with respect to enzyme conformation (see Results, section VII C). The results presented in Figs. 40 & 41 show the responses of similar DNA

nucleotidyltransferase preparations from calf thymus tissue and Landschutz ascites-tumour cells to Mg^{2+}, K^{+} and Na^{+} ions. The salient feature of these figures is that neither enzyme shows an even response to the ionic variable, all curves showing irregular shoulders of activity. Fig. 40 shows the ratio of the activity of the calf thymus preparation to that of the Landschutz preparation over a range of Mg^{2+} ion concentration. Above 8mM there is a peak of thymus enzyme activity over that of the Landschutz enzyme. Allowing for the fact that the peak of DNA nucleotidyltransferase activity for both preparations lies in the region 4 - 6mM, it might be suggested that the excess of thymus activity over Landschutz activity at 8mM could be due to the presence of a greater amount of a certain form of the enzyme in the former preparation. (This subsidiary peak of activity in thymus extracts was very marked and was at one time regarded as being due to terminal enzyme activity. Indeed, as described above the concept of intact and altered forms may be modified to reconcile this view). Fig. 41 indicates that as before (see Fig. 22 and Results, section II B) both enzymes are stimulated by a univalent cation. However, the responses are not uniform nor do the peaks of activity correspond e.g., the K^{+} ion responses for both enzymes have shoulders of activity but they occur on opposite sides of their respective peaks, which themselves are not coincident. These variations have been found to occur widely in the DNA nucleotidyltransferase extracts used in this work, and they may be regarded as evidence in support of the concept that there may be more than one form of the enzyme in cell free extracts. The fact that

variation in ionic conditions in the assay system may itself hasten conversion of the enzyme from the intact to altered forms during incubation is suggested by the evidence of Kirshner & Tanford (1964), which shows that haemoglobin is dissociated into two subunits by increasing concentrations of sodium chloride, calcium chloride, magnesium chloride and ammonium sulphate. The last of these compounds may well account for the low stimulation of activity on prior incubation of the Landschutz 20 - 45% ammonium sulphate fraction (see Fig. 38) and for the loss of activity on prolonged incubation by the same enzyme fraction (see Fig. 15).

The interpretation of the results of preliminary kinetic studies presented in Figs. 42 & 43 is described in the Results section VII D. DNA nucleotidyltransferase from Landschutz ascites-tumour cells appears to bind two molecules of single-stranded DNA-primer, when there are high concentrations of DNA in the assay medium. At low DNA concentrations the possibility is that only one DNA strand is bound by the enzyme. In the context of the theory of intact and altered forms of DNA nucleotidyltransferas these results have two implications. The first is that they may lend support to the suggestion that the intact form is made up of two subunits which can replicate DNA efficiently. The second implication is that the two subunits may not have undergone complete separation in the altered form, which predominates in the pH 5 precipitate fractions used in these assays because, although it cannot utilise native DNA as it might be expected to do in the intact form, the altered form appears to retain the ability to bind

simultaneously two single -stranded primer molecules.

Actinomycin D inhibition of DNA nucleotidyltransferase also exhibits a non-linear relationship when presented in the 1/v against 1/s plots (see Fig. 37). As is the case for non-inhibited preparations (see Fig. 43) a linear relationship was obtained when the reciprocal of the velocity was plotted against the reciprocal of the square of the substrate concentration for the same results (see Fig. 44). Indeed, some physical studies on DNA have suggested that Actinomycin D, when bound to single-stranded DNA, has the ability to attract laterally another strand of DNA (Cavalieri & Nemchin, 1964). Therefore, it may be that Actinomycin D favours the binding of two strands of DNA to the enzyme molecule although the resultant complex does not lead to DNA synthesis. This interpretation reinforces the concept that both the <u>intact</u> form and the predominant <u>altered</u> form of DNA nucleotidyltransferase present in these preparations are capable of binding two strands of DNA per molecule.

The observations presented above in support of the concept of <u>intact</u> and <u>altered</u> forms of DNA nucleotidyltransferase are all open to the one serious objection that the fractions employed were contaminated with nuclease. While this possibility must be considered, it is very simple to overestimate the effect of interference exercised by this enzyme in these studies. For example, the prior incubation technique although carried out in the presence of DNA, was most often performed in the absence of the other components of the reaction mixture (see

Experimental, section IV A). This means that Mg^{2+} ions, which are essential for DNaseI activity, were absent from the prior incubation medium. Therefore, negligible formation of 3'-hydroxy-terminal groups would occur in the primer during a 2 - 6 min. prior incubation period, and little stimulation of DNA nucleotidyltransferase from this source could be expected. Modification of DNA nucleotidyltransferase activity as a result of the production of 3'-hydroxy-terminal groups in the primer by DNaseI activity at certain concentrations of Mg^{2+} or K^{+} or Na^{+} ions is perhaps a more serious objection in view of the small magnitude of the changes of activity involved. The kinetic studies, however, are less open to these criticisms, since during incubation, provided that at low substrate concentrations all the DNA is not digested, the production of 3'-hydroxy-terminal groups in the primer DNA and consequent stimulation of DNA nucleotidyltransferase activity will be constant for all assays.

Thus, in conclusion, it can be said that these results, taken into account with the observations, which led to the formulation of the concept of the intact and altered forms of DNA nucleotidyltransferase, suggest that in its broadest form this theory may well contain the seeds of truth. It would certainly be unwise at this stage to talk with conviction about "template subunit altered form" and "base subunit altered form", but with a view to developing new techniques to investigate this concept it remains very useful to propose such molecular models.

II General Discussion of DNA Biosynthesis

In this section it is proposed to discuss some of the conceptual problems which currently face the research worker employed in the investigation of DNA biosynthesis. As the mechanism of DNA synthesis has been characterised most extensively in bacteria, more reference will be made to these systems. However, evidence will also be included from mammalian and viral systems in a general survey of the present state of knowledge of the mechanism of chromosome replication. Throughout this discussion it is assumed that the replicative DNA nucleotidyltransferase from the various systems previously mentioned is the enzyme responsible *in vivo* for DNA replication.

A The mechanism of DNA Replication

The results of the ultracentrifugal studies of Meselson & Stahl (1958) indicated that DNA replication in *E. coli* was accomplished by a semi-conservative mechanism (see Introduction, section VI A and Fig. 9). Initially it was assumed that the conserved unit was the single strand of the model of Watson & Crick (1953). However, work with intact cells and *in vitro* work with *E. coli* DNA nucleotidyltransferase (Cavalieri & Rosenberg, 1961a, b & c; Cavalieri, 1963; Cavalieri & Rosenberg, 1963) was interpreted to mean that the conserved unit in DNA replication was the double-helix. The proposal was reconciled with the earlier proof of the semi-conservative nature of replication by evidence which suggests that the DNA of bacteria, in the process of DNA synthesis, was four-stranded, i.e., consisted of two laterally-bound, crosslinked double helices. Semi-conservative replication, according

to this view, proceeds by the formation of two new double-helices on the four-stranded template, followed by segregation of the resulting four double-helices into two sets of two double-helices, one of which is newly synthesised and the other is derived from the parental template. Therefore, the overall replication is semi-conservative and the double-helix is the conserved unit. However, evidence from an experiment, which was an extension of the ultracentrifugal studies of Meselson & Stahl (1958), has tended to favour the former interpretation (Baldwin & Shooter, 1963). Thymine-requiring E. coli mutants were grown synchronously in the presence of thymine or 5-bromouracil. Growth of the bacteria was controlled in such a manner that DNA molecules containing variable quantities of thymine and 5-bromouracil were obtained. Thus, DNA molecules in which the conserved units both contained thymine or 5-bromouracil or in which one of the conserved units contained thymine and the other 5-bromouracil were obtained. The hybrid molecule was shown, using ultracentrifugal techniques, to dissociate at a temperature intermediate between the melting temperatures of DNA molecules whose conserved units both contained thymine or 5-bromouracil. These results would seem to indicate conclusively that the conserved unit in DNA biosynthesis is probably the single-strand of a double-helix. Other evidence in support of this mechanism includes the radioautographic studies of chromosome replication in E. coli (Cairns, 1963a, b) using a tritium label. It has been found that the amount of tritium per unit length of newly synthesised DNA is consistent with the presence of only one newly synthesised strand in a daughter chromosome. Rolfe (1962) has also shown that the

subunits in a hybrid molecule are not separated after sonic breakage indicating that they are not linked end to end. Nevertheless, a firm conclusion on this controversy awaits more experimental data on the nature of the chromosome *in vivo*.

Assuming the single-strand to be the conserved unit in DNA replication, the problem of how the enzyme carries out the synthetic process now presents itself. The terms *complementation* and *replication* have already been defined (see Discussion, section I E 1). It can be seen that for the production of an exact replica of a double-helical DNA molecule in preparation for cell division DNA replication must take place. However, replication can be regarded as two separate operations involving the complementation of each of the two strands. After the elucidation of the double-helical structure of DNA (Watson & Crick, 1953) proposals for the mechanism of semi-conservative replication were advanced by Delbruck & Stent (1957). One of these involved the passage of the enzyme along the chromosome with complementation of both strands of the DNA taking place simultaneously at the site of synthesis. Thus a Y -shaped model was drawn up in an attempt to describe this operation. The arms of the Y represented the newly complemented strands of the parent molecule, while the trunk represented that part of the parent molecule still to be replicated. Synthesis was pictured as taking place at the junction of the arms and the trunk. As synthesis proceeded the junction moved down the trunk until complementation of both strands of the parent molecule was complete, resulting in semi-conservative replication of the parent molecule. However, this

concept fell out of favour because it requires two mechanisms for polymerisation due to the antipolar nature of the strands of the double-helix. The *in vitro* work of Kornberg indicated that the mechanism of polymerisation was that of the addition of deoxyribonucleoside 5'-triphosphates to the 3'-hydroxyl ends of primer chains (see Fig. 8). The corresponding mechanism for the complementation of the other strand would involve the addition of deoxyribonucleoside 3'-triphosphates to its 5'-hydroxyl end. Failure to detect deoxyribonucleoside 3'-triphosphates in cell extracts seemed to eliminate this mechanism. An alternative mechanism for the simultaneous complementation of the second chain would be the addition of the 3'-hydroxyl group of a deoxyribonucleoside 5'-triphosphate on to a 5'-triphosphate group at the growing point. However, the apparent conceptual difficulties in envisaging an enzyme simultaneously catalysing two polymerisation reactions with different mechanisms caused this theory to be discarded. It was then proposed that synthesis began at both 3'-hydroxyl ends of the double-helix, each strand being complemented individually. Conceptual difficulties are also encountered with this theory, since the problem of control of replication at two separate points on the chromosome becomes much more complex (see Discussion, section II B). The Y-model of Delbruck & Stent (1957) was revived by the work of Cairns (1963a, b), Nagata (1963) and Yoshikawa & Sueoka (1963), which showed that the replication of the circular chromosomes of *E. coli* and *B. subtilis* was sequential and unidirectional from a fixed point. Therefore, it became apparent that, in the bacterial system at least, both strands of the

chromosome must be complemented simultaneously, thus ruling out initiation of synthesis at more than one point. This revelation has brought the nature of the DNA nucleotidyltransferase molecule back into prominence. Results of this work have indicated that the DNA nucleotidyltransferase from Landschutz ascites-tumour cells may well have two active centres, and other evidence from both mammalian and bacterial systems suggests that the DNA nucleotidyltransferase molecule studied in in vitro systems may be considerably modified from the structure of the enzyme within the cell. Finally there has been a recent and extremely important report of the existence in B. subtilis of an enzyme, which catalyses the phosphorylation of deoxythymidine 3'-diphosphate to deoxythymidine 3'-triphosphate, ATP being required for the reaction; the enzyme does not catalyse the corresponding reaction with 5'-phosphates (Canellakis, Kammen & Morales, 1965). This may be the first positive evidence in support of the theory that deoxyribonucleoside 3'-triphosphates have a function in DNA biosynthesis.

Thus, current evidence shows that DNA nucleotidyltransferase must complement both strands of DNA simultaneously from a fixed point in vivo. This almost certainly involves a very complex operation, which may include the simultaneous complementation of two strands by different mechanisms. The complementation process itself involves the interplay of the template, the enzyme and one of the four bases, the last of which may be accepted or rejected by the template. Therefore, the enzyme may also have a specific binding site for each of the base substrates (base altered form subunit).

Indeed, it seems very reasonable to expect that an enzyme with such a function must have a very complex structure, which when removed from its natural environment may very readily undergo collapse. The conclusions concerning the structure and mode of action of DNA nucleotidyltransferase obtain some support from the theoretical considerations of Butler (1963), Sibatani & Hiai (1964) and Sibatani (1964). Butler suggests that the DNA nucleotidyltransferase molecule has two active sites, while Sibatani & Hiai favour the controlled simultaneous replication of both strands by two separate enzyme molecules.

The observations of Cavalieri and his co-workers that DNA nucleotidyltransferase and RNA nucleotidyltransferase from E. coli are the same molecule or are derived from a single parent molecule (Cavalieri & Rosenberg, 1963; Lee-Huang & Cavalieri 1963, 1964) are interesting in view of the complex structure, which has been proposed for the DNA nucleotidyltransferase molecule in this work. However, work in this laboratory suggests that in Landschutz and Krebs II ascites-tumour cells the RNA nucleotidyltransferase appears to be totally located in the nuclear fraction (Eason & Smellie, 1965) indicating that the enzymes may be two distinct molecules. However, the possibility still remains that during cell disruption a hybrid DNA - RNA nucleotidyltransferase is dissociated into two component molecules, one of which (the DNA nucleotidyltransferase) becomes soluble, while the other (the RNA nucleotidyltransferase) remains particulate within the nucleus.

B) Control of DNA Biosynthesis

The problem of cellular organisation is one which has increasingly employed the energies of biologists over the last fifteen years or so, as

more detailed knowledge of individual biological reactions has become available. Because of its central role in the life cycle of the cell, the problem of the control of cellular DNA biosynthesis is probably one of the best examples of this trend. Many pages of discussion could be devoted to this subject, which has been reviewed in some detail by Lark (1963), but it will be limited to a brief description of the possible sites along the DNA biosynthetic pathway at which control might be exerted. Lark (1963) subdivides the various mechanisms of control into two types - one quantitative, the other temporal - because the amount of DNA synthesised is always equal to that initially present, and the time of synthesis and the duration of synthesis are always restricted to a defined period during the growth cycle (see Introduction, section VI). The various possible mechanisms of control discussed in this section will also be considered within this type of classification.

The first region in which a control mechanism may exert an influence is on the supply of the four deoxyribonucleoside 5'-triphosphate precursors of DNA (Lark, 1963). Control may be effected by modification of the activity or supply of the enzymes which catalyse the reactions leading to the formation of these precursor molecules. The more complex biosynthetic pathway involved in the formation of dTTP (see Fig. 6) makes the supply of this precursor more readily amenable to control of the type proposed above. Thus, dTTP would appear to occupy a unique position in the synthesis of the precursor molecules and afford scope for effective control of DNA biosynthesis (Davidson, 1962; Grav, 1964). However, control of the

formation of the other three deoxyribonucleoside 5'-triphosphates is probably also exerted as a means of controlling DNA synthesis (Reichard, Canellakis & Canellakis, 1960; Davidson, 1962). These effects would appear to be mainly temporal in their control, as they might be expected to trigger off DNA synthesis when, and only when, the intracellular concentration of deoxyribonucleoside 5'-triphosphate precursors, which was sufficient to complete replication of the chromosome, was achieved. These changes of enzymic activity might be due to stimulation or inhibition of specific protein synthesis through gene repression or activation (Monod, Jacob & Gros, 1962), or to simple positive or negative feedback control by terminal products of the reaction sequence (Potter & Auerbach, 1959; Potter, 1962).

The second site at which control of DNA synthesis may occur is at the initiation of the process. This operation has been shown by several groups of workers to require prior protein synthesis in bacterial systems at least (Maaløe, 1963; Lark et al., 1963; Pritchard & Lark, 1964; Lark & Lark, 1964). Their work also indicates that the initiation and termination of synthesis are widely separated events in the division cycle, and that once initiated, synthesis will continue until completion of the chromosome even although protein synthesis is inhibited. Much of this work is very elegant and has led to several hypotheses on the nature and control of DNA replication in the bacterial cell. These hypotheses involve the action of protein molecules to initiate and control the process of replication along the strand (Jacob & Brenner, 1963; Jacob, Brenner & Cuzin, 1963;

Sibatani & Hiai, 1964). These molecular models of the replication of the chromosome will be useful for the design of future experiments. Maaløe (1963) showed that variation in growth conditions, which altered the mean generation time of bacterial cultures, failed to alter the length of the DNA synthetic phase, suggesting that once the synthetic process has been initiated neither the DNA nucleotidyl-transferase molecule nor the intracellular environment can influence the rate of replication. However, the work of Lark *et al.*, (1963), which showed that amino acid deprivation of some amino acid requiring bacterial mutants reduces the rate of replication initiated before deprivation, suggests that the replication process, once initiated, is not entirely independent of the intracellular environment.

The DNA template in the DNA synthetic system of a cell has the potential to exert control over DNA synthesis in both a quantitative and a temporal manner. There is massive evidence indicating that the DNA template is probably the sole determinant of the total amount of DNA synthesised during the DNA synthetic period. Indeed, this would necessarily be the case if an exact replica of the template is to be formed. Evidence that the nature of the template itself may play some role in the initiation of replication is obtained from work, which suggests that there may be *in vivo* metastable forms of DNA, which readily prime *in vitro* synthesis of DNA (Frankel, 1963; Rolfe, 1963; Sampson, Katoh, Hotta & Stern, 1963; Rosenberg & Cavalieri, 1964). Another factor which may determine the initiation of DNA synthesis is the nature of the

association of the DNA with the histone content of the chromosome in mammalian systems. Association or dissociation of histones with DNA may determine the ability of the DNA to initiate and maintain replication (see Introduction, section VIB). These factors may also play some role in the direction of the synthesis of the proteins responsible for the initiation and control of DNA synthesis, if they exist in mammalian systems.

Another factor which may control the DNA synthetic process of the mammalian cell is the location of DNA nucleotidyltransferase within the cell. Current views on the possibility that DNA nucleotidyltransferase moves to and fro between nucleus and cytoplasm depending on whether or not DNA synthesis is taking place have been discussed in the Introduction, section V D. The suggestion of Littlefield et al., (1963) that the enzyme is particulate while in the nucleus performing the function of DNA synthesis, and is soluble while inactive in the cytoplasm, may indicate that some form of modification of the enzyme occurs at or near the termination of synthesis. Perhaps the molecule may be split into two subunits (template subunits) on completion of replication, and as such be able to pass through the nuclear membrane into the cytoplasm. Before re-initiation of synthesis can take place return of the subunits and their reassembly into active DNA nucleotidyltransferase molecules must occur, and these operations may well offer sites at which control is effected. However, whether or not this mechanism is operative with the cell, the general principle still holds that the location of the enzyme within the

complexity of the nucleus may well furnish a means by which control of the synthetic process could be exercised.

The observations in this section indicate the salient points along the DNA synthetic pathway at which cellular control of replication may be most readily exerted. The processes of DNA replication and subsequent cell division are necessarily the resultant of all these factors, which are themselves products of the cellular environment.

SUMMARY

SUMMARY

1) DNA nucleotidyltransferase (E.C. 2.7.7.7.) was extracted in soluble form, from Landschutz ascites-tumour cells and purified approximately 12-fold by conventional fractionation procedures.

2) Small quantities of the DNA nucleotidyltransferase were obtained free from deoxyribonuclease by chromatography of the 12-fold purified fraction on a column of hydroxylapatite.

3) The various enzyme fractions investigated exhibited negligible phosphatase activity under the conditions optimal for DNA nucleotidyltransferase activity.

4) DNA nucleotidyltransferase from Landschutz cells exhibited an absolute requirement for DNA as primer, heat-denatured DNA being 5 - 10 times more effective than native DNA in this respect.

5) The presence of a bivalent cation in the *in vitro* assay system was also found to be essential for enzymic activity, the enzyme being much more active in the presence of Mg^{2+} ions than in the presence of Mn^{2+} ions. The univalent cations, K^{+} and Na^{+}, were shown to stimulate the reaction.

6) The pH for optimal enzymic activity was in the range 7.2 - 7.5.

7) The presence of EDTA at 0.4mM in the assay system markedly stimulated enzymic activity and preparation of the enzyme in 0.001M EDTA-0.005M-2-mercaptoethanol allowed storage for two months at 0°.

8) Optimal activity was attained in the presence of equal amounts of all four deoxyribonucleoside 5'-triphosphates, (dATP, dGTP, dCTP and dTTP).

Replacement of one, two or three of these by the corresponding mono- or diphosphates, the fourth being (α-^{32}P)-dTTP, gave a reduction of synthesis; the residual level of incorporation is suggested to be attributable to the action of enzymes capable of catalysing trans-phosphorylation reactions.

9) The product of enzymic activity was shown to be high molecular weight polynucleotide material, and studies on the incorporation of (α-^{32}P)-dTTP, - dGTP, and - dATP suggest that the pattern of base incorporation into product DNA depends on the base ratios of the DNA-primer. In addition, the rate of DNA synthesis in these preparations appears to be commensurate with the <u>in vivo</u> DNA synthetic rate calculated from data cited in the literature. Therefore, it would seem probable that the enzyme studied in this work is well qualified to fulfil the function of DNA replication <u>in vivo</u>.

10) Concurrent studies were conducted with DNA nucleotidyltransferase preparations from calf thymus gland for purposes of comparison with the enzyme derived from the tumour cells. The thymus enzyme, when subjected to the same fractionation techniques behaved in a manner similar to the Landsohutz enzyme; it also exhibited similar requirements for optimal DNA nucleotidyltransferase activity.

11) A survey of the <u>replicative</u> DNA nucleotidyltransferase and <u>terminal</u> addition activities from several mammalian tissues revealed that calf thymus exhibited much more <u>terminal</u> addition activity relative to <u>replicative</u> activity than did the following rodent tissues, rabbit spleen,

liver, thymus and appendix, and rat spleen and liver. Preparations of DNA nucleotidyltransferase from Landschutz ascites-tumour cells displayed very low levels of terminal addition activity.

12) Actinomycin D was shown to inhibit the action of DNA nucleotidyltransferase from Landschutz ascites-tumour cells in a competitive manner. Replicative activity was much more sensitive in this respect than was terminal addition

13) The inhibition exerted on DNA nucleotidyltransferase from Landschutz ascites-tumour cells by iodoacetamide, iodoacetate, p-hydroxymercuribensoate and sarkomycin was found to be non-competitive. Inhibition by iodoacetamide and iodoacetate was irreversible and that by p-hydroxymercuribensoate was reversed by glutathione or 2-mercaptoethanol, while inhibition by sarkomycin was only partially released by the same procedures.

14) The terminal addition reaction catalysed by the Landschutz enzyme preparations was somewhat less sensitive to the inhibition effect of these compounds.

15) Other studies were undertaken in an attempt to characterise the DNA nucleotidyltransferase molecule.

a) Prior incubation of the enzyme for 0.2 min. at 45° stimulated activity. Longer periods of prior incubation led to progressive denaturation of the enzyme.

b) Preliminary kinetic studies showed that the enzyme did not exhibit a linear 1/v against 1/s relationship, when assayed in the presence of varying amounts of primer DNA. However, if 1/v was plotted against $1/s^2$

for the same experiments a linear relationship was demonstrated. This is interpreted to mean that the enzyme may contain two active sites.

c) More detailed investigation of the ionic requirements of different DNA nucleotidyltransferase preparations indicated that there could be a wide variation in response of the enzyme to Mg^{2+}, K^{+} and Na^{+} ions, depending on such factors as method and duration of the preparation and source of the enzyme.

Collectively, these studies offer some support for the concept, formed during this work, that <u>in vivo</u> DNA nucleotidyltransferase may be a highly organised entity containing two or more independently active, easily dissociable subunits.

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