THYMIDINE NUCLEOTIDES IN NUCLEIC ACID BIOSYNTHESIS

by

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ABBREVIATIONS

The following abbreviations will be employed in this thesis:-

RNA	ribonucleic acid
s-RNA	"soluble" ribonucleic acid
DNA	deoxyribonucleic acid
UR	uridine
CR	cytidine
AR	adenosine
GR	guanosine
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
S-AMP	adenylosuccinic acid
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
XMP	xanthosine 5'-monophosphate
GMP	guanosine 5'-monophosphate
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
IMP	inosine 5'-monophosphate

5-Me-CMP	5-methylcytidine 5'-monophosphate
IDP	inosine 5'-diphosphate
ITP	inosine 5'-triphosphate
UdR	2'-deoxyuridine
CdR	2'-deoxycytidine
AdR	2'-deoxyadenosine
GdR	2'-deoxyguanosine
dUMP	deoxyuridine 5'-monophosphate
dUDP	deoxyuridine 5'-diphosphate
dUTP	deoxyuridine 5'-triphosphate
dCMP, deoxycytidylate	deoxycytidine 5'-monophosphate
dCDP	deoxycytidine 5'-diphosphate
dCTP	deoxycytidine 5'-triphosphate
5-Me-dCMP	5-methyldeoxycytidine 5'-monophosphate
5-MeOH-dCMP	5-methylhydroxydeoxycytidine 5'-monophospha
5-MeOH-dCDP	5-methylhydroxydeoxycytidine 5'-diphosphate
5-MeOH -dCTP	5-methylhydroxydeoxycytidine 5'-triphosphat
dAMP, deoxyadenylate	deoxyadenosine 5'-monophosphate
dADP	deoxyadenosine 5'-diphosphate
dATP	deoxyadenosine 5'-triphosphate
dGMP, deoxyguanylate	deoxyguanosine 5'-monophosphate
dGDP	deoxyguanosine 5'-diphosphate
dGTP	deoxyguanosine 5'-triphosphate
TdR , thymidine	2'-deoxyribose thymine
TMP, TMP-5'	thymidine 5'-monophosphate
TMP-3'	thymidine 3'-monophosphate

TDP	thymidine 5'-diphosphate
TTP	thymidine 5'-triphosphate
PRPP	5-phosphoribosyl-1-pyrophosphate
AICAR	4-amino-5-imidazole carboxamide ribonucleo
FAICAR	5-formamino-4-imidazole carboxamide ribonucleotide
PRA	5-phosphoribosylamine
GAR	glycinamide ribonucleotide
FGAR	formylglycinamide ribonucleotide
AIR	5-aminoimidazole ribonucleotide
(^{3}H) TdR	thymidine labelled with tritium
(^{3}H) CdR	deoxycytidine labelled with tritium
(^{32}P) TMP	thymidine 5'-monophosphate labelled with a radioactive phosphorus atom
CEP	2-cyanoethylphosphate
DCC	dicyclohexylcarbodiimide
DCU	dicyclohexylurea
NAD	nicotinamide-adenine dinucleotide
NADH	reduced nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphat
NADPH	reduced nicotinamide-adenine dinucleotide phosphate
P_i	inorganic orthophosphate
$P\text{-}P_i$	inorganic pyrophosphate
tris	2-amino-2-hydroxymethylpropane-1, 3-diol
EDTA	ethylenediaminetetra-acetate

Enzymes: Trivial enzyme nomenclature was used throughout this thesis. The term thymidine and thymidylate kinases is employed to describe the set of phosphokinases required to phosphorylate thymidine to thymidine-5'-triphosphate. The term thymidylate kinase(s) refers to phosphokinases capable of phosphorylating thymidine 5'-monophosphate to the products thymidine 5'-di- and thymidine 5'-triphosphate. The term TMP kinase refers exclusively to the phosphokinase catalysing the formation of thymidine 5'-diphosphate from thymidine 5'-monophosphate. (For other definitions, see text, Section 3.1).

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"True wisdom is not to be gained by measuring out the boundless world..... It is rather to be attained by the thorough investigation of any individual thing for thus we seek to arrive at a full knowledge and understanding of its true and peculiar nature."

Schopenhauer.

CHAPTER I

GENERAL INTRODUCTION

In the 17th and 18th centuries any competent scientist had reasonable expectation that he might adequately cover the whole range of scientific knowledge and experience within a lifetime. The ever widening scope and growing effort expended on science throughout the 19th and into the 20th century has resulted in massive proliferation and subdivision of scientific disciplines so that his present-day counterpart may only aspire to mastery of an infinitesimal part of the scientific knowledge available to him. Nevertheless, the acquisition of a unified vision of the natural processes thus made more difficult for the individual remains as desirable to-day as it was 300 years ago, but the restrictions imposed by rigid and narrowly based specialization allied to failure of communication between practitioners of related sciences has too often led to a frustration of these efforts. In retrospect, it is not difficult to perceive that the remedy lay in the removal of artificially created inter-disciplinary barriers thus giving due recognition to the fundamental principle of the interdependence of all scientific disciplines. Nowhere have the advantages of co-operation and concerted effort been better illustrated than within the biological sciences and, in particular, in the progress of the research on the nucleic acids over the last decade. Indeed, such vast progress had been inconceivable without the participation of groups of scientists having at their command a wide array of disciplines ranging from physics and chemistry through biochemistry to genetics, all seeking differing primary objectives but simultaneously channelling their efforts into a quest for the true nature of "the genetic material".

Many aspects of the transmission of heredity remain obscure, but a momentary culmination of these various researches was attained with the identification of the two types of nucleic acid DNA and RNA with, respectively, the cell carrier of genetic information and the agent responsible for translation of this information into the regulation of cell metabolism.

The occurrence of these high molecular weight polymers in all living tissues including the viruses is now well established as is their close association with protein, and a vast body of evidence indicates that their precise structure is of signal importance in the expression of the biological function of the nucleic acids.

1. 1. Chemical and physical structure of the nucleic acids

(a) RNA

RNA consists of the nucleoside 5'-monophosphates of adenine, cytosine guanine and uracil joined together in long chains through the 5'-phosphate group on one nucleotide to the 3'-hydroxyl group on the neighbouring nucleotide. In addition, certain ribonucleic acids such as S-RNA contain a relatively high proportion of a derivative of uracil, 5-ribofuranosyl-uracil (pseudouridine) (Cohn, 1960). Various ribonucleosides derived from methylated purines and pyrimidines, including thymine, 5-methylcytosine, 1-methyladenine, 2-methyladenine, 6-methylaminopurine, 6-dimethylaminopurine 1-methylguanine, 6-hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethyl-aminopurine, occur in small amounts (Adler, Weissman and Gutman, 1958;

Dunn, Smith and Spahr, 1960; Dunn, 1959, 1961). Recently, the occurrence of inosine and 1-methylinosine has been reported in yeast S-RNA (Hall, 1963). The type of linkage and the identity of the nucleotides were determined by studies on the products of alkaline and enzymic hydrolysis of ribonucleic acids extracted from various bacterial and mammalian sources (Cohn, 1950, 1951; Cohn and Volkin, 1953; Whitfield, Heppel and Markham, 1955). Early studies on RNA had suggested that it was a tetranucleotide since the methods then in use gave rise to fragments of this order of complexity. This hypothesis was soon abandoned when, with the advent of more precise analytical techniques, such as chromatography, the base composition of RNAs from different sources was observed to vary very widely. Furthermore, with the introduction of milder methods of extraction it became possible to isolate RNAs containing up to several hundred nucleotide units. Recent studies have also shown that the cell content of RNA is heterogeneous and RNAs with molecular weights ranging from 10,000 to 20,000 for S-RNA (Zamecnik, Stephenson and Hecht, 1958) to complex polymers of 10^6 or more have been described.

The secondary structure of RNA remains somewhat obscure and is probably related to the specific function of the different types of RNA. According to Doty (Doty et al., 1959; Doty, 1961) it appears that the larger molecules behave as irregularly coiled, relatively compact, single polymeric chains involving up to one-half of the molecule.

(b) DNA

DNA has recently been identified as the genetic substance (Avery,

MacLeod and McCarty, 1944; Hershey, 1953; Beadle, 1957, and the expression of its functions has received an admirable definition by Kornberg (1962) in the following words: "In its role DNA must have two functions; it must contain information, in chemical code, to direct the development of the cell according to its inheritance, and it must be reproducible in exact replica for the transmission of this inheritance to future generations".

In its chemical composition DNA differs from RNA in that it contains thymine in place of uracil and 2-deoxy-D-ribose in place of D-ribose. Moreover, in addition to the four major bases, certain others occur in small amounts. The most widely distributed of the unusual bases is 5-methyl-cytosine which is particularly abundant in wheat germ DNA (Wyatt, 1950, 1951 and also is present in DNAs from calf thymus, fish and insect sources. T-even bacteriophages have been found to contain 5-hydroxymethyl cytosine where it replaces cytosine (Wyatt and John, 1952; Kornberg, Zimmerman, Kornberg and Josse, 1959); 6-methylaminopurine has been discovered as a constituent of DNA extracted from certain strains of Escherichia coli (Dunn and Smith, 1958).

The DNA molecule is built up from deoxyribonucleotide units linked together in a manner similar to that existing in RNA. In contrast to RNA, analysis of samples of DNA from various sources have revealed that certain regularities in its base composition generally apply. It was found that the purine content always equalled the pyrimidine content, and while among the purines the adenine content may differ considerably from the guanine, and among the pyrimidines, the thymine from the cytosine, there is always

equivalence among the bases with an amino group in the 6-position in the ring to the bases with a keto group in the 6-position (Chargaff, 1950, 1951, 1955).

That DNA is a very large molecule became evident after the introduction of mild methods of extraction, and with the aid of recently perfected physical techniques, such as light scattering and ultracentrifugation, it has become possible to obtain a measure of its molecular weight. The values so obtained have varied between 2×10^6 and 16×10^6 for DNAs from different sources and it is possible that even molecules of this size are degraded forms of still more complex molecules (Hershey and Bungi, 1960; Levinthal and Davison, 1961). For the T2 bacteriophage DNA, molecular weights of about 100×10^6 have been quoted and it was suggested that a molecule of this order of complexity represented the total quota of DNA in each phage particle (Rubenstein, Thomas and Hershey, 1961).

Although the DNA has been regarded as the least variable of all cell components and the total DNA content has been utilised to calculate the total number of cells in a given portion of tissue (Davidson and Leslie, 1950), a considerable body of evidence is available to indicate that within a single cell nucleus there is physical heterogeneity of DNA molecules. Chromatographic fractionations of DNA on ECTEOLA cellulose (Bendich et al., 1958), have indicated that the various fractions so obtained exhibit base ratios that differ widely from those predicted by the Chargaff rules; similar results were obtained in studies using the ultracentrifuge (Butler, Laurence, Robins and Shooter, 1959).

On the basis of stereochemical models, X-ray crystallographic measurements accumulated by Wilkins and collaborators (Wilkins, 1957) and chemical data, Watson and Crick (1953a,b) proposed a structure for DNA in which two polynucleotide chains are wound about a common axis in a helical manner. The physical measurements had indicated that DNA exists in the form of long stiff rods, 10,000 or more nucleotide units in length, and Watson and Crick further proposed that the deoxypentose rings linked by 3', 5'-phosphate residues formed the backbone of the chain with the purine and pyrimidine rings emerging at right angles from the main axis of the chain on the inside. To explain the exceptional stability of the DNA molecule it was suggested that the purine and pyrimidine bases on one strand are bonded to the pyrimidine and purine bases on the complementary strand by hydrogen bonds. X-ray measurements have indicated that the space between opposing strands in the model agrees with the calculated value for a purine hydrogen bonded to a pyrimidine, thus accounting for the equivalence of the purines to the pyrimidines and the hydrogen bonding of adenine to thymine and cytosine to guanine. Fig. 1 shows how the nucleotide units are linked together in DNA and also indicates that the two complementary strands are of opposite polarity; Fig. 2 represents a space-filling molecular model of DNA.

Collapse of the rigid helical structure to a random coil form may be brought about when DNA is in aqueous solution under the influence of such factors as temperature, pH, ionic strength and dielectric constant of the medium or by agents such as urea that are known to favour dissociation of

Fig. 1

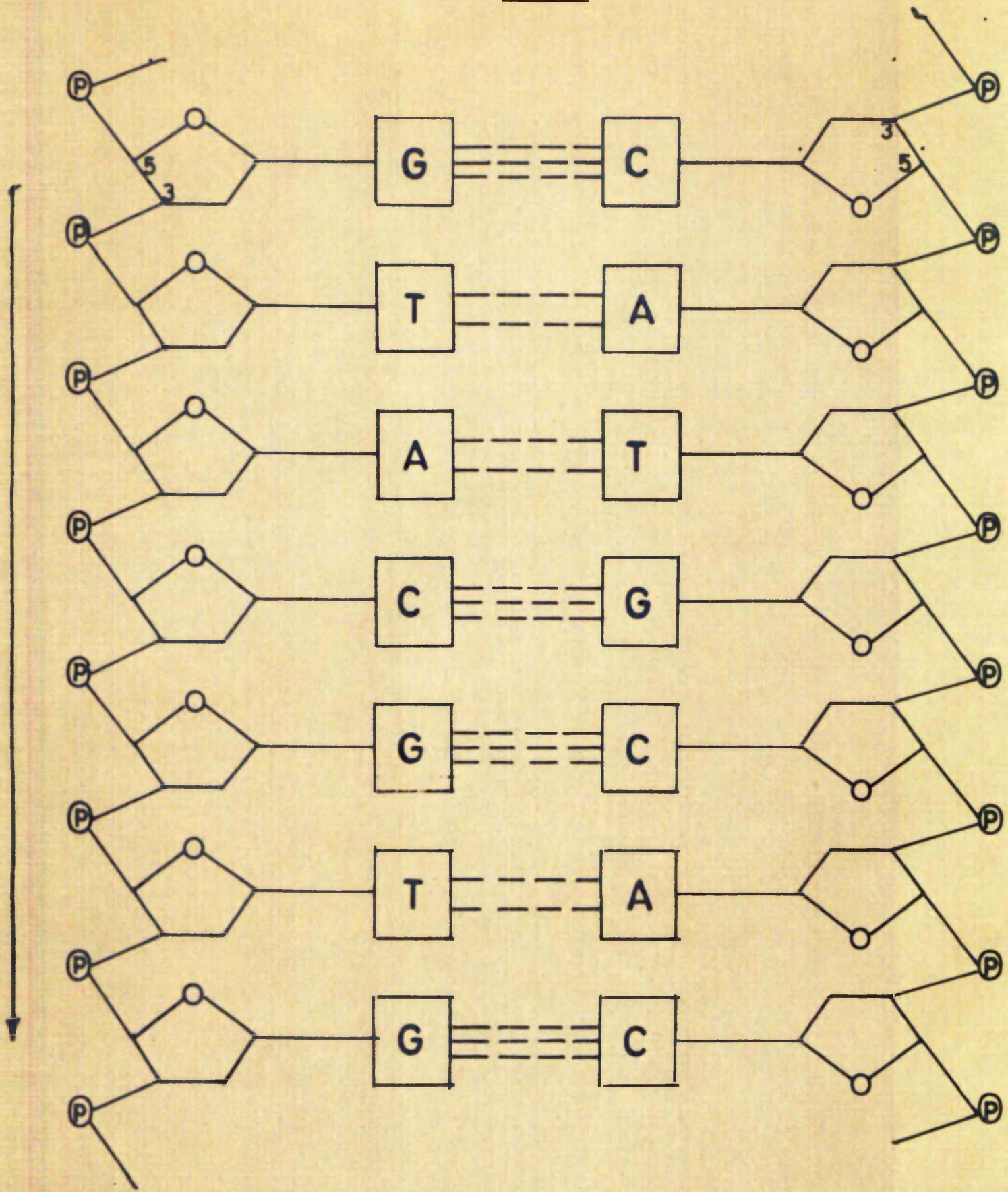


Fig. 1. Schematic representation of a section of the DNA molecule.

(Adapted from Kornberg, A. (1960) Science, 131, 1503)

Fig 2

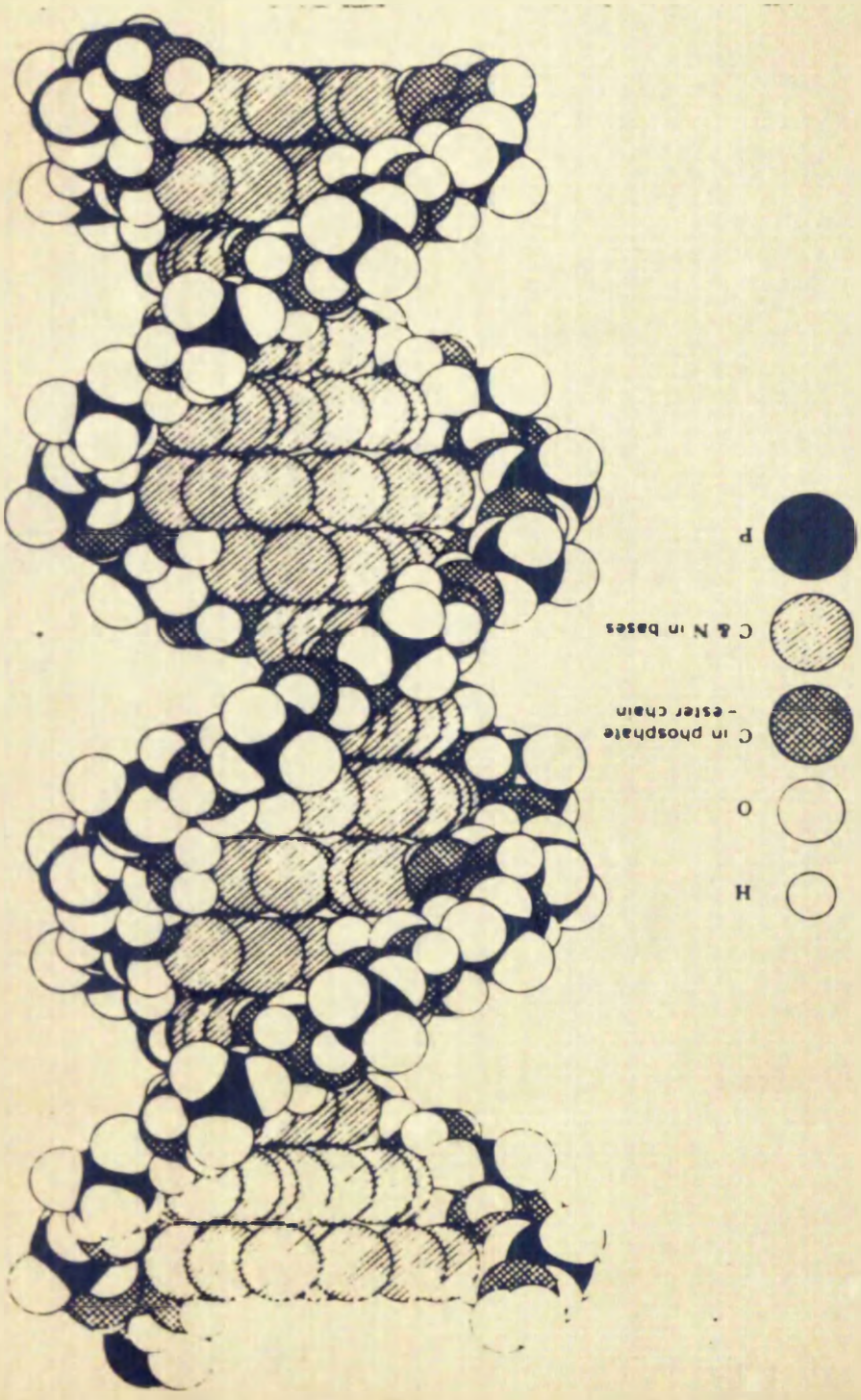


Fig. 2. Three-dimensional representation of a portion of the DNA molecule.

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

hydrogen bonds. This phenomenon is referred to as denaturation. The precise structural changes induced in the DNA molecule by denaturation are not well understood and conflicting views have been expressed as to the molecular weight of denatured DNA. It was suggested (Doty, Marmur, Eigner and Schildkraut, 1960) that DNA is converted to single stranded material upon denaturation since the data from sedimentation and diffusion measurements had indicated that the molecular weight had been halved. On the other hand, Rice and Doty (1957) and Cavalieri, Deutsch and Rosenberg (1961) failed to detect differences in the molecular weight of DNA before and after denaturation when the progress of denaturation was followed by light-scattering techniques. The available evidence seems to suggest that there is a degree of strand separation associated with the denaturation process while some hydrogen bonds remain unbroken.

The association of DNA with the chromosomes when cells are undergoing mitosis is supported by an impressive body of evidence, but the secondary structure of DNA or the precise arrangement of the polynucleotide double helix in morphologically recognisable structures remains an unsolved problem (Taylor, 1963; Thomas, 1963). However, Cairns (1963) provides evidence which suggests that the circular "chromosome" of E. coli (Kellenberger, 1960) consists of a single DNA unit with a molecular weight of 4×10^9.

1. 2. Biosynthesis of the Nucleic Acids

Self-replication is a unique property of the living cell and since its hereditary message has been found to reside in its polynucleotide

complement, the cell must possess efficient mechanisms for the precise duplication of this polynucleotide material. While the individual stages of this process may not be easily discernible in the intact cell, studies on broken cells and cell-free systems have shown that the general characteristics of the biogenesis of the polynucleotides makes it convenient to consider the process as proceeding through the following four main phases:- (1) An initial phase involving the assembly of the purine and pyrimidine ring systems, the biosynthesis of the carbohydrate moieties, and the convergence of the synthetic pathways of the purine and pyrimidine bases on the one hand and the pentose moieties on the other in the formation of nucleoside monophosphates; (2) the conversion of ribonucleotides to deoxyribonucleotides; (3) the phosphorylation of ribo- and deoxyribonucleoside monophosphates to the corresponding di- and triphosphates; (4) polymerisation of ribonucleotides and deoxyribonucleotides to polynucleotide material in presence of an appropriate polynucleotide primer.

Although the phases outlined above appear to apply universally irrespective of the origin of the cells, the precise sequence of anabolic events is a property of the individual cell types and, in addition to de novo biogenesis, some cells can utilise preformed (see Section 2. 1. (c)) purine and pyrimidine bases and can thus dispense with one or more of the obligatory stages in the formation of the polynucleotides.

(a) Biosynthesis of purine ribonucleoside monophosphates

Although it has been known for a long time that many organisms are capable of producing purines de novo from small molecules, the

establishment of the complete sequence of events leading to the assembly of the purine ring has come about primarily as a result of the efforts of Buchanan and Greenberg and their colleagues over the last 15 years. The initial attack on the problem of purine biosynthesis was carried out by administering small molecules labelled (^{13}C) to live pigeons and examining the distribution of this isotope in the excreted uric acid. This was achieved by selective degradation of the recovered uric acid in such a way that each individual carbon atom could be assayed for its isotope content (Buchanan, Sonne and Delluva, 1948). On the basis of these preliminary studies with labelled precursors of uric acid, it was shown that carbons 2 and 8 were derived from formate, carbon 6 came from CO_2 and that the ultimate precursor of carbon 4 was C_1 of glycine (Sonne, Buchanan and Delluva, 1948; Elwyn and Sprinson, 1950). Simultaneous studies on the incorporation of nitrogenous precursors into uric acid had revealed that nitrogen 7 was derived from the amino group of glycine (Shemin and Rittenberg, 1947). In further investigations it was found that nitrogens 3 and 9 arose from the amide group of glutamine and that either glutamic acid or aspartic acid donated the nitrogen in position 1 of the purine ring structure (Sonne, Lin and Buchanan, 1953, 1956). Buchanan et al. (1957) recently demonstrated that aspartic acid was the specific donor of nitrogen atom 1.

At a very much earlier date it had been shown that lack of the enzyme xanthine oxidase prevented the formation of uric acid in pigeon liver slices and that the product which accumulated was instead hypoxanthine (Edson,

Krebs and Model, 1936), and this observation was used by Greenberg (1948, 1950) to demonstrate that the same small molecules which provided the carbon and nitrogen atoms in uric acid were also precursors for hypoxanthine. Analogous studies with mammals and microorganisms had indicated that an identical assortment of small molecules was the precursor pool for the purine rings of the guanine and adenine of the nucleic acids (Heinrich and Wilson, 1950), and the conclusion was, therefore, inescapable that the various purines had a common precursor in their biosynthesis from small fragments. Greenberg (1951a) studied the incorporation of ^{14}C-formate and $^{14}CO_2$ into hypoxanthine with the use of pigeon liver homogenates and made the important discovery that hypoxanthine was not a primary product but was derived from the ribonucleotide of hypoxanthine - inosinic acid. This compound was well established as a deamination product of adenylic acid and although there was little evidence to indicate that it was a precursor of AMP, IMP attracted much attention and has since remained a focal point in purine metabolism.

In the search for possible intermediates in purine biosynthesis, an observation made by Stetton and Fox (1945) proved pertinent. They found that when Escherichia coli was grown in presence of sulphonamides, a diazotisable amine could be isolated from the culture medium and this amine was later identified by Shive et al.(1947) as 5-amino-4-imidazole carboxamide. The probable relationship of this compound to purine synthesis was immediately recognised since it is equivalent to hypoxanthine with carbon 2 missing. Although this material was shown to be incorporated into the nucleic acids

(Miller, Gurin and Wilson, 1950), experiments conducted with pigeon liver extracts (Greenberg, 1951b; Shulmann and Buchanan, 1952) indicated that the true intermediate in hypoxanthine synthesis was not 4-amino-5-imidazole carboxamide itself but rather its ribonucleotide derivative AICAR. Further in vitro studies using the pigeon liver system revealed that AICAR was converted to 5-formamino-4-imidazole carboxamide ribonucleotide (FAICAR) (Greenberg, 1954) by the transfer of a formyl group from N^{10}-formyltetrahydrofolic acid (Hartman and Buchanan, 1959). This reaction is followed by ring closure to yield inosine 5'-monophosphate (IMP) (Warren, Flaks and Buchanan, 1957). There now remained the elucidation of the pathway leading to the production of AICAR and again the pigeon liver extract proved an invaluable tool in that it represented a source from which all the enzymes of purine biosynthesis could be obtained in a soluble form and in high activity.

The discovery that the phosphoribosyl derivatives were involved in purine biosynthesis (Goldthwait, Peabody and Greenberg, 1954) had led to a series of investigations into the intermediates involved in the synthesis of these compounds. From such studies an enzymic mechanism was discovered which involved ribose-5-phosphate and ATP to give 5-phosphoribosyl-1-pyrophosphate (PRPP) in a reaction first described by Kornberg, Lieberman and Simms (1954, 1955a, b). In what is now considered the first step proper in purine synthesis de novo, PRPP was shown to be utilised by a purified pigeon liver system with glutamine in the formation of 5-phosphoribosylamine (PRA) (Goldthwait, 1956), which in turn condensed with glycine to give

glycinamide ribonucleotide (GAR). The next step was shown to be the formylation of GAR to yield formylglycinamide ribonucleotide (FGAR) (Goldthwait, Peabody and Greenberg, 1956; Warren and Flaks, 1956) by the intervention of N^5, N^{10}-anhydroformyltetrahydrofolic acid as donor of the formyl group (Hartman and Buchanan, 1959). The stage which follows involves the reaction of FGAR with glutamine and ATP to form irreversibly formylglycinamidine ribonucleotide (Levenberg and Buchanan, 1957b). However, with less purified enzyme preparations, the appearance of this compound is transient and the product which then accumulates was identified as 5-aminoimidazole ribonucleotide (AIR) (Levenberg and Buchanan, 1957a). Lukens and Buchanan (1957) discovered that the next step in purine biosynthesis was the reaction of AIR with CO_2 (or HCO_3^-) to yield 5-amino-4-imidazolecarboxylic acid ribonucleotide followed by reaction with aspartic acid and ATP to give 5-amino-4-imidazole-(N-succinyl)-carboxamide ribonucleotide, which in turn is cleaved to fumarate and AICAR (Miller, Lukens and Buchanan, 1957). Thus a complete reaction sequence in the enzymic formation of IMP from small precursors has been established using the system derived from pigeon liver, and although information from other sources is not impressive, all the available evidence points to a similar sequence of events in all cells which possess the ability to synthesise purines de novo. The probable reaction mechanism is illustrated in Fig. 3.

Experiments on bone marrow extracts demonstrated that IMP was a precursor of both GMP and AMP (Abrams and Bently, 1955a) of the RNA in that tissue, and further in vitro studies with bone marrow (Abrams and Bently, 195

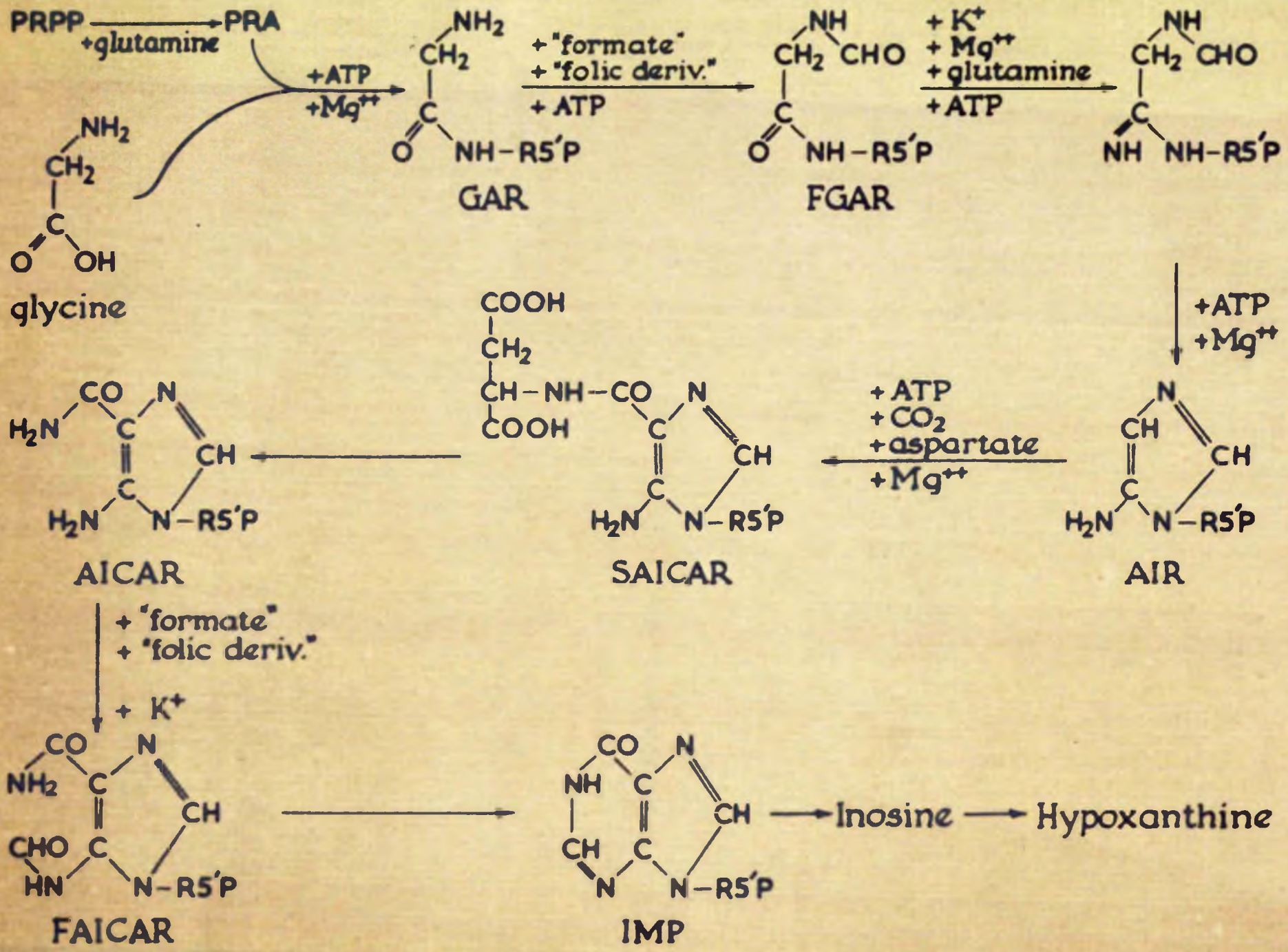

PRPP
+glutamine
PRA
+ATP
+Mg++
GAR
+"formate"
+"folic deriv."
+ATP
FGAR
+K+
+Mg++
+glutamine
+ATP
glycine
+ATP
+Mg++
+ATP
+CO2
+aspartate
+Mg++
AIR
SAICAR
AICAR
+"formate"
+"folic deriv."
+K+
FAICAR
IMP
Inosine
Hypoxanthine

Fig. 3

Fig. 3. The enzymic synthesis of inosinic acid *de novo*.

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

and with pigeon liver (Carter and Cohen, 1956; Lieberman, 1956) have established that the initial reaction in the conversion of IMP to AMP involves the condensation of IMP with L-aspartic acid in presence of GTP as co-factor. The adenylosuccinic acid thus formed is then enzymically cleaved to give, reversibly, AMP and fumarate.

The first step in the enzymic conversion of IMP to GMP involves the oxidation of IMP by an NAD-dependent enzyme to give XMP. This compound is subsequently aminated by the amide nitrogen of glutamine in the formation of GMP; an ATP requirement was demonstrated for systems from bone marrow (Bently and Abrams, 1956) and from pigeon liver (Lagerkvist, 1955). The relevant conversions are shown in Fig. 4.

(b) Biosynthesis of pyrimidine ribonucleoside monophosphates

On account of the clear structural similarity between the purine and pyrimidine ring systems it was thought for a long time that there was an intimate biochemical relationship between their respective biosynthetic pathways and that the purines were precursors of the pyrimidines or *vice versa* since both types of compound could be built up from similar types of small molecules. Thus it was shown that ammonia, CO_2 and aspartic acid were incorporated into the pyrimidines of avian liver polynucleotides. However, when methods became available for specific degradation of biosynthetically labelled pyrimidines, it appeared that nitrogen atom 1 was derived from ammonia (Lagerkvist, 1953), carbon 2 from CO_2 (Heinrich and Wilson, 1950) and the remainder of the pyrimidine ring was derived from aspartic acid, *i.e.* N-3, C-4, C-5 and C-6 (Reichard and Lagerkvist, 1953).

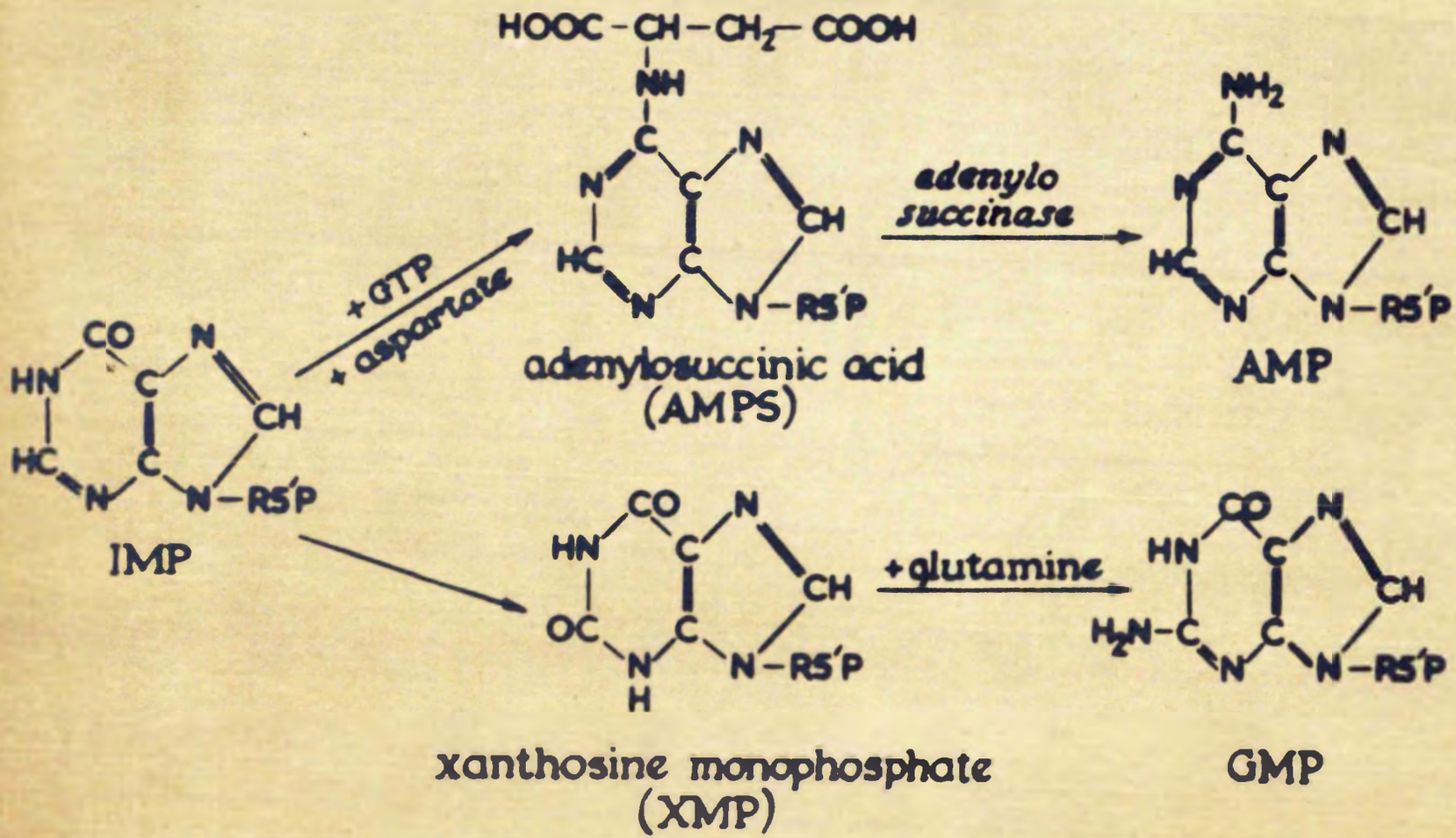

HOOC - CH - CH2 - COOH
NH
+GTP
+aspartate
adenylo
succinase
adenylosuccinic acid
(AMPS)
AMP
IMP
+glutamine
xanthosine monophosphate
(XMP)
GMP

Fig. 4

Fig. 4. Enzymic synthesis of adenylic and guanylic acids from inosinic acid.

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

More striking evidence for the existence of a unique pathway for pyrimidine synthesis *de novo* was furnished by studies on mutant strains of *Neurospora* which require, for growth, the nucleoside uridine; some of these mutants were found to utilise the pyrimidine orotic acid in place of uridine (Mitchel Houlahan and Nyc, 1948). Similarly, orotic acid is a growth factor for *Lactobacillus bulgaris* 09 and when ^{14}C-labelled orotic acid was provided in the medium, radioactivity could be recovered in the UMP and CMP of the bacterial nucleic acids while the AMP and GMP remained unlabelled (Wright *et al.*, 1951). In addition to orotic acid, 5,6-dihydroorotic acid was shown to support growth in this organism and labelled ureidosuccinic acid was utilised for pyrimidine synthesis. Experiments on extracts from yeast (Edmonds, Delluva and Wilson, 1952), rat liver (Hurlbert and Potter, 1952) and various mammalian tissues (Weed, 1951) similarly appeared to implicate ureidosuccinic acid and orotic acid in a biosynthetic pathway leading to uridine 5'-monophosphate (UMP).

In bacterial preparations the preliminary step in this sequence was shown to be the interaction of NH_3 and CO_2 in presence of ATP to form carbamyl phosphate while in rat liver systems this compound was apparently produced via an alternative route requiring the presence of an acyl-L-glutami acid (Jones, Spector and Lipmann, 1955; Reichard and Hanshoff, 1956). In what is now regarded as the initial step in pyrimidine biosynthesis *de novo* carbamyl phosphate reacts with aspartic acid to yield ureidosuccinic acid (or carbamyl-L-aspartic acid) in a reaction which is essentially irreversible (Reichard, 1954, 1957). Lieberman and Kornberg (1954,

demonstrated that cell-free extracts of Zymobacterium oroticum were capable of catalysing the interconversion of ureidosuccinic acid and orotic acid through the intermediate 5,6-dihydroorotic acid; ring closure was effected by the enzyme dihydroorotase followed by oxidation of the ensuing dihydro-orotic acid under the influence of a flavin nucleotide linked dehydrogenase (Friedman and Vennesland, 1958). The two enzymes also appeared to be present in rat liver (Wu and Wilson, 1956) and in extracts of E.coli (Yates and Pardee, 1956a) where both reactions were shown to be freely reversible.

The formation of the first nucleotide in the pyrimidine sequence, orotidine 5'-phosphate was accomplished by the reaction of orotic acid with PRPP in presence of the highly specific orotidylic acid pyrophosphorylase, thus the enzyme purified from yeast was without action on other pyrimidines (Lieberman, Kornberg and Simms, 1955). Orotidine 5'-phosphate was in turn converted to uridine 5'-monophosphate (UMP), irreversibly, by orotidine 5'-phosphate decarboxylase (Hurlbert and Reichard, 1955). The probable complete pathway is shown in Fig. 5.

Up to the present no pathway of de novo pyrimidine synthesis has emerged other than that leading to the production of UMP; all the other pyrimidines being formed either directly or indirectly from UMP and, as far as is known, UMP may be said to perform a role analogous to that played by IMP in purine nucleotide metabolism. Thus UMP is a precursor of the CMP and, ultimately, of the TMP of the nucleic acids.

Detailed pathways leading from UMP to the other pyrimidine nucleoside monophosphates have been elucidated in recent years and several detailed

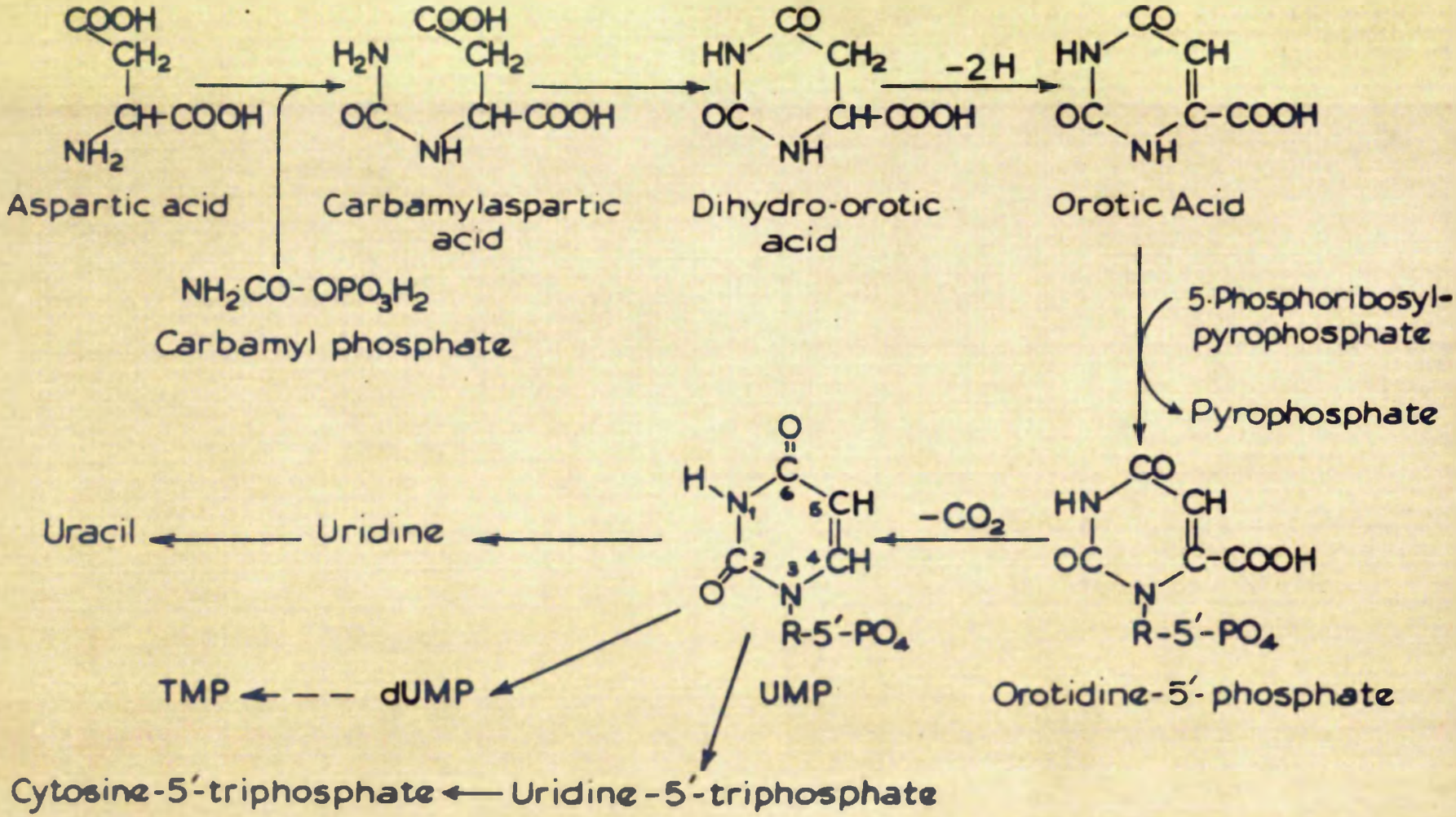

Aspartic acid
Carbamylaspartic acid
Dihydro-orotic acid
Orotic Acid
-2 H
NH2CO-OPO3H2
Carbamyl phosphate
5-Phosphoribosyl-pyrophosphate
Pyrophosphate
Uracil
Uridine
-CO2
R-5'-PO4
TMP
dUMP
UMP
Orotidine-5'-phosphate
Cytosine-5'-triphosphate
Uridine-5'-triphosphate

Fig. 5

Fig. 5. The enzymic synthesis of uridylic acid *de novo*.

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

studies have emphasized that a number of alternative routes may operate to effect these nucleotide interconversions (see Section 2. 1. (b)). The precise conditions under which cells can utilise one route in preference to others remain uncertain, neither has the question of possible species preference in the selection of such pathways been satisfactorily answered (see reviews by Crosbie, 1960 and Beesman, 1963).

That a pathway existed to convert UMP to GMP was indicated by various metabolic studies in which the extent of labelling of the nucleic acid pyrimidines was followed as a function of time after the injection of various pyrimidine precursors. Incorporation of ^{14}C-labelled orotic acid into the nuclear RNA of rat liver and rat liver slices (Hecht and Potter, 1956, 1958) showed that the ratio of the specific activity of GMP to the specific activi of UMP from the same samples eventually approached unity. Thus it would appear that part of the UMP formed by the de novo pathway is converted to GMP before being incorporated into the RNA. The exact pathway for the conversion of UMP to GMP was investigated by Lieberman (1956) with extracts of E.coli and by Kammen and Hurlbert (1959) using a cell-free supernatant fraction from Novikoff hepatoma. Both systems appeared to catalyse a direc amination at the level of UDP or UTP in the presence of ATP, amination at the triphosphate level being favoured by both sets of data, but the systems differed in that the E.coli extract required ammonia for the amination while the mammalian system utilised glutamine as donor of the amino group.

The biosynthesis of the thymidine derivatives has attracted much attention in recent years. The presence of a methyl group in the 5-positio

of the thymine ring created special problems of biosynthesis, and further interest was stimulated by the observation (Reichard and Estborn, 1951) that the thymidine nucleotides appeared to function almost exclusively as precursors of DNA.

Early studies on whole cell systems had pointed to the role of uracil and cytosine derivatives as precursors of DNA thymine; thus E.coli cells were found capable of incorporating orotic acid into DNA thymine residues (Reichard, 1949) and uracil was utilised in the synthesis of DNA thymine by thymine-less mutants of E.coli (Green and Cohen, 1957). Uridine (Ames and Magasanik, 1957) and cytidine (Rose and Schweigert, 1953) have likewise been shown to be incorporated intact into DNA thymine residues. From these and similar studies it was suggested that the de novo conversion of uracil derivatives to thymine derivatives was conducted entirely on the nucleotide level without at any stage implicating free pyrimidine bases. This conclusion was confirmed by the demonstration that ^{14}C-labelled deoxycytidine (CdR) appeared in both cytosine and thymine residues of DNA when injected into rats (Reichard and Estborn, 1951). In experiments designed to illuminat the nature and origin of the one-carbon unit which constitutes the difference between the uracil and thymine ring systems, the β-carbon of serine as well as the α-carbon of glycine were found to be incorporated extensively into the 5-methyl groups of DNA thymine in the adult rat (Elwyn and Sprinson, 1954 In addition, formate and formaldehyde could also serve as precursors in rat tissues (Totter, 1954). The evidence from such incorporation experiments pointed to a folic acid derivative as the donor of the one-carbon unit in the

formation of thymidylic acid; more specifically, there were reasons for believing N^5, N^{10}-methylenetetrahydrofolic acid to be the active intermediate in this reaction. This derivative was reported to be the product of non-enzymic interaction of formaldehyde and tetrahydrofolic acid and of serine hydroxymethylase action on serine (Jaenicke, 1956). Independent evidence to this effect was derived from the fact that low concentrations of the folic acid antimetabolite aminopterin blocked the utilisation of deoxyuridine for thymine synthesis (Friedkin and Roberts, 1956). Evidence pertaining to the nature of the one-carbon acceptor in thymidylate synthesis have come from comparisons of incorporation rates of various possible precursors into polynucleotides. Such experiments have revealed that deoxyuridine like thymidine was utilised almost exclusively for DNA synthesis while deoxyuridine itself was not incorporated into the DNA of regenerating rat liver (Reichard, 1955). Friedkin and Kornberg (1957), using cell-free extracts of E.coli, described the formation of thymidine 5'-triphosphate (TTP) from deoxyuridine 5'-phosphate (dUMP) in presence of N^5, N^{10}-methylenetetrahydrofolic acid, ATP, Mg^{++} and thymidylate kinase, thus confirming the role of deoxyuridine derivatives as the immediate precursors of TMP. The missing links in one possible route leading from UMP to TMP were provided by the discoveries of enzyme systems which catalyse the reduction of CDP to dCDP (Reichard and Rutberg, 1960) and of the specific deaminases responsible for the conversion of dCMP to dUMP (Scarano, 1958; Maley and Maley, 1959) (see Section 2. 1. (b)).

(c) Biosynthesis of the pentose moieties of the nucleic acids

D-ribose (Levene and Jacobs, 1909) and 2-deoxy-D-ribose (Levene, Mikeska and Mori, 1930) were early identified as the carbohydrate components of RNA and DNA, respectively. However, the intracellular formation of these compounds remained obscure until after the Embden-Meyerhof scheme of anaerobic glycolysis had received general recognition. At that time, an alternative pathway of glucose oxidation (Warburg, Christian and Griese, 1935; Dickens, 1938) which led to the formation of a pentose phosphate with 6-phosphogluconic acid as one of the intermediates had been discovered.

Actual investigation of the aerobic biosynthesis of pentoses was initiated about 1950 when Cohen and Horecker undertook intensive studies of the "hexose-monophosphate shunt" and identified the pentose intermediates. Preliminary studies led to the conclusion that D-ribulose-5-phosphate occupies a key position in the formation of D-ribose-5-phosphate from glucose (Scott and Cohen, 1951; Horecker, 1951). The first reaction involved was shown to be the oxidation of D-glucose-6-phosphate by $NADP^+$ in presence of the widely distributed glucose-6-phosphate dehydrogenase to form 6-phospho-D-gluconic acid (Glock and McLean, 1951; Horecker and Smyrniotis, 1953). Further studies indicated that yeast, bacteria (Scott and Cohen, 1953) and mammalian tissues (Seegmiller and Horecker, 1952) contain the enzyme 6-phosphogluconic acid dehydrogenase which in presence of $NADP^+$ catalyses the oxidative decarboxylation of 6-phosphogluconic acid and leads to the formation of D-ribulose-5-phosphate; the precise mechanism of this reaction is still obscure but it seems clear that a decarboxylation occurs at carbon atom 1

and a dehydrogenation at carbon atom 3 (Glock, 1955). The conversion of ribulose-5-phosphate to D-ribose-5-phosphate is reversibly catalysed by the widely distributed enzyme ribose-5-phosphate isomerase (Horecker and Smyrniotis, 1951; Horecker, Smyrniotis and Seegmiller, 1951).

While these studies were in progress, a number of investigations pointed to a series of anaerobic reactions in which pentose phosphates were reconverted to hexose phosphates and triose phosphates. These reactions were shown to be reversible and to involve the intermediate formation of both tetrose and heptulose intermediates. Horecker et al. (1953) implicated the thiamine pyrophosphate dependent enzyme transketolase in these reactions and showed that it catalysed the cleavage of xylulose-5-phosphate by the transfer of a ketol group to a suitable acceptor aldehyde; moreover, it was shown that ribose-5-phosphate qualified as a reaction partner giving glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate (Horecker and Smyrniotis, 1952). Confirmation of the connection between the aerobic and anaerobic pathways of pentose phosphate biosynthesis was provided by the demonstration that animal tissues and micro-organisms contain an enzyme which catalyses the isomerisation of ribulose-5-phosphate and xylulose-5-phosphate (Hurwitz and Horecker, 1956; Dickens and Williamson, 1956). In the presence of glyceraldehyde-3-phosphate, the heptulose phosphate was shown to be cleaved by the enzyme transaldolase in such a manner that its dihydroxyacetone moiety was transferred to the triose phosphate with the formation of fructose-6-phosphate and erythrose-4-phosphate (Horecker and Smyrniotis, 1955). Fructose-6-phosphate may finally be converted to glucose-6-phosphate under

the influence of phosphohexoisomerase, thus completing the pentose phosphate pathway.

The precise interactions between the transaldolase and transketolase systems and the stoichiometry of the various reactions involved have been further elucidated by Horecker and his collaborators (Horecker et al., 1956), but knowledge of the relative importance of the various alternative pathways in the production and reutilisation of ribose-5-phosphate is still rudimentary and current investigations (Ghosh and Bernstein, 1963) indicate that in almost no instance is one pathway utilised to the total exclusion of others.

Although ribose-5-phosphate may be considered as the ultimate product in the pentose phosphate pathway, this sugar requires further modification to complete the connection between de novo synthesis and the ribose of the nucleotides and the nucleic acids. Formation of ribose-1-phosphate has been shown to occur in two ways. It can be formed reversibly from ribose-5-phosphate by the action of phosphoribomutase, an enzyme which has been demonstrated in muscle (Guarino and Sable, 1955), or it can arise by the action of the enzyme nucleoside phosphorylase (Kalckar, 1947) which catalyses a reaction of the type:

$$\text{Base-ribonucleoside} + \text{inorganic phosphate} \rightleftharpoons \text{Base} + \text{ribose-1-phosphate}$$

However, as pointed out by Kalckar (1947), there are reasons for believing that the reaction favours utilisation rather than formation of ribose-1-phosphate (see Section 2. 1. (a)). In E.coli (Long, 1955) and in rat liver (Agranoff and Brady, 1956) ribose-5-phosphate can also be formed from D-ribose by the enzyme ribokinase in presence of Mg^{++} and ATP.

The discovery by Kornberg, Lieberman and Simms (1954, 1955 a, b) of the reversible reaction leading to the formation of 5-phosphoribosyl-1-pyrophosphate (PRPP) from ribose-5-phosphate has proved to be of widespread significance in the biosynthesis de novo of purine (Section 1. 2. (a)) and pyrimidine (Section 1. 2. (b)) nucleotides and also at the level of the utilisation of the preformed bases (Section 2. 1. (a)). The reaction involves transfer of the terminal pyrophosphate group of ATP to the carbon 1 of the ribose-5-phosphate, in which position the pyrophosphate unit apparently assumes the α-configuration (Remy, Remy and Buchanan, 1955). Some of the relevant reactions in the pentose phosphate pathway are illustrated in Fig. 6.

A pathway for the formation of 2-deoxyribose, not involving the intermediate formation of ribose or nucleoside derivatives, was described by Racker (1951, 1952), who demonstrated a condensation of glyceraldehyde-3-phosphate and acetaldehyde to produce 2-deoxyribose-5-phosphate. The enzyme catalysing this reaction was termed deoxyriboaldolase and was reported to occur in E.coli (Racker, 1951) and in rat liver (Boxer and Shonk, 1953). Further utilisation of the deoxyribose-5-phosphate so formed could be visualised through the formation of deoxyribose-1-phosphate by a phosphodeoxyribomutase described by Manson and Lampen (1951) and the coupling of this reaction with the nucleoside phosphorylase of Friedkin and Kalckar (1950) to form deoxyribonucleosides in the following manner:

Deoxyribose-1-phosphate + purine $\rightleftharpoons$ purine deoxyriboside + inorganic phosphate

Similar phosphorylases, present in rat liver, react with the pyrimidine thymine and uracil, though apparently not with cytosine (De Verdier and

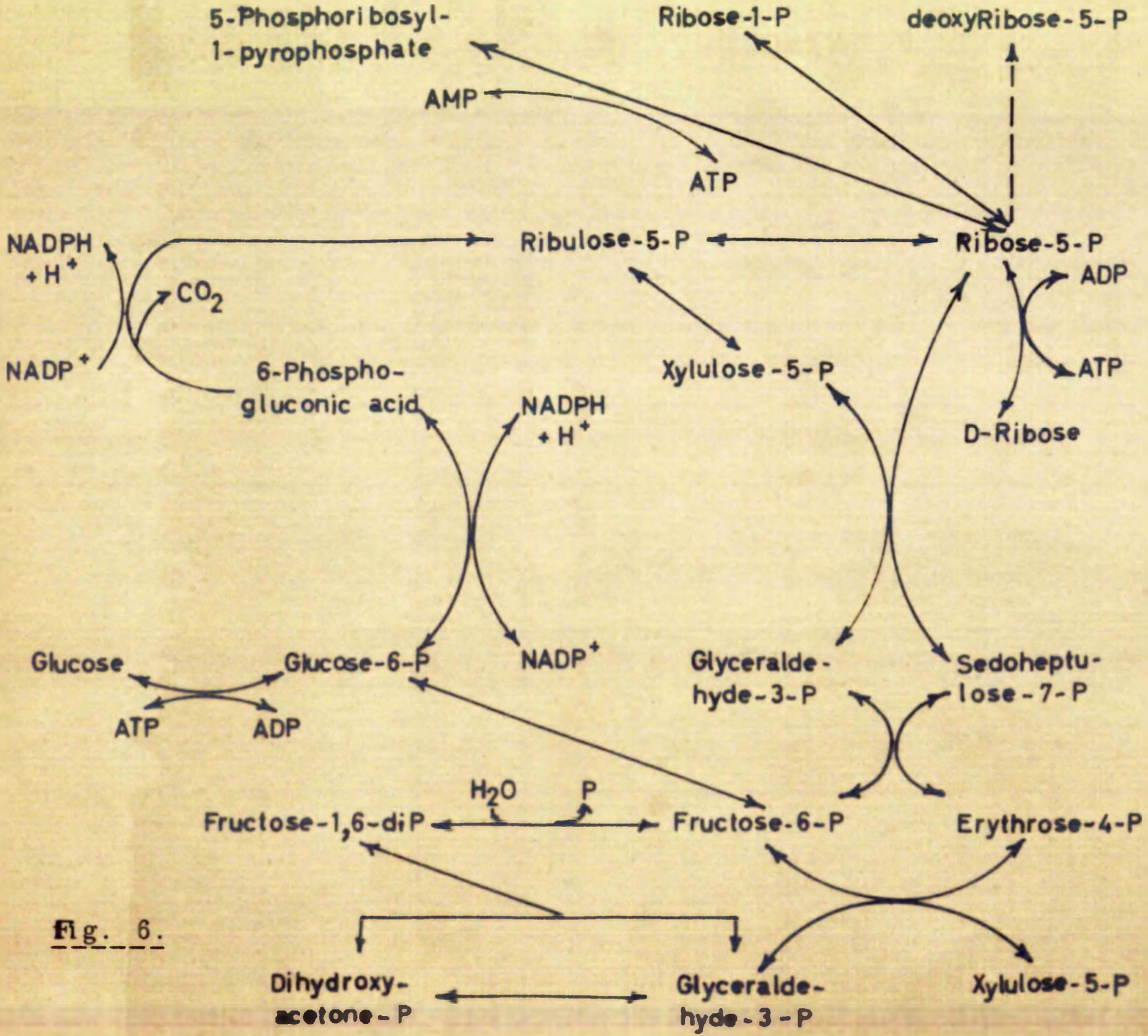

5-Phosphoribosyl-
1-pyrophosphate
Ribose-1-P
deoxyRibose-5-P
AMP
ATP
NADPH
+H+
CO2
NADP+
Ribulose-5-P
Ribose-5-P
ADP
ATP
6-Phospho-
gluconic acid
Xylulose-5-P
D-Ribose
NADPH
+H+
NADP+
Glucose
Glucose-6-P
ATP
ADP
Glyceralde-
hyde-3-P
Sedoheptu-
lose-7-P
H2O
P
Fructose-1,6-diP
Fructose-6-P
Erythrose-4-P
Dihydroxy-
acetone-P
Glyceralde-
hyde-3-P
Xylulose-5-P

Fig. 6.

Fig. 6. Some reactions in the formation *de novo* and the metabolism of ribose phosphates.

The symbol P represents ester linked orthophosphate.

(adapted from Fruton, J. S. and Simmonds, S. (1958) "General Biochemistry", 2nd Ed., Ch. 21, New York: John Wiley and Sons Ltd.)

Potter, 1960). Finally, *in vivo* studies on some growing bacterial (Lenning and Cohen, 1955; David and Jaymond, 1958) and mammalian (Ghosh and Bernstein, 1963) cell populations have been interpreted as indicating a direct reduction of ribose to deoxyribose.

The significance of the Reeker pathway for the formation of the deoxyribosyl moiety of the nucleic acids remains doubtful, particularly since the equilibrium in the transaldolase reaction is unfavourable towards synthesis (Allende and Reeker, 1963) and there are strong reasons for believing the pathway involving deoxyribose phosphates to be exclusively concerned with the degradation of the pentose molecule. Moreover, Kornberg (1957) failed to find any trace of a deoxyribosyl derivative analogous to PRPP, the active intermediate in ribonucleotide biosynthesis. The most powerful evidence against the participation of *de novo* formed deoxyribose in DNA synthesis has been the large number of reports that a direct reduction of ribose to deoxyribose can occur while in ribosidic linkage in nucleosides or nucleotides. This conclusion is supported by two main lines of evidence; the first of which concerns experiments performed with ribonucleosides or ribonucleotides labelled with ^{14}C in both the nitrogenous base and in the pentose moiety. Experiments of this type have shown that in microorganisms and in mammalian tissues such precursors were incorporated into the DNA without apparent alteration in the relative specific activities of sugar and base (Rose and Schweigert, 1953; Roll, Weinfeld and Carroll, 1956). The second line of evidence concerns attempts by enzyme studies in isolated systems to determine the precise level at which such reductions occur.

Reichard (1958) presented evidence in favour of the reduction of both cytidine and uridine at the ribonucleotide level with cell-free extracts from chick embryos, and in subsequent studies with purified enzymes from E.coli and from chick embryos it was demonstrated that the reduction of cytidine and uridine phosphates occurs preferentially at the diphosphate level (Reichard, Baldesten and Rutberg, 1961; Bertani, Häggmark and Reichard, 1961 The actual mechanism of the reduction is still a matter of some dispute, but the E.coli enzyme appears to require the presence of Mg^{++} and ATP with reduced lipoic acid as cofactor (More and Reichard, 1963). The question whether the action of this enzyme is restricted to the reduction of pyrimidin ribonucleotides is as yet unresolved, but it appears likely that the purine deoxyribonucleotides may be produced via a similar direct reduction mechanism (MacNutt, 1958); thus Reichard (1960) has presented evidence for the preferred reduction of GDP to dGDP in chick embryo extracts.

(d) Production of nucleoside di- and triphosphates

The primary products of the de novo synthesis of purine and pyrimidine nucleotides are in each case the nucleoside monophosphates; however, all the known systems that produce polynucleotides utilise as ultimate precursors either the nucleoside diphosphates or the nucleoside triphosphates and it is therefore necessary to consider the reactions that convert the monophosphates to their anabolically more useful polyphosphate derivatives.

The presence in cells of the most important of the nucleoside poly-phosphates - ATP - can largely be accounted for by such well-known sources as oxidative phosphorylation and by transfer of phosphate from 1, 3-diphospho

glycerate and phospho(enol)pyruvate to ADP. However, the free ribo- and deoxyribonucleoside polyphosphates of the purine and pyrimidine series have been discovered in tissues (see Section 2. 1. (a)) and the means by which nucleoside polyphosphates other than ATP acquire their phosphate have attracted considerable attention in recent years.

The findings of such studies have pointed to a series of transphosphorylations which can account for the distribution of phosphate from ATP to all the nucleotides, and in general, two classes of enzymes, which have been termed nucleoside monophosphate kinases (I) and nucleoside diphosphate kinases (II), may be considered as responsible for the legitimate de novo formation of nucleoside di- and triphosphates. Such reactions involve the transfer of a terminal phosphate from ATP and a requirement for magnesium ions has been demonstrated.

(I) nucleoside monophosphate + ATP $\rightleftharpoons$ nucleoside diphosphate + ADP

(II) nucleoside diphosphate + ATP $\rightleftharpoons$ nucleoside triphosphate + ADP

Muscle adenylate kinase, or myokinase, specific for adenine nucleotides was the first nucleoside monophosphate kinase to be discovered (Colowick and Kalckar, 1943; Kalckar, 1943) and was shown to catalyse the reaction:-

$$AMP + ATP \rightleftharpoons ADP + ADP$$

In the years following this discovery a large number of nucleotide phosphokinases were described exhibiting various specificities toward nucleotide acceptors; thus an enzyme partially purified from yeast (Stroming Heppel and Maxwell, 1954; 1959) catalysed the phosphorylation of AMP, GMP and UMP to the corresponding diphosphates, and a nucleoside diphosphokinase

has been investigated in yeast, muscle and intestinal mucosa (Berg and Joklik, 1954) where it was shown to convert UDP and IDP to UTP and ITP, respectively, in presence of ATP. The incorporation of deoxyribonucleoside monophosphates into DNA indicates that such tissues are capable of phosphorylating the monophosphates to the triphosphate level, presumably under the influence of deoxyribonucleotide kinases. A number of these have been described.

At present there is considerable evidence that many tissues enclose separate kinase systems specific for each of the purine and pyrimidine nucleoside monophosphates although the ability of such enzymes to distinguish between ribosyl and deoxyribosyl derivatives of a particular base remains a matter of some dispute (Bessman, 1963). In either case, the kinases that act on the thymidine nucleotides would appear to be placed in a special position in that the corresponding ribosyl derivatives have not been detected (For a more detailed discussion of the phosphokinases, see Section 3. 1.).

(e) Biosynthesis of ribonucleic acids

A number of systems have been discovered which catalyse the formation of polyribonucleotides from ribonucleotideprecursors (Grunberg-Manago, 1962; Smellie, 1963). Of the enzyme systems which have so far been described, the great majority appears to be involved in three general types of reaction; the first forms polynucleotides from ribonucleoside 5'-diphosphates with the elimination of inorganic phosphate, the second type, utilising ribonucleoside 5'-triphosphates, adds a limited number of nucleotide units to existing RNA chains, the third type which also uses the 5'-triphosphates results in

incorporation of nucleotide residues into internucleotide linkage in polyribonucleotides.

The enzyme which promotes the incorporation of ribonucleoside 5'-diphosphates into polynucleotide material, polynucleotide phosphorylase, was discovered in 1955 by Grunberg-Manago and Ochoa in extracts from Azotobacter vinelandii and upon subsequent partial purification it was shown to catalyse the formation of high molecular weight polynucleotides indistinguishable from naturally occurring RNA (Singer, Heppel, Ochoa and Gill, 1959) when examined under the scrutiny of chemical and enzymic methods of analysis. The enzyme appears to be widely distributed in bacteria and in plants (Grunberg-Manago, Ortiz and Ochoa, 1956; Littauer and Kornberg, 1957; Brummond, Stachelin and Ochoa, 1957), while the evidence for its occurrence in animal tissues is unimpressive. Extensively purified enzymes from A. vinelandii (Singer, Heppel and Hilmoe, 1960) and from E. coli (Littauer and Kornberg, 1957) have been shown to be dependent for activity on magnesium ions. Such preparations are capable of catalysing the polymerisation of ribonucleoside diphosphates in the absence of RNA primer (Beers, 1957) and they have been employed extensively for the preparation of artificial polynucleotides (Ochoa and Heppel, 1957). The function of the polynucleotide phosphorylase in bacterial cells has not yet been established.

Enzymes from a variety of sources have been shown to add nucleotide units on to the ends of existing polynucleotide chains. This reaction is now relatively well understood and appears to involve the addition of a

few ribonucleoside 5'-phosphoryl units derived from the corresponding triphosphates on to the 3'-hydroxyl ends of pre-existing ribonucleic acids of rather low molecular weight. The original observation was made by Heidelberger et al. (1956) who discovered that ^{32}P-labelled AMP was incorporated intact into the rat liver cytoplasm, and in subsequent experiments it was shown that in RNA so labelled the terminal AMP residue was preferentially linked through a cytidyl-3', 5'-cytidyl grouping to the main polynucleotide chain (Hecht, Zamecnik, Stephenson and Scott, 1958; Harbers and Heidelberger, 1959). Similar systems have been shown to exist in several other biological sources (Paterson and LePage, 1957; Herbert, 1958; Hurwitz, Bresler and Kaye, 1959). Important contributions to the understanding of the cellular function of these systems have been made by Zamecnik and his collaborators (Hoagland et al., 1960; Hecht et. al., 1959), who have shown that the end unit containing AMP provides a functional grouping in soluble RNA (s-RNA) necessary for its role as carrier of activated amino acids.

A contrasting type of reaction involves the incorporation of ribonucleoside triphosphates into the interior of polynucleotide chains. Canellakis (1957a) using a rat liver system, observed that a UMP unit derived from UTP could be incorporated into a non-terminal position, thus providing indication of extensive polynucleotide synthesis. Similarly, a system from the soluble fraction of chick embryos which incorporates AMP residues from ATP into RNA has been described (Chung and Mahler, 1959). Subsequent experiments on this system (Chung, Mahler and Enrione, 1960,

have indicated that the incorporation of adenylate residues is stimulated by the addition of GMP, UMP and CMP. The presence of systems of this type has further been demonstrated in pigeon liver microsomes (Straus and Goldwasser, 1961), ascites carcinoma (Burdon and Smellie, 1961) and in Micrococcus lysodeikticus (Nakamoto and Weiss, 1962).

While such systems show requirement for various types of RNA primer, other systems have been described dependent on added DNA and the presence of all four ribonucleoside 5'-triphosphates for optimal activity. Although such enzymes appear to be widely distributed among bacteria (Hurwitz, Bresler and Diringer, 1960; Furth, Hurwitz and Goldmann, 1961; Ochoa, Burma, Kröger and Weill, 1961; Stevens, 1961) reports from animal sources are relatively few and include only a particulate fraction from calf thymus nuclei (Abrams, Edmonds and Biswas, 1962) and a system derived from ascites tumour nuclei (Burdon and Smellie, 1962).

In the bacterial systems, at least, it appears that the added DNA plays an important part in the polymerisation, since the base ratios of the products show a close resemblance to those of the added DNA (Weiss and Nakamoto, 1961a) as do the respective nearest neighbour frequences (Weiss and Nakamoto, 1961b). The generally accepted interpretation of these results is that such systems provide a mechanism for the transfer of genetic information from DNA to a complementary strand of RNA.

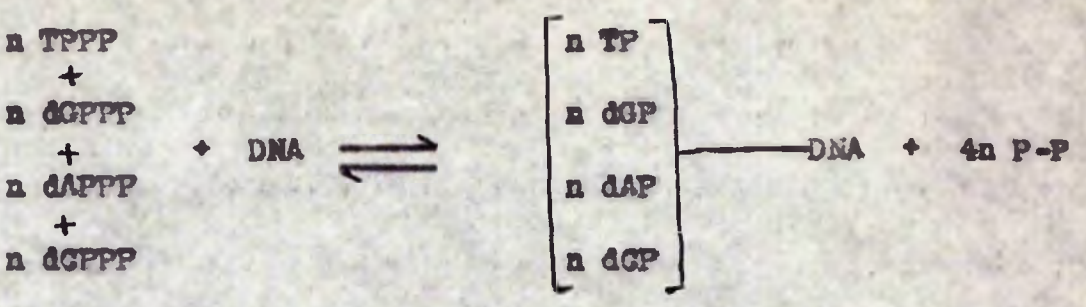
(f) Biosynthesis of deoxyribonucleic acids

DNA synthesis appears to be a process almost exclusively associated with cell proliferation and independent of the rate of cellular metabolism.

Although the strict limits of definition would tend to confine such synthesis to the ultimate polymerisation step, it seems probable that at its inception the point of diversion from the nonspecific pool of nucleotide precursors is the reduction of ribosyl to deoxyribosyl derivatives; thereafter the process proceeds as directed through the specific sequence of reactions that leads to the formation of the DNA molecule (Canellakis, 1962 ; Bessman, 1963).

Enzyme systems which catalyse the formation of polydeoxyribonucleotides appear to occur very widely and the existence of such enzymes in all cells capable of mitosis seems probable. Such enzymes have shown a strict requirement for all the main four deoxyribonucleoside triphosphates, Mg^{++} and highly polymerised primer DNA. DNA nucleotidyltransferase (DNA polymerase) was first discovered in extracts of E.coli by Kornberg, Lehman and Simms in 1956 and, subsequently, enzyme systems performing a similar role have been reported in tissue extracts from a variety of animal, plant and bacterial sources (Bollum and Potter, 1958; Herford and Kornberg, 1958; Smellie, Keir and Davidson, 1959; Monastavinos and Canellakis, 1959 ; Smellie, et al., 1960). Of all such systems, only the nuclear enzyme from calf thymus (Krakow, Coutsogeorgopoulos and Canellakis, 1961 ; Keir and Smith, 1963) which incorporates a few ribo- and deoxyribonucleotides on to the ends of existing DNA chains appears to be markedly different in properties from those ascribed to the E.coli enzyme by Kornberg and his associates. The unique properties of the latter enzyme were intensively investigated on preparations purified several thousand-fold over the original E.coli extract (Lehman, Bessman, Simms and Kornberg, 1958) and it was shown that the general

characteristics of the reaction and the requirement for net synthesis of DNA (Bessman, Lehman, Simms and Kornberg, 1958) could be expressed as follows:



The mechanism of the reaction catalysed by the E.coli DNA polymerase is viewed by Kornberg (1960) as the formation of a 3', 5'-diester linkage between two nucleotide units by an enzyme catalysed nucleophilic attack on the nucleotidyl phosphorus atom of the deoxyribonucleoside 5'-triphosphate by the terminal 3'-hydroxyl at the growing end of the new polynucleotide chain with the elimination of inorganic pyrophosphate (Fig. 7).

However, it was seen that when one of the substrates was omitted from the reaction mixture a small but significant number of deoxyribonucleotides were added on to the nucleoside ends of some DNA chains (Adler et al., 1958) whereas the presence of the full complement of deoxyribonucleoside triphosphates resulted in a ratio of product to primer of 20:1. The end product so formed proved to be indistinguishable from high molecular weight, double stranded DNA as isolated from natural sources (Schachman et al., 1958). A somewhat similar situation appears to obtain when the E.coli enzyme is incubated with either TTP and dATP or with dCTP and dGTP in the absence of primer DNA resulting in the formation of high molecular weight d-AT or d-GC polymers (Schachman et al., 1960). Subsequent product analysis has shown that the d-AT polymer so formed is composed exclusively of dAMP and TMP

Fig. 7

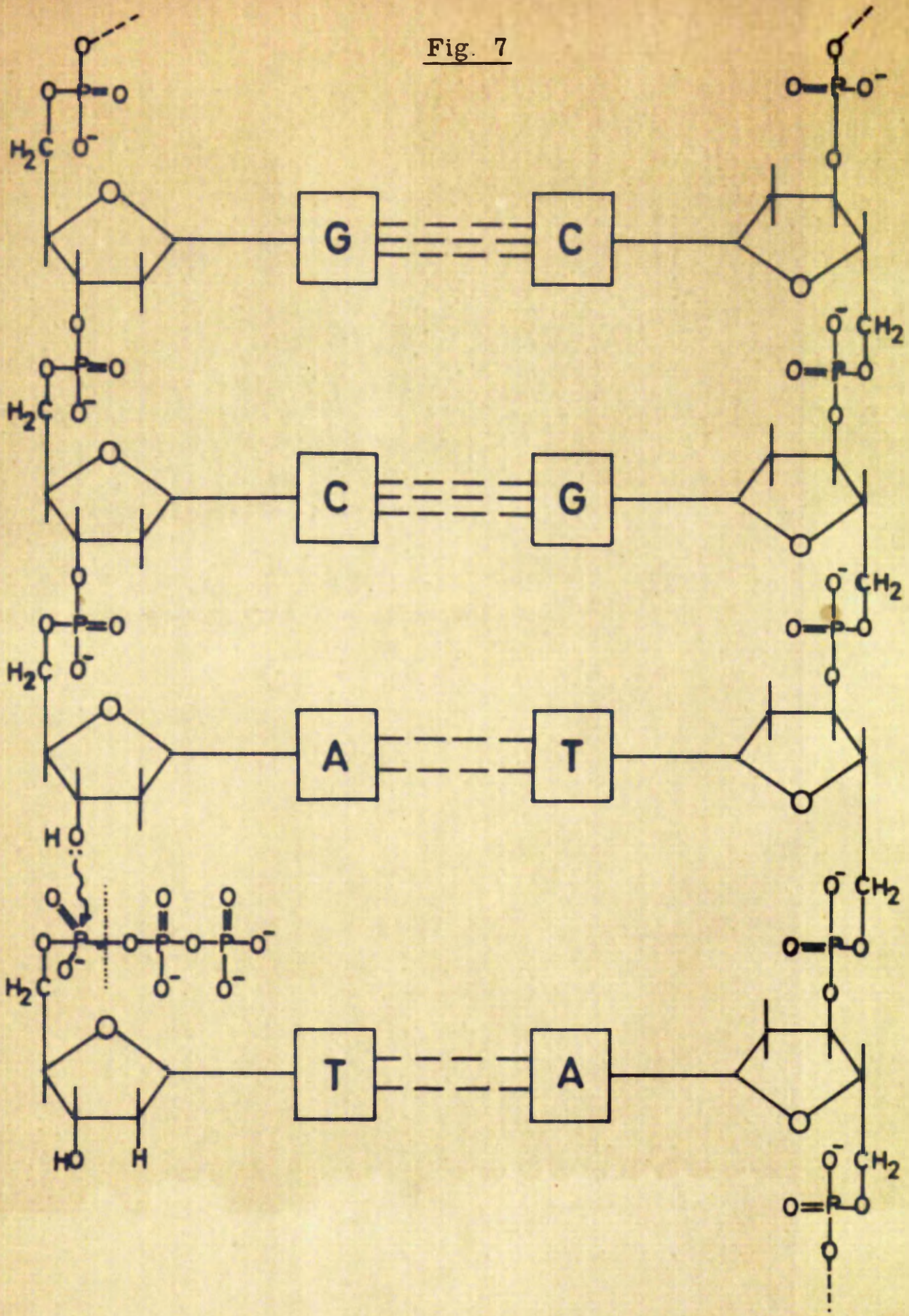


Fig. 7. Postulated mechanism for extending a DNA chain.

(adapted from Kornberg, A. (1960) Science, 131, 1503)

residues arranged in a strictly alternating sequence on both strands with adenine hydrogen-bonded to thymine as in the primed reactions. In the case of the polymer composed of dGMP and dCMP residues, the reaction again proceeds exponentially in the absence of primer DNA but the product formed differs from the d-AT polymer in that it is made up of separate strands containing dGMP or dCMP (Josse, Kaiser and Kornberg, 1961).

Subsequent investigations have focussed attention on the role played by the DNA primer in these reactions, and the findings not only helped to elucidate the mechanism of DNA replication in E.coli but they provided powerful independent support for the predictions following from the Watson and Crick (1953b) concept of the structure of DNA. Thus it appeared that the enzyme DNA polymerase receives directions from a template in the form of the DNA primer in such a way that it matches the particular purine or pyrimidine substrate which will form a hydrogen-bonded pair with a base on the template, the directions being dictated by the relationship between adenine and thymine and between guanine and cytosine. The net result appears to be that DNA functions as a template directing the synthesis of exact replicas of itself.

These conclusions were based on several lines of enzymic evidence (Kornberg, 1962); firstly, the double stranded nature of the product, secondly, it was seen that the incorporation of purine and pyrimidine analogues into DNA followed the pattern predicted by the hydrogen-bonding capabilities of these unnatural bases, thirdly, the chemical base composition of the primer DNA and the enzymic product were identical, fourthly, a

procedure developed for the determination of the 16 possible nearest neighbour base sequences showed that different DNAs direct the synthesis of unique and non-random patterns of such sequences and that the two complementary strands were of opposite polarity (Josse, Kaiser and Kornberg, 1961), and finally, the requirement of TTP, dGTP, dCTP, dATP and DNA primer for DNA synthesis. The evidence is clearly consistent with the contention that the DNA polymerase provides an appropriate mechanism for the replication of DNA.

Much less information is available on the replication of DNA in animal systems, but a DNA polymerase purified from calf thymus (Bollum, 1959, 1963) has been shown to be similar to the E.coli enzyme in many important respects. Partial purification of a DNA polymerase from ascites tumour cells has also been reported (Keir, 1962).

As to the physical state of DNA for optimal priming capacity, it was observed that when DNA is heated to 100° for 10 minutes it becomes twice as effective as a primer for the E.coli enzyme (Lehman, 1959) and, even more strikingly, when E.coli is infected with the bacteriophage T2 the native, double stranded DNA is relatively inert as a primer unless it is modified (Aposhian and Kornberg, 1962). Similarly, the single stranded DNA of the bacteriophage ϕX174 has proved to be an excellent primer for the E.coli polymerase (Lehman, 1959). It seems probable that although complete strand separation is not required for priming activity, part of the chain, at least, must be single stranded and available for base pairing with incoming precursor molecules.

It seems probable also that the replication process, in bacteria at least, proceeds sequentially in a predetermined direction starting from one end of the DNA chain (Nagata, 1963; Yoshikawa and Sueoka, 1963).

In addition to the *in vitro* studies described above, a number of investigations on whole cells have lent support to the Watson-Crick concept of replication. The experiment of Meselson and Stahl (1958) showed clearly that when *E.coli* divides, each daughter cell receives two DNA units, one old (or "conserved") unit and one newly synthesised unit. This was interpreted as indicating "semi-conservative" replication. However, in subsequen experiments with this organism Cavalieri and Rosenberg (1961, 1962) suggested that the conserved unit was not a single strand as could be expected from the *in vitro* studies, but a double helix; thus each daughter cell was presumed to receive one new and one conserved double helix joined together as a pair. It is somewhat difficult to reconcile this finding with the known behaviour of the *E.coli* polymerase unless some mechanism of strand unwinding, separation and recombination is visualised prior to mitosis. In contrast, examination of the pH melting behaviour of hybrid DNA where 5-bromouracil was employed to label one subunit has revealed that the bonds which hold the subunits together are specific and consistent with the properties expected of the hydrogen bonds of two strands of a single double helix (Shooter and Baldwin, 1962; Baldwin and Shooter, 1963), thereby suggesting that the replicating subunits are single polynucleotide strands.

1. 3.. Catabolism of the nucleotides and their substituents

In addition to the enzyme systems that catalyse the formation of

polynucleotides there appears to exist, in constant competition with the anabolic forces, a large number of catabolic systems operating at all levels of the synthetic pathways. The equilibria between the opposed forces may be modified in response to the immediate requirement of the cell with the involvement of physical as well as biological changes in the environment. Separate cellular locationsof the enzyme systems of anabolic and catabolic function can be visualised to account for specifically directed synthesis and degradation of the polynucleotides since the equilibria of both nucleotide kinases and polymerases favour synthesis.

Currently, there is reason to believe that the polynucleotides are initially degraded by ribonucleases and deoxyribonucleases, of which there are many types, to nucleoside monophosphates with an unknown number of oligonucleotide intermediate products. Little is known of the precise situations in which these degradations manifest themselves and hence the function of the enzyme systems which catalyse them, but some are undoubtedly involved in the breakdown of nucleic acid fragments so that the ensuing nucleotide units may be reutilised for polynucleotide synthesis. The ultimate products of nuclease action appear to be nucleoside monophosphates with the phosphate group either in the 5'- or the 3'-position thereby re-emphasising the role played by the nucleoside monophosphates as pivotal points in nucleotide metabolism.

The dephosphorylation of nucleoside 5'- or 3'-monophosphates and the nucleoside 5'-polyphosphates is catalysed by a number of phosphatases of low specificity abundant in many tissues. Such enzymes as intestinal phosphatase

exhibit low specificity towards both the nucleoside part and the number of phosphate groups. In such cases, the hydrolysis of polyphosphates probably occurs in a stepwise fashion, e.g., ATP → ADP → AMP → adenosine. In addition, several types of enzyme are known with relatively selective action; thus the mammalian 5'-nucleotidases when purified are capable of hydrolysing only the nucleoside 5'-monophosphates while no action can be detected on 3'-monophosphates or polyphosphates (Heppel and Hilmoe, 1951). 3'-Nucleotidases have also been reported in extracts of certain leaves and plant seeds (Heppel and Hilmoe, 1953). The nucleosides, ultimate products of phosphatase action on nucleotides, are further split to give the free base and pentose moieties, presumably by the reactions catalysed by (a) nucleoside phosphorylase or (b) by the irreversible hydrolysis effected by nucleoside hydrolase.

(a) nucleoside + inorganic phosphate ⇌ base + ribose-1-phosphate (or deoxyribose-1-phosphate)

(b) nucleoside + H_2O → base + ribose (or deoxyribose)

Both types of enzyme appear to occur widely in forms exhibiting varying degrees of specificity towards base and sugar moieties and it seems certain that purine nucleoside phosphorylases differ from pyrimidine nucleoside phosphorylases (Friedkin and Kalckar, 1961). Some of the ways in which the pentose moiety may be degraded have been outlined elsewhere (see Section 1. 2. (c)). At the level of the free bases, adenine appears to lack a deaminase in the animal body while guanine is deaminated to xanthine. Hypoxanthine is readily converted to xanthine in rat liver and xanthine, as the exit to the catabolic pathway for the purines, is converted to uric acid

(Schmidt, 1955). Further degradation does not appear to occur in man and other primates and in birds, while in other mammals allantoin is the chief excretion product. The conversion of uric acid to allantoin has been studied in detail in rat liver (Canellakis and Cohen, 1955 ; Canellakis, Tuttle and Cohen, 1955). An appraisal of the purine nucleotide interconversions relevant to the degradation of purines is shown in Fig. 8.

The catabolism of the pyrimidine bases appears to be channelled exclusively through uracil and thymine, cytosine not being metabolised vigorously in either direction, and the present evidence suggests that cytosine derivatives are catabolised via uracil derivatives in the way shown in Fig. 8 (Potter, 1960; Lichtenstein, Barnum and Cohen, 1960).

The degradation of uracil and thymine in mammalian systems (Fink, Cline, Henderson and Fink, 1956; Canellakis, 1956 , 1957b) has been shown to involve an initial reduction step with the formation of the 5, 6-dihydro-derivatives followed by ring opening to give the appropriate ureido-acid. Cleavage of the latter compounds produced β-amino acids, CO_2 and NH_3. The possibility that the amino acids so formed may obtain entry to the citric acid cycle has been visualised by Kupiecki and Coon (1957). In contrast to the pattern obtaining in mammalian cells, the catabolism of thymine and uracil in selected bacteria appears to involve an initial oxidation leading to the production of 5-methylbarbituric acid and barbituric acid, respectively (Heyaishi and Kornberg, 1958; Wang and Lampen, 1958). The pathway of further degradation in bacterial systems has remained obscure until recently (Biggs and Doumas, 1963) when it was shown that 5-methylbarbiturate was

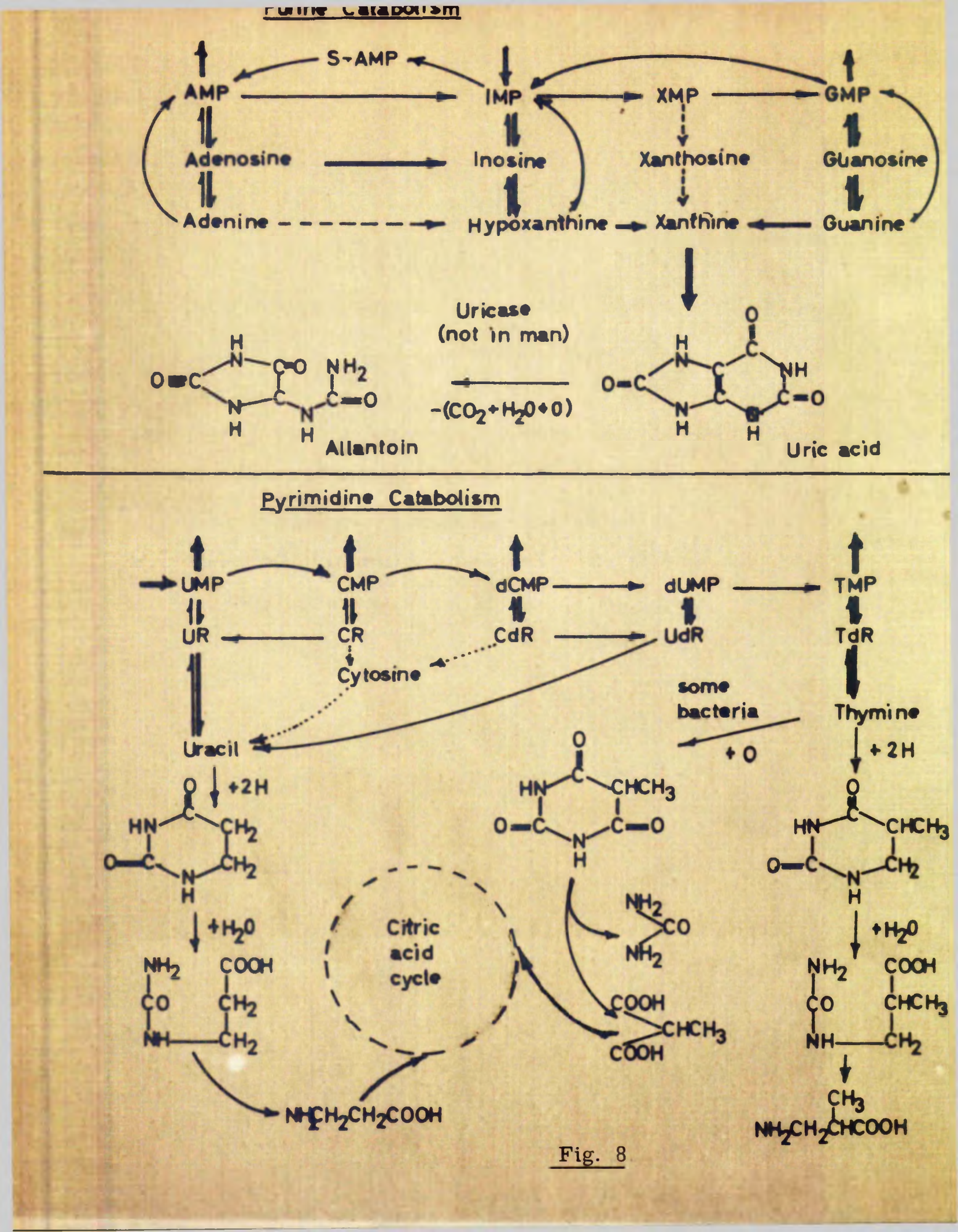

Purine Catabolism
S-AMP
AMP
IMP
XMP
GMP
Adenosine
Inosine
Xanthosine
Guanosine
Adenine
Hypoxanthine
Xanthine
Guanine
Uricase
(not in man)
-(CO2+H2O+O)
Allantoin
Uric acid
Pyrimidine Catabolism
UMP
CMP
dCMP
dUMP
TMP
UR
CR
CdR
UdR
TdR
Cytosine
some
bacteria
Thymine
Uracil
+2H
+O
+H2O
Citric
acid
cycle
NH2CH2CH2COOH

Fig. 8

Fig. 8. The major pathways in the catabolism of purine and pyrimidine nucleotides.

Thick arrows indicate exit points to anabolic and catabolic pathways. Thick half-arrows indicate the bias of the system when actively catabolising. Broken arrows represent postulated reactions or reactions of doubtful significance in higher organisms.

(adapted from Potter, V. R. (1960) "Nucleic Acid Outlines", Vol. I, Ch. VIII, Minneapolis: Burgess Publishing Company)

hydrolytically cleaved to urea and methylmalonate thus affording a possible alternative entry to the citric acid cycle.

1. 4. The control of DNA synthesis

The unique molecular properties and nicely circumscribed functions of DNA as the cellular store of genetic information has resulted in a number of important conclusions related to the nature of events in the life cycle of the cell. The property of exceptional metabolic stability, alone, predicts the existence of efficient mechanisms directed towards the protection and preservation of the cellular complement of DNA so as to ensure the viability of such progeny as receive its hereditary message. Moreover, it seems clear that the process of DNA replication, an essential prerequisite to this latter function, must be subject to an extremely rigorous set of cellular controls. Thus it is postulated that DNA synthesis must be so regulated that it provides exact replicas, identical with respect to composition, base sequence and amount to the original cellular quota of DNA. In addition to this quantitative type of control, several lines of evidence point to the existence of control mechanisms directly related to temporal events in the life cycle of the cell. This conclusion is partly based on the fact that demonstrable DNA synthesis seem to be confined to cell populations actively engaged in cell division while such synthesis appeared unrelated to the rate of general intracellular metabolism.

By the use of several recently developed techniques, such as autoradiography, quantitative microphotospectrometry and synchronous culture

techniques, it has become possible to determine to what extent intracellular DNA synthesis is subject to control; moreover, such studies have made it evident that in animal, plant and some bacterial cells the process of DNA synthesis is restricted to a discrete period in their life cycle. The evidence from studies of this type clearly points to the existence of a DNA synthesis cycle associated with the cell division cycle and the evidence clearly admits the possibility that the DNA cycle can be subdivided into certain defined periods (Howard and Pelc, 1953). In cells of higher organisms, at least, there appears to exist a period following mitosis during which no DNA synthesis takes place (G_1), a subsequent period during which DNA is actively synthesised resulting in a doubling of the nuclear DNA content (S), a second period during which no DNA synthesis can be detected (G_2) and, finally, the period of mitosis (M) (Lajtha, Oliver and Ellis, 1954; Prescott, 1960; Vendrely and Vendrely, 1956; Lark and Maalpe, 1956; Hotta and Stern, 1961; Terasima and Tolmach, 1963). In terms of the conventional concept of the cell division cycle, the S period coincides with the latter part of interphase (See Fig. 9). The duration of these periods vary from system to system, but is often remarkably constant from cell to cell in the same population. It is also possible to obtain variation in the duration of periods of the DNA cycle by various chemical and physical treatments without disturbing the sequence of events (Lark, 1963). Thus it seems clear that a temporal control mechanism exists to allow DNA replication at a stage prior to the morphologically observed preparations for mitosis and since cellular DNA synthesis appears to be a discontinuous process, this control mechanism must be capable of deciding when to initiate

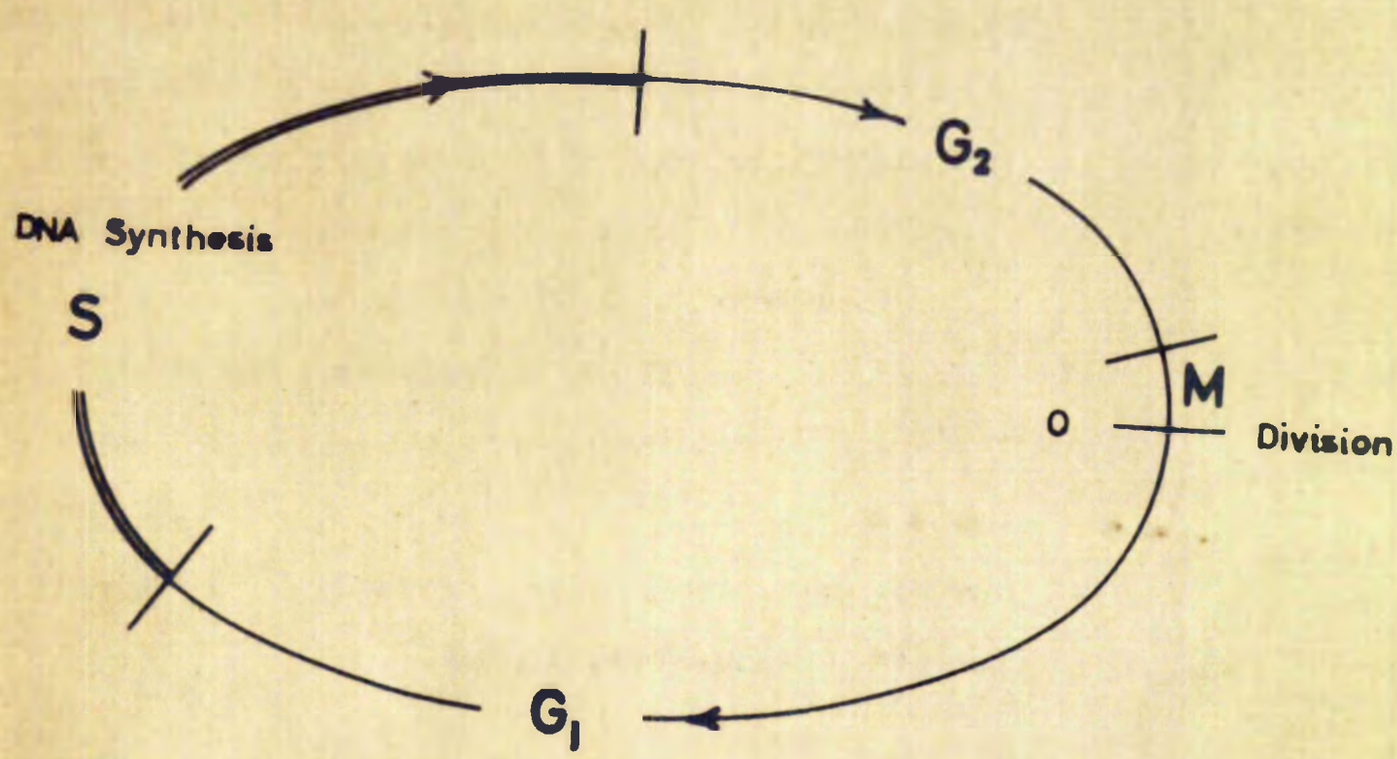


Fig. 9.

Fig. 9. A representation of the DNA synthetic cycle in relation to the cell division cycle.

In this representation, the cycle is presumed to commence at the end of mitosis (O). The early period of the cycle (G_1) in which no DNA synthesis takes place is followed by a period of DNA synthesis (S). When the replication of the nuclear DNA has been completed, there follows a period of preperation for mitosis (G_2), the cycle being concluded by the period of mitosis (M).

(Adapted from Stanmers, G. F. and Till, J. E. (1960) Biochim. biophys. Acta, 37, 406.

such synthesis. Recent evidence suggests that the importance of this type of control may lie in its ability to impose a decision which, once taken, irrevocably commits the cell to proceed towards mitosis or, alternatively, towards specialisation (Defendi and Manson, 1963).

In the search for the molecular basis of such regulatory mechanisms certain types of cellular control system have been discovered which, apart from their intrinsic significance, provide a series of basic models applicable to several different kinds of metabolic processes. These discoveries originated from studies on the fundamental interrelationship between DNA, RNA and protein; more particularly, the way in which information stored in the DNA genome is transcribed onto a strand of RNA, transferred via the "messenger" RNA to ribosomes and finally "translated" by synthesis of specific proteins (Astrachan and Volkin, 1958; Brenner, Jacob and Meselson, 1961). Studies of this type on the production of certain bacterial enzymes led Jacob and Monod (1961) to propose a general hypothesis of the intracellular regulation of protein synthesis. In their view the DNA genome is divisible into several structural genes, each responsible for controlling the formation of an appropriate transcript in the form of messenger RNA which subsequently leads to the production of a specified protein. It is further assumed that transcription is initiated only at certain points or segments on the DNA strand. These points are termed operator genes, each of which may direct the function of several structural genes. In addition, the genome is assumed to involve a gene known as the regulator which has a nucleotide sequence similar to that of the operator. The regulator is

presumed to function by forming an RNA transcript, termed the repressor, which tends to associate reversibly with the operator thus blocking the initiation of transcription with resulting inhibition of the protein forming mechanism. Such a situation would seem to imply that the system is normally repressed. The function of the repressor is, however, presumed to be effected by the presence of certain small molecules, called effectors, so as to either impede or facilitate the combination repressor-operator. In cases where the presence of the effector leads to active protein synthesis, Monod, Jacob and Gros (1962) postulate that a repressor so modified can no longer associate with the operator and the system therefore permits transcription to take place. In other systems only the effector modified repressor is functional and the presence of the effector inhibits transcription. These two types of effector are termed inducers and metabolic repressors, respectively.

The major outlines of this scheme is now firmly based on experimental evidence, at least as far as bacterial systems are concerned, while the actual molecular mechanisms involved remain purely speculative. Superficially, it would appear that the type of control outlined above is more directly concerned with the biological expression of DNA function than with the biogenesis of DNA; however, since such a system would be responsible for regulating the production of the enzymes required for DNA synthesis, it seems possible that the two mechanisms are intimately connected.

This view is supported by current knowledge of the cellular control of enzyme catalysed reactions. A number of investigations have focussed

attention on a special type of regulatory mechanism, termed feedback control, which involves cases where the reaction substrate either activates the enzyme or induces the formation of new enzyme by its influence on the enzyme forming system (positive feedback) or, similarly, where a product either inhibits the initial enzyme involved in the reaction (negative feedback inhibition) or represses the formation of the enzyme (feedback repression)(Potter and Auerbach, 1959; Potter, 1962). Induction and repression are therefore essentially positive and negative feedback mechanisms. Feedback inhibition normally refers to the situation in which accumulation of the end metabolite causes a specific inhibition of enzymes catalysing an early step in the metabolic sequence leading to its formation. The net result of this type of regulation is that excessive accumulation of a particular end product will result in decreased activity of the overall metabolic pathway leading to its synthesis. It is thought (Davidson, 1962) that this type of control mechanism may play a significant role in regulating the formation of the deoxyribonucleoside triphosphates, the immediate precursors of DNA. Negative feedback control also appears to influence the operation of the pathways for de novo synthesis of the purine and pyrimidine nucleotides. Thus both ANP and GMP were found to inhibit the initial enzyme in purine synthesis, PRPP aminotransferase (Wyngaarden and Ashton, 1959; Magasanik and Karibian, 1960; Henderson, 1962); similarly, CMP (Yates and Pardee, 1956b), CTP (Gerhart and Pardee, 1962) and pyrimidine deoxyribonucleosides (Bresnick, 1963; Ennis and Lubin, 1963) have been found to exert negative feedback control over the action of aspartate

transcarbamylase.

The control of cellular proliferation in mammalian tissues is currently being investigated in terms of the basic biochemical processes leading to the synthesis of DNA and the means by which critical steps in the biosynthetic sequence may be regulated by interaction of products. Although some relationships between enzymic reactions examined in growing and non-growing tissues have been observed, there is yet no indication of the critical point at which control is initiated or a mechanism by which it is operated. However, a considerable body of evidence appears consistent with the view that part of the regulatory mechanism, at least, is associated with the synthesis of deoxyribonucleoside triphosphates and there are some indications that the synthetic system responsible for the formation of thymidine triphosphate (TTP) may be rate limiting in the production of substrates for the DNA polymerase. Under these circumstances, it seemed of value to re-examine some aspects of the enzymic reactions leading to the formation of TTP from thymidine and TMP using extracts from rapidly proliferating mammalian cells.

C H A P T E R II

"STUDIES ON THE FORMATION OF THYMIDINE 5'-TRIPHOSPHATE WITH ENZYMES FROM LANDSCHUTZ ASCITES TUMOUR CELLS."

2.1. Introduction

(a) The existence of cellular pools of free nucleotides

The introduction and development in the early 1950s of chromatographic techniques made it possible to isolate and determine extremely small quantities of cellular components. The classical experiments of Cohn (1950, 1951) introducing column ion-exchange chromatography to the field of nucleic acids led to the identification and separation of the major nucleotide components of these polymers. In the following few years, these techniques were further developed by others and applied not only to nucleic acid digests but also to dilute acid extracts of cellular material. As a result of such studies, it became apparent that, in addition to the already well documented adenine nucleotides (Embden and Zimmerman, 1927), there existed in many cells cytoplasmic pools composed of a large variety of free nucleotides. Thus the 5'-mono-, the 5'-di- and the 5'-triphosphates of cytidine, guanosine and uridine were found to occur in the acid soluble extracts of rat tissues (Schmitz, Hurlbert and Potter, 1954; Hurlbert, Schmitz, Brumm and Potter, 1954) and in extracts of other types of tissue (Schmitz, 1954; Bergkvist, 1956; Ballio, Cesinovi and Serlupi-Crescenzi, 1956). In the course of these investigations there appeared a number of other hitherto unsuspected nucleotides, the majority of which were coenzymes, so that by 1958, Henderson and LePage could report over 110 separate nucleotides isolated from biological sources. Measureable quantities of the pyrimidine deoxyribonucleoside 5'-mono, 5'-di- and 5'-triphosphates were found in rat thymus tissue (Potter, Schlesinger, Beuttner-Janusch and Thompson, 1957) and in extracts of other rat tissue (Schneider, 1955;

Ord and Stocken, 1958) while no trace of purine deoxyribonucleotides could be detected. Determination of the pool size of a purine deoxyribonucleotide has been recorded by LePage (1957) who found small quantities of dATP in extracts of Flexner-Jobling carcinoma.

Potter (1960) considers that the nucleotides so far discovered follow certain rules and fall into a limited number of categories. In general, it appears that every nucleotide that occurs as a major component of nucleic acids will also be found as the 5'-monophosphate, 5'-diphosphate and the 5'-triphosphate in the acid soluble fraction, and that there is also likely to occur a series of compounds that can be considered as derivatives of these three forms and are likely to possess coenzyme function.

It seems clear that the presence of pools of free nucleotides in various parts of the cell may facilitate the synthesis of nucleic acids. Cells, therefore, need not depend solely on de novo synthesis of nucleotides to sustain polymerisation but can command a store of nucleotide material from which nucleic acid precursors may be directed to the site of synthesis. However, it seems apparent that while material from such a nucleotide pool could be expected to support relatively extensive RNA synthesis, the cellular deoxyribonucleotide pool, frequently of the order of 0.1% of the total nucleotide content of the DNA, is very small in comparison and extensive de novo synthesis or conversion of ribonucleotides to deoxyribonucleotides is required to sustain the process of DNA replication. The measure of control of DNA synthesis exercised by the size of the deoxyribonucleotide pool has been intensively investigated on synchronously growing cells of various types.

Stern (1961), using a system from Trillium erectum, has shown that the deoxyribonucleotides of guanine, adenine, cytosine and thymine appear in the fluid surrounding the microspores shortly before DNA synthesis commences. A similar situation appears to exist in developing amphibian embryos (Kuriki and Okazaki, 1959). However, in some rapidly dividing synchronous cell populations such as regenerating rat liver no significant accumulation of deoxyribonucleotides is observed (Rotherham and Schneider, 1958) so that the evidence for control of DNA synthesis by the size of the deoxyribonucleotide pool remains unconvincing.

(b) Internucleotide metabolism

The intracellular pools of nucleotides (see above) are composed of nucleotides in various states of phosphorylation, nucleosides and, sometimes, of free purine and pyrimidine bases; all must be capable, when required, of transformation into a form suitable for incorporation into the nucleic acids. Present evidence suggests that these conversions occur in a non-random fashion and that the cell can direct nucleotide material entering the pool via the de novo sequences through certain specified pathways leading finally to the nucleoside 5'-triphosphates.

The existence of kinases capable of phosphorylating AMP, GMP, dAMP, dGMP, UMP, CMP, dCMP and TMP (see Sections 1. 8 (d). and 3. 1.) to their respective triphosphates have now been described and it is generally presumed that the reactions occur in a stepwise manner involving the intermediate formation of nucleoside diphosphates. In addition to such sequential phosphorylations, several pathways for the interconversion of nucleotides

have been described, the primary function of which is to ensure the production of distal nucleic acid precursors at the prescribed site and at the required time. Excessive production of any one precursor appears to result in an increase in the catabolic activity relative to that precursor (Roth, 1963).

Much less is known about the interconversions occurring among the purine than pyrimidine nucleotides. Particularly as regards the formation of the purine deoxyribonucleotides present knowledge is scanty and the scheme presented in Fig. 10 must therefore be regarded as somewhat tentative. However, the formation of the two RNA precursors ATP and GTP proceeds through well documented routes from IMP itself formed de novo, and interconversion between AMP and GMP through the cycles where the two nucleotides are reconverted to IMP has been demonstrated in many tissues (Guarino and Yuregir, 1959; Mager and Magasanik, 1960). Several investigations have pointed to the occurrence of deoxyadenosine kinases capable of forming dATP from AdR (Klenow and Lichtler, 1957; Maley and Maley, 1960), but no indication emerged from such studies as to the way in which deoxyadenosine derivatives are derived from the ribonucleotide pool. In a recent report, Larsson (1963) has shown that purified enzyme preparations from E. coli and from Novikoff hepatoma which catalyse the reduction of CDP, UDP and GDP, also reduce adenosine ribonucleotides to deoxyribonucleotides, presumably at the ADP level, but the evidence seems to suggest that ADP is not a primary substrate for the reduction system so that interconversion of adenine and AdR under the influence of a purine deoxyribonucleoside phosphorylase remains the only well established pathway (DeVerdier and Gould, 1963). Direct reduction of guanosine ribonucleotides to deoxyribonucleotides at the diphosphate level

Fig. 10. The metabolism of purine ribonucleotides and deoxyribonucleotides in relation to the synthesis of nucleic acids.

s-AMP = adenylosuccinic acid (6-(succinylamino)-9-(ribofuranosyl 5'-monophosphate) purine).

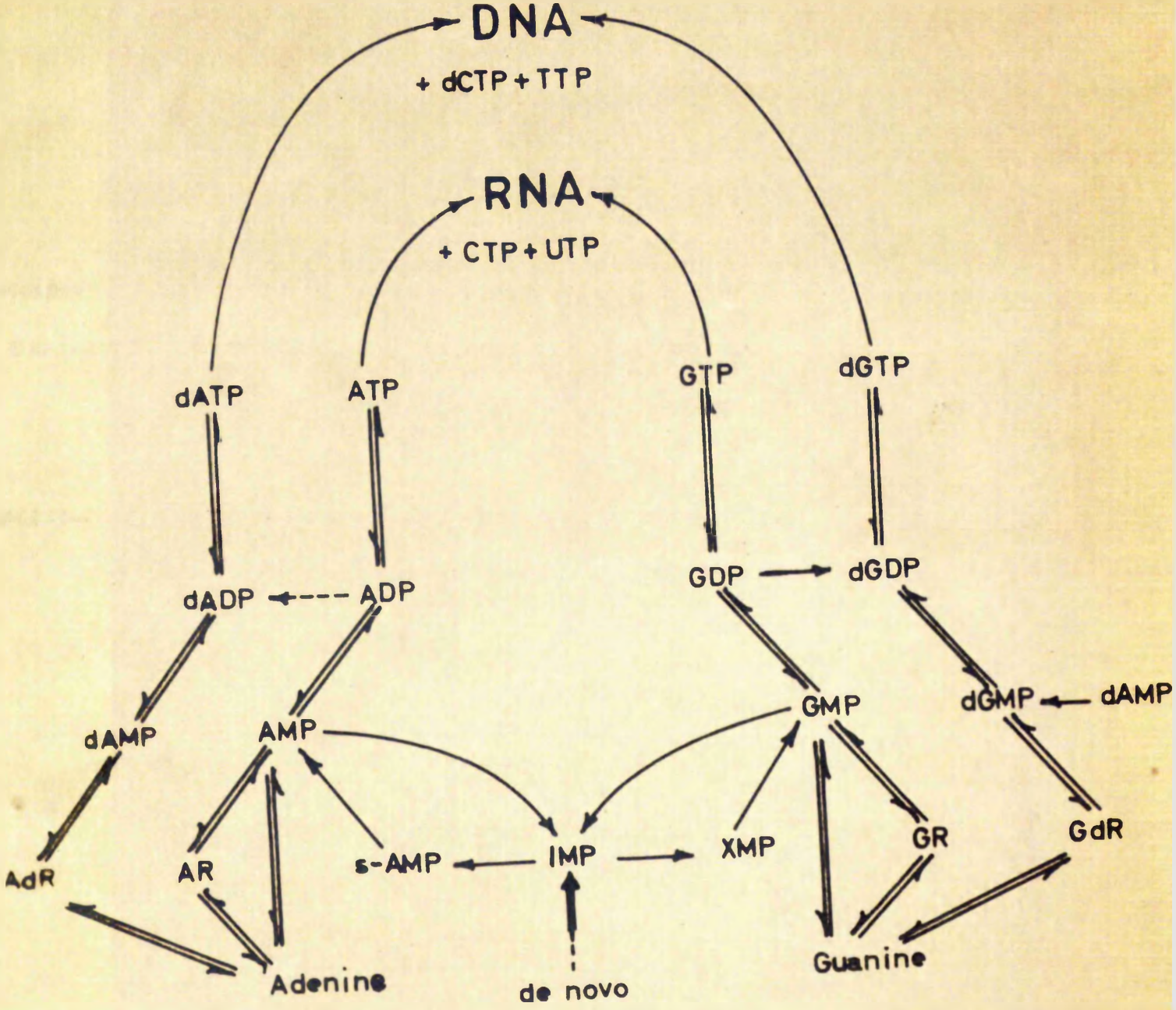

DNA
+ dCTP + TTP
RNA
+ CTP + UTP
dATP
ATP
GTP
dGTP
dADP
ADP
GDP
dGDP
dAMP
AMP
GMP
dGMP
dAMP
AdR
AR
s-AMP
IMP
XMP
GR
GdR
Adenine
de novo
Guanine

Fig. 10.

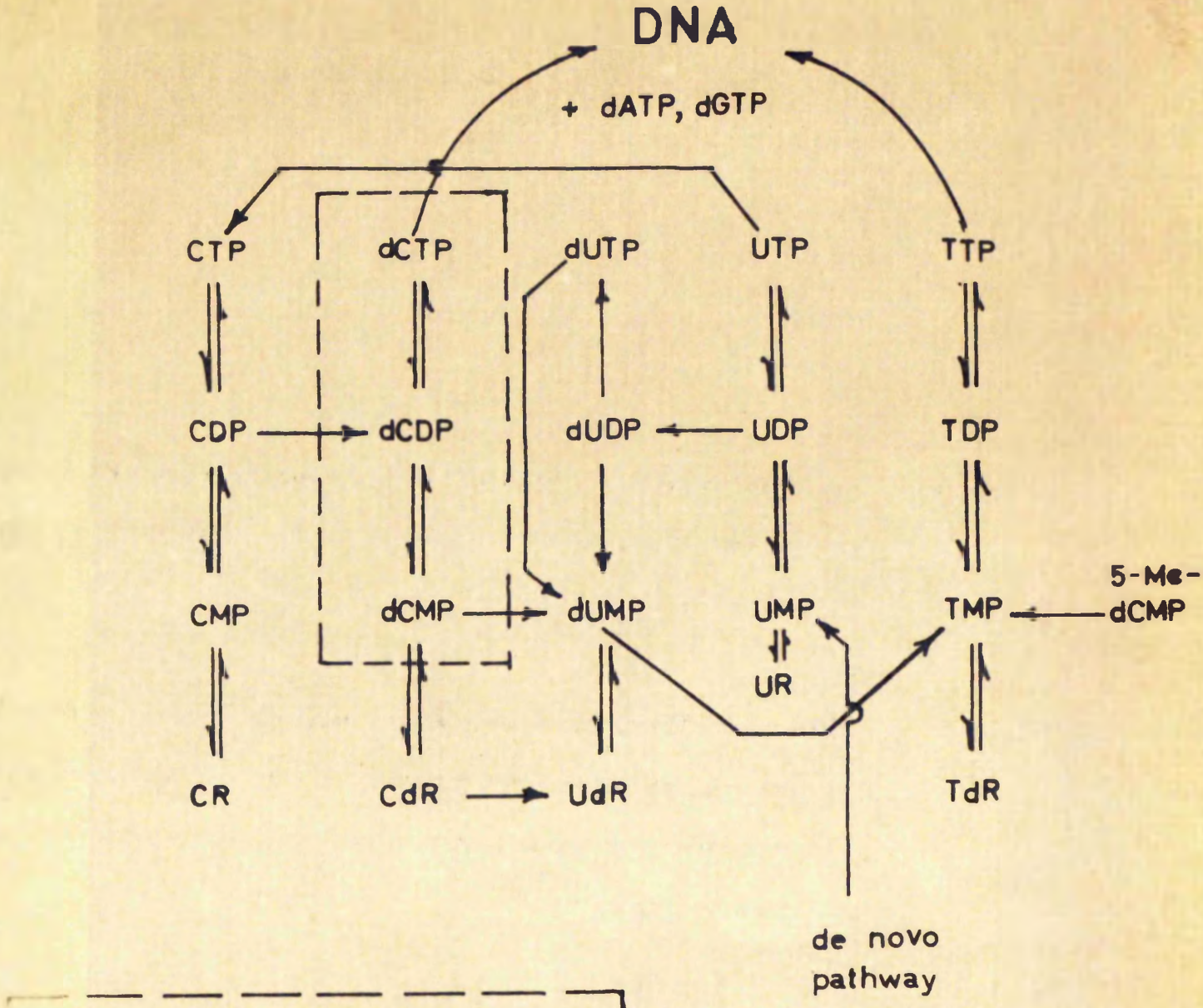
has been demonstrated with extracts of chick embryos and preparations from E.coli (Reichard, 1961) and deoxyribonucleoside phosphorylases catalysing the conversion of guanine to GdR have been reported. Finally, Maley and Maley (1961a) have found evidence of a direct conversion in chick embryo mince of dAMP to dGMP. At present, there is little indication as to which of these interconversions, if any, represent the main intracellular pathway for the formation of the purine deoxyribonucleotides.

A somewhat clearer picture has emerged over recent years of the interconversions operating in the pyrimidine nucleotide pool. Several pathways appear to promote the formation of the pyrimidine deoxyribonucleotides, all arising by ramifications of the route leading from UMP formed de novo, and the deoxyribonucleotides so formed are subsequently phosphorylated to the level of phosphorylation required by the polymerising system (see Fig. 11). The conversion of UMP to CMP by amination at the UTP level has already been described (see p.16). However, in many systems, it seems probable that the conversions of ribonucleotide to deoxyribonucleotide are critical steps and responsible for the bulk of the cellular DNA precursor material. Enzymes catalysing the reduction of CDP to dCDP have been demonstrated in a variety of bacterial (Reichard, 1962) and mammalian (Moore and Hurlbert, 1962; Abrams, Libenson and Edmonds, 1960)(see also p.24 cell extracts. Conversion of dCMP to dUMP has been shown to be prominent in several types of rapidly growing mammalian cells (Scarano, 1960; Schneider and Rotherham, 1961; Maley and Maley, 1961b) while some recent evidence points to the possibility that embryonic tissues may utilise an alternative

Fig. 11. The metabolism of pyrimidine ribonucleotides and deoxyribonucleotides in relation to the synthesis de novo of DNA.

Several alternative pathways are illustrated, only a limite number of which may operate in any one type of cell, whi reactions of mainly catabolic significance have been disregarded.

The insertion illustrates the changes in the metabolism of cytosine deoxyribonucleotides resulting from infection of Escherichia coli with bacteriophage T2.


DNA
+ dATP, dGTP
CTP
dCTP
dUTP
UTP
TTP
CDP
dCDP
dUDP
UDP
TDP
CMP
dCMP
dUMP
UMP
TMP
5-Me-
dCMP
UR
CR
CdR
UdR
TdR
de novo
pathway

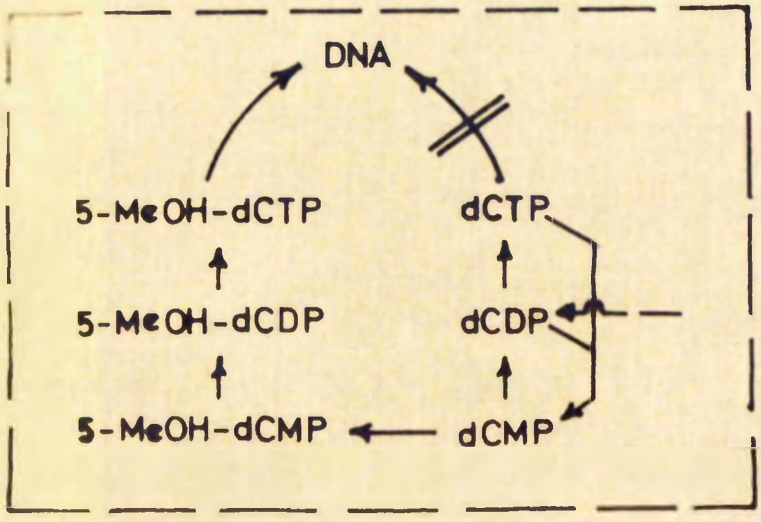

DNA
5-MeOH-dCTP
dCTP
5-MeOH-dCDP
dCDP
5-MeOH-dCMP
dCMP

Fig. 11.

deamination reaction. Maley and Maley (1962a) have shown that extracts of rat embryos catalyse the incorporation of ^{14}C from ^{14}C-labelled CdR into DNA thymine to a greater extent than into DNA cytosine and in subsequent experiments they (Maley and Maley, 1962b) have indicated that the utilisation of CdR in several organisms involve a direct deamination of CdR to UdR followed by a phosphorylation step to give dUMP. Bacteria appear to lack the ability to form dUMP from dCMP. Beesman et al. (1958) observed that the purified E.coli DNA polymerase could incorporate dUTP into DNA in place of TTP, but since no uracil was found in the "natural" DNA it was thought that its absence was due to the lack of kinases capable of phosphorylating dUMP to dUTP. Using cell-free extracts of the same organism, Reichard and Rutberg (1960) provided evidence for a direct reduction of uridine ribonucleotides to uridine deoxyribonucleotides thus indicating the existence of a third pathway for the production of dUMP. The purified CDP $\longrightarrow$ dCDP reductase system from E.coli has recently been shown to act also on UDP resulting in the formation of dUDP (Bertani, Hüggmark and Reichard, 1961, 1963). In the view of Hüggmark (1963) the dUDP so formed is rapidly transformed by a kinase to dUTP followed by a specific pyrophosphatase catalysed breakdown to dUMP. A series of kinases capable of phosphorylating dUMP to dUDP and dUTP has recently been reported in T2-infected E.coli, but again production of dUTP was controlled by the presence of a dUTP specific pyrophosphatase (Greenberg and Somerville, 1962). The precise sequence and mechanism of these reactions remain uncertain, but they appear to provide an efficient means of producing dUMP in systems where deaminases catalysing

either dCMP → dUMP or CdR → UdR are lacking.

That de novo formation of the thymidine nucleotides occur principally through the methylation of dUMP to TMP is now well established and the mechanism of the reaction has been elucidated in great detail by the use of highly purified enzyme preparations and sophisticated analytical techniques. Early studies on calf thymus extracts (Blakley, 1957) suggested that UdR was a better substrate than dUMP in the reaction, while more highly purified preparations (Greenberg, Nath and Humphreys, 1961) established dUMP as the acceptor of the one-carbon unit; formaldehyde and tetrahydrofolate were required as co-factors. The purification of an E.coli thymidylate synthetase with similar properties has been described (Wahba and Friedkin, 1962).

An alternative pathway which at one time was thought to contribute to the thymidine nucleotide pool was the direct formation of TMP from 5-Me-dCMP by deamination. In experiments with sea urchin eggs, Scarano, Bonaduce and DePetrocellis (1960) showed that 5-Me-dCMP was the preferred substrate for the dCMP deaminase of that tissue. Together with earlier methylation experiments (Scarano and Maggio, 1959) which demonstrated the tetrahydrofolate dependent formation of 5-Me-dCMP from dCMP, the evidence suggested an alternative to the dUMP → TMP pathway. However, several subsequent experiments by Maley and Maley (1961c) have failed to reveal the presence of a 5-Me-dCMP synthetase in other similar tissues and they conclude that the pathway can at best be a very minor one in comparison with the dCMP → dUMP → TMP conversion. It is also important to note that formation of the 5-Me-dCMP of wheat germ DNA has been demonstrated

to occur by methylation at the polynucleotide level (Gold, Hurwitz and Anders, 1963a).

Some of the changes in the sequence and direction of nucleotide interconversions that can occur in response to viral infection are nicely demonstrated by the example of the T2 bacteriophage-infected E.coli system. In the T2 phage DNA, the dCMP residues are replaced by 5-MeOH-dCMP residues and it seems clear that the latter are not the result of hydroxymethylation in situ but are instead incorporated from the corresponding 5'-triphosphate. A series of new enzymes is sequentially induced in the infected system to secure the formation of this material while excluding dCTP from being incorporated. In this new situation, dCTP is being continuously degraded to dCMP by a newly formed specific pyrophosphatase (Kornberg, Zimmerman, Kornberg and Josse, 1959). This is followed by the hydroxymethylation of dCMP by another new enzyme which requires tetrahydrofolate (Flaks and Cohen, 1959) and the ensuing 5-MeOH-dCMP further leads to the synthesis of 5-MeOH-dCTP under the influence of a new kinase system (Somerville, Ebisuzaki and Greenberg, 1959). The main alterations in internucleotide metabolism due to T2 infection of an E.coli system is shown in the insertion on Fig. 11.

(c) Formation of nucleotides by preformed pathways

Even before the discovery of the de novo pathways of purine and pyrimidine ribonucleotide synthesis, it was well known that many types of cell were capable of utilising exogenously supplied purine and pyrimidine bases and their nucleosides for nucleic acid synthesis. Bacteria were generally observed to be more active in this respect than higher organisms and later

experiments have shown that animal cells frequently cause rapid breakdown of such compounds (Brown and Roll, 1955).

Present evidence admits the existence of at least three types of enzymic reaction which together constitute the pathways for utilisation of preformed purine and pyrimidine bases. Using adenine as the example, the following reactions appear to apply:-

(a) Ribonucleoside phosphorylase

Adenine + ribose-1-phosphate $\rightleftharpoons$ adenosine + orthophosphate

(b) Ribonucleotide pyrophosphorylase

Adenine + PRPP $\rightleftharpoons$ adenosine 5'-monophosphate + pyrophosphate

(c) Ribonucleoside phosphokinase

Adenosine + ATP $\rightleftharpoons$ adenosine 5'-monophosphate + ADP

In general, the equilibria of these reactions appear to favour degradation, but under special circumstances when the de novo pathways are not effective these reactions offer an alternative means by which the cell can acquire nucleotide precursors for nucleic acid synthesis (see review by Friedkin and Kalckar, 1961).

The specificity of nucleoside phosphorylases is not yet well documented but there can be no doubt that the purine phosphorylases differ from the pyrimidine phosphorylases. Thus the purified purine ribonucleoside phosphorylase from horse liver (Korn and Buchanan, 1956) formed adenosine, guanosine and xanthosine from adenine, guanine and xanthine, respectively, but was without action on pyrimidines. Several relatively specific nucleoside phosphorylases have been reported to act on uracil and thymine without being

active on purines (Friedkin and Roberts, 1954; Pontis, Degerstedt and Reichard, 1961). Ribonucleotide pyrophosphorylase activity has been demonstrated with adenine, guanine and hypoxanthine as substrates (Kornberg, Lieberman and Simms, 1955b) and a similar enzyme catalysing the production of UMP from uracil has been reported (Kornberg, 1957); cytosine and thymine were not utilised (see also Fig. 8).

A further two reactions of a somewhat more tenuous nature utilising the free bases have been reported. One is the deoxyribosyl phosphorylase observed in extracts of rat liver (Friedkin, 1950) (d) and the other, the trans-N-deoxyribonucleosidase described by MacNutt (1958) (e) which catalyses an exchange of base moieties of deoxyribonucleosides without forming the free deoxyribose-1-phosphate.

(d) Deoxyribonucleoside phosphorylase

Adenine + deoxyribose-1-phosphate + ⇌ deoxyadenosine + orthophosphat

(e) Trans-N-deoxyribonucleosidase

Adenine + deoxyuridine ⇌ deoxyadenosine + uracil

The importance of reaction (e) may be to offer yet another alternative pathway for nucleotide interconversion. This was illustrated by the use of bacterial extracts (Beck and Levin, 1962) where vitamin B_{12} deprived cultures showed greatly enhanced trans-N-deoxyribonucleosidase activity between deoxyadenosine and thymine, presumably because the lack of vitamin B_{12} caused an inhibition of the direct reduction of ribonucleotides to deoxyribonucleotides. DeVerdier and Potter (1960) described a similar activity in rat liver but could not resolve the relation between transferase activity and that due to

reaction (d). Their work was continued by Zimmerman (1962, 1963) who purified extensively the thymidine phosphorylase from rat liver and showed that the ratio of transferase to phosphorylase activity did not alter throughout the purification thus suggesting that the two activities were the property of a single enzyme.

(d) Thymine ribonucleoside and thymidine 5'-diphosphate coenzymes

A large number of investigations have rested on the assumption that the thymidine nucleotides operate exclusively as precursors of DNA. However, the recent observation that thymine occurs in two other classes of compounds has drawn attention to the possibility of alternative thymine utilisation in some organisms. These two types of derivative are the thymine present in S-RNA and the thymidine 5'-diphosphate coenzymes. From the point of view of the thymidine derivatives available at any time for incorporation into DNA, it is clearly important to consider to what extent such alternative utilisation may affect the progress of replicative DNA synthesis.

Trace amounts of thymine was reported (Dunn, 1959) in bacterial RNA preparations and it has subsequently been found in several other types of tissue (Dunn, 1961) principally in association with S-RNA. Enzymic degradation of such preparations revealed that thymine appeared in non-terminal positions in the polyribonucleotide chain and that the nucleotide unit was in fact derived from thymine ribonucleoside. The biosynthesis of this latter compound had been reported by DeVerdier and Potter (1960) in an exchange reaction between thymine and uridine catalysed by extracts of rat

liver and Dunning hepatoma and thus indicating the existence of a trans-N-ribonucleosidase; with liver preparations this reaction was more prominent that the phosphorylase activity. A similar type of reaction has been observed with thymine requiring mutants of E.coli (Maestavinos and Lamenhof, 1961). The further utilisation of thymine ribonucleoside remains a mystery since it seems clear that no kinases analogous to the thymidine kinases capable of forming the thymine ribonucleoside 5'-triphosphate can be demonstrated in extracts of E.coli. The preformed 5'-triphosphate, on the other hand, has been shown to be incorporated into RNA specifically in place of UMP using a purified DNA dependent RNA polymerase (Kahan and Hurwitz, 1962).

Another approach to the problem was adopted by Mandel and Borek (1961) who in the course of studies on the methionine requiring auxotroph E.coli $K_{12}W$-6 observed that the methyl group of RNA thymine appeared to be derived from methionine without involving the intervention of dUMP or derivatives of tetrahydrofolic acid. In subsequent experiments, they (Mandel and Borek, 1963a) showed that when the methionine requiring auxotroph was grown in a medium containing ^{14}C-methyl labelled methionine, the specific activity of the methionine used and the specific activity of the methylated components ultimately derived from the s-RNA, were identical, thus demonstrating a direct transfer of the methyl group from methionine to the RNA. Thus it would appear that the thymine in RNA and the same base intended for incorporation in DNA are produced by entirely different pathways which would therefore not compete for the cellular pool of thymidine nucleotides.

The origin of all the methyl groups of s-RNA from the same pool and

the synthesis, under certain conditions (Mandel and Borek, 1963b), of S-RNA lacking these minor components pointed to the possibility that methylation occurs *in situ* after synthesis of the polynucleotide. The existence of enzyme systems which catalyse the methylation of S-RNA at the polynucleotide level has been demonstrated in bacterial extracts (Fleissner and Borek, 1962; Svensson, Boman, Eriksson and Kjellin, 1963). Although a specific function has not yet been ascribed to the minor, methylated base, component of S-RNA, it now seems certain that methylation proceeds in a non-random manner and exhibits clear species specificity thus suggesting that the methylated bases may be another criterion for distinguishing S-RNA molecules (Srinivasan and Borek, 1963; Gold, Hurwitz and Anders, 1963b). Recent work has indicated that the nucleolus may be the site of S-RNA methylation (Birnstiel, Fleissner and Borek, 1963).

A further class of thymine derivative was discovered in extracts of *E.coli* in 1959 by Okazaki, Okazaki and Kuriki. The isolated compounds appeared to be thymidine-linked sugars and the first two were tentatively identified as TDP-D-glucose and TDP-D-rhamnose (Okazaki, 1959). Following the discovery of these new thymidine nucleotides, a number of additional TDP-linked sugars and aminosugars were identified from several types of bacteria and plants while none could be detected in animal cells. A number of enzyme systems have recently been described catalysing the formation of thymidine-linked sugars from thymidine 5'-triphosphate and a variety of phosphorylated sugars, including α-D-glucose-1-phosphate (Kornfeld and Glaser, 1961; Pazur and Shuey, 1961; Kalina and Avigad, 1963),

α-D-galactose-1-phosphate (Pazur and Anderson, 1963; Frydman, Neufeld and Hassid, 1963) and α-D-glucosamine-1-phosphate (Kornfeld and Glaser, 1962). In addition, it has been shown that interconversion of sugars may occur while linked to TDP indicating the coenzyme function of the nucleotide; thus both TDP-glucose (Okazaki, Okazaki, Strominger and Michelson, 1962) and TDP-galactose (Pazur and Anderson, 1963) are convertible to TDP-rhamnose through certain relatively well defined pathways. Almost in every case the sugars linked to thymidine are the same as those reported as linked to uridine in other organisms and it is probable that these coenzymes are in some way involved in forming the cell wall lipopolysaccharides of those organisms (Okazaki, Strominger and Okazaki, 1963).

Several features of these reactions remain very doubtful but it is evident that in organisms where such TDP-linked compounds occur, there will exist alternative pathways competing for the cellular pool of thymidine nucleotides. However, no TDP coenzymes have yet been reported to occur in mammalian cells and it seems clear that such cells, at least, the thymidine nucleotide pool functions specifically in the role of providing precursors for DNA synthesis.

(e) Formation of thymidine nucleotide phosphates

Current concepts on the synthesis of DNA prescribe the deoxyribonucleoside 5'-triphosphates of adenine, guanine, cytosine and thymine as substrates in the polymerisation reaction. However, in many systems purine and pyrimidine deoxyribonucleosides and deoxyribonucleoside 5'-monophosphates have been shown to be utilised in the synthesis of polydeoxyribonucleotides

provided the requirements for ATP and Mg^{++} are fulfilled, and it seems probabl that deoxyribonucleoside and deoxyribonucleotide precursors present in such systems are converted to deoxyribonucleoside triphosphates prior to incorporation into the DNA. The formation of triphosphates in such systems have been attributed to a series of phosphokinases which catalyse the transfer of phosphate from ATP to nucleoside and nucleotide substrates (see Section 1.2 (d)). In recent years, considerable interest has centred on the cellular activities of the deoxyribonucleotide kinases as possible controlling or rate regulating factors in DNA synthesis.

Kinases capable of phosphorylating dGMP and TMP to dGTP and TTP have been described in extracts of regenerating rat liver (Hecht, Potter and Herbert, 1954) and Canellakis and Manstavinos (1958) reported that such extracts also catalysed the formation of dATP and dGTP from dAMP and dGMP. However, the interesting observation was made that while regenerating rat liver clearly could supply the full complement of deoxyribonucleotide kinase activities necessary for the formation of all the four triphosphates, normal liver appeared to be deficient or lacking in its ability to phosphorylate TMP (Manstavinos and Canellakis, 1959). The regenerating rat liver system of Bollum and Potter (1959) and cell-free supernatant fluids derived from ascites tumour cells (Smellie, Keir and Davidson, 1959) have been shown to utilise thymidine in the synthesis of DNA <u>in vitro</u> thus indicating the presence of thymidine kinase, in addition to deoxyribonucleotide kinases (Keir and Smellie, 1959), in these tissues.

The ability of enzyme preparations from ascites tumour cells to catalys

the formation of TTP from TdR or TMP is very limited (Keir and Smellie, 1959; Gray et al., 1960; Ives, Morse and Potter, 1962) particularly in comparison with their capacity to form dATP, dGTP and dCTP (Keir and Smellie, 1959; Gray et al., 1960). Similarly, while extracts of non-proliferating tissues contain active enzymes catalysing the formation of dATP, dGTP and dCTP from the corresponding deoxyribonucleoside 5'-monophosphates, the activity of the kinase system responsible for the formation of TTP is invariably low (Gray et al., 1960). However, the universality of this type of correlation is in some doubt since the TMP kinase activity in extracts of E.coli was found to be independent of the growth rate of the culture (Bessman, 1959).

Fractionation experiments on the thymidine and thymidylate kinases of ascites cells led Weissman, Smellie and Paul (1960) to suggest that the formation of TTP from TdR occurred by the stepwise addition of orthophosphate forming successively TMP, TDP and TTP thus:-

$$TdR \rightleftharpoons TMP \rightleftharpoons TDP \rightleftharpoons TTP$$

However, from experiments on the time course of phosphorylation of TdR, Bianchi, Butler, Crathorn and Shooter (1961) have proposed that the mechanism involves the addition of orthophosphate to TdR to yield TMP followed by the addition of pyrophosphate to yield TTP directly. TDP observed in the reaction products was considered to arise from the degradation of TTP after prolonged incubation. This mechanism may be illustrated as follows:-

$$TdR \rightleftharpoons TMP \rightleftharpoons TTP \longrightarrow TDP$$

The present investigation represents an attempt to characterise relatively fully the thymidine kinase and thymidylate kinase systems derived from cell-free extracts of Landschutz ascites tumour cells and to distinguish between the two reaction mechanisms postulated for the enzymic conversion of TdR and TMP to TTP.

2.2. Experimental

2.2.1. Preparation of crude cell-free extracts from Landschutz ascites tumour cells

Crude extracts of Landschutz ascites-tumour cells were prepared by the procedure of Smellie, Keir and Davidson (1959) with some minor modifications. Landschutz ascites carcinoma was maintained by serial transplantation in albino mice of the departmental colony and harvested 8 - 9 days after inoculation. The ascitic fluid was withdrawn from several animals under aseptic conditions and pooled in an ice-cooled flask. The fluid was centrifuged at 200 - 300 x g for 5 minutes to separate the cells from the plasma. The resulting sediment was resuspended in 5 - 10 volumes of chilled 0.1 M phosphate buffer, pH 8.1, and again centrifuged at 200 - 300 x g to separate the tumour cells from erythrocytes. This washing procedure was repeated several times until the sediment of tumour cells was free from erythrocyte contamination. The washed cells were again resuspended in phosphate buffer and centrifuged at 600 x g for 10 minutes to pack them tightly in the centrifuge tube. To this pellet of cells was added 10 - 15 volumes of ice-cold distilled water and the cells were then disrupted by gentle passage in a Potter-type homogeniser (Potter and Elvehjelm, 1936). 3 - 4 Passes of the material were sufficient for adequate breakdown of cells. Microscopic examination with the aid of a solution of crystal violet in citric acid was used as control of the efficiency of the process. The procedure effected rupture of most cells without destroying many nuclei.

The resulting suspension was centrifuged in a Spinco Model L ultracentrifuge at 105,000 x g for 90 minutes to yield a clear extract. The extract thus prepared had a protein concentration of 3 - 6 mg. per ml. In some experiments, this crude extract was used immediately as a source of thymidine and thymidylate kinases but, in general, these enzyme activities were preserved by rapid lyophylisation of the extract and storage at - 50°.

2.2.2. Chemical preparation of (^{32}P) - labelled thymidine 5'-phosphates

(a) Preparation of (^{32}P) - labelled thymidine 5'-monophosphate ((^{32}P) TMP)

(^{32}P) TMP was prepared by the method of Tener (1961) from thymidine and (^{32}P) - labelled 2-cyanoethylphosphate. The latter compound was either obtained commercially (The Radiochemical Centre, Amersham) or prepared in the laboratory from carrier-free (^{32}P) - labelled orthophosphoric acid and 2-cyanoethanol in the presence of dicyclohexylcarbodiimide (DCC). An outline of the reaction is given on Fig. 12, Stage I.

Preparation of (^{32}P) 2-cyanoethylphosphate ((^{32}P) CEP)

A solution containing 50 mc of carrier-free (^{32}P) - labelled orthophosphate was mixed with 1 mmole of unlabelled phosphoric acid in dilute acid solution to permit randomisation of (^{32}P). This solution was concentrated to dryness *in vacuo* in a rotary evaporator at 40° to remove traces of hydrochloric acid. The resulting labelled phosphoric acid was dissolved in 10 ml. of anhydrous pyridine (freshly distilled and stored over calcium hydride) and 1 ml. of 2-cyanoethanol was added. The solution was then evaporated to an oil *in vacuo* at 40°. The residue was redissolved in a

Fig. 12.

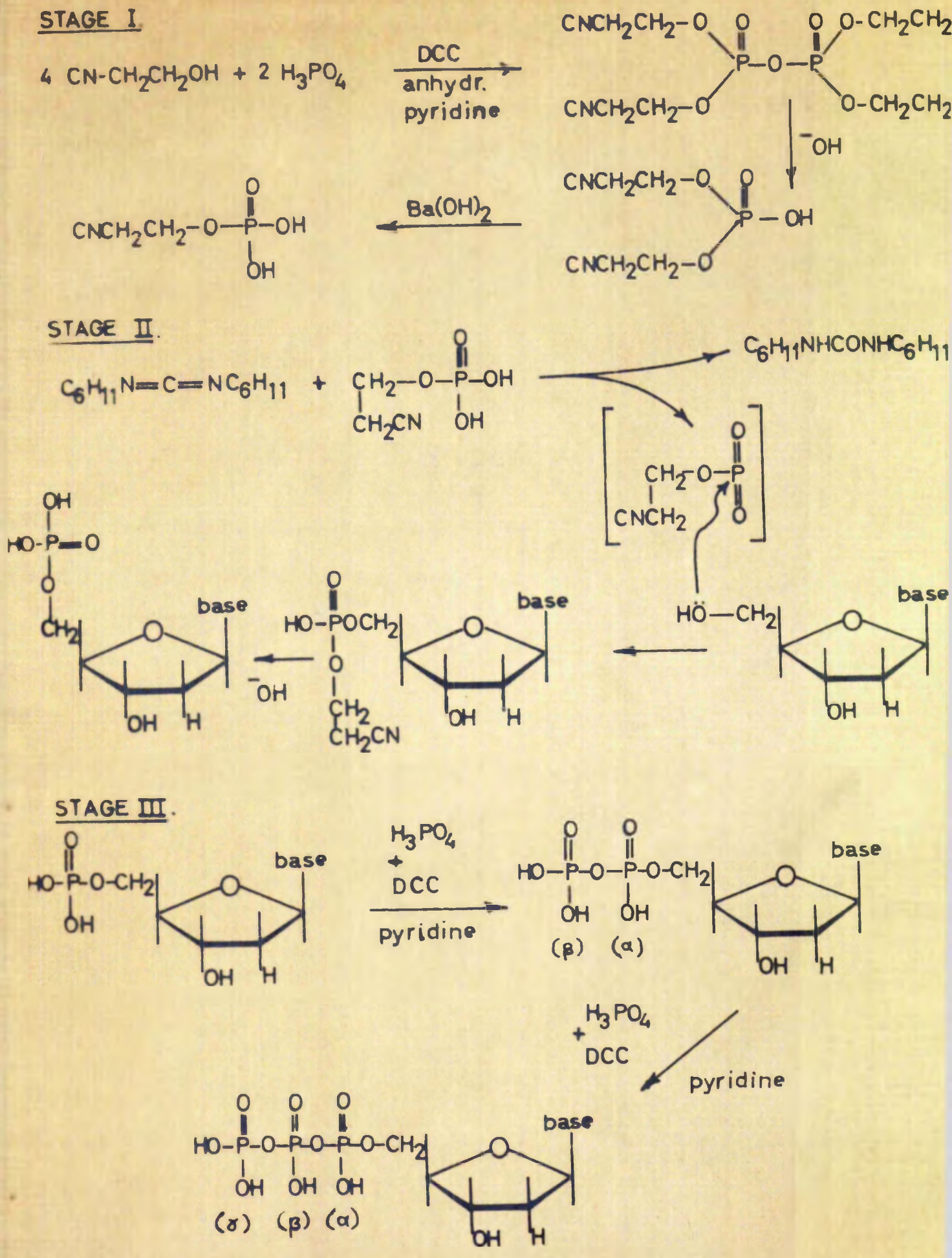

STAGE I
4 CN-CH2CH2OH + 2 H3PO4
DCC
anhydr.
pyridine
Ba(OH)2
STAGE II.
C6H11N=C=NC6H11
C6H11NHCONHC6H11
base
STAGE III.
H3PO4
DCC
pyridine
(γ) (β) (α)

Fig. 18. Outline of the chemical synthesis of deoxyribonucleoside 5'-polyphosphates.

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-cyanoethylphosphate.

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.

further 10 ml. of anhydrous pyridine and again concentrated to an oil.

This oily material was dissolved in 5 ml. anhydrous pyridine and 2.1 gm. of dicyclohexylcarbodiimide (DCC) was added. The reactants were carefully mixed and the securely stoppered reaction flask left overnight at room temperature.

Distilled water (5 ml.) was added to terminate the reaction and the resulting mixture heated on a boiling water-bath for 30 minutes. The contents of the flask was then taken to dryness in vacuo at 40° and 10 ml. of distilled water and 10 ml. of saturated barium hydroxide added to the residue. After standing for 10 minutes at room temperature, the pH of the solution was adjusted to 7.5 with glacial acetic acid. The mixture was then filtered at the pump to remove the precipitate containing dicyclohexylurea (DCU) and barium phosphate. The precipitate on the filter was thoroughly washed with distilled water and the washings added to the bulk of the solution. Two volumes of 95% ethanol was added to the solution to precipitate the (^{32}P) barium 2-cyanoethylphosphate. The solution was stirred and allowed to crystallise for one hour at 0°. The CEP crystals so obtained were collected by centrifugation and the ethanol supernatant was discarded. The crystals were redissolved in 5 ml. of distilled water with the addition of a few drops of dilute acetic acid. The resulting solution was neutralised with saturated barium hydroxide and any trace of undissolved material removed by centrifugation. The CEP was reprecipitated with two volumes of ethanol and the mixture transferred to a pre-weighed centrifuge tube. The CEP was then washed with ethanol, acetone and finally ether and allowed to dry at room temperature. The amount of CEP so

prepared (Ba salt, monohydrate) as determined by reweighing of the test tube was 800 mg. This represents a yield of 65 per cent.

The barium salt of CEP must be converted to the free acid prior to its use in the synthesis of TMP. This was achieved by redissolving the crystals in a small volume of distilled water (10 ml.) using acetic acid to assist solution . This solution was then passed through a column of Dowex-50-H^+ (10 x 1 cm., dia.) (for preparation, see Section 2.2.8 (c)) and the column washed with water until most of the radioactivity was in the effluent. This effluent now contained the free acid of CEP contaminated with some acetic acid. The solution was finally taken to dryness *in vacuo* at 40° to remove the acetic acid and the residue dissolved in pyridine.

Preparation of (^{32}P) TMP

The formation of TMP from thymidine and 2-cyanoethylphosphate is outlined on Fig. 12, Stage II. For the preparation of (^{32}P) TMP, the following reaction mixture was set up:

Thymidine (2 mmoles) was dissolved in pyridine and 1 mmole of (^{32}P) CEP (prepared as described above) in pyridine was added. When only smaller amounts of 2-cyanoethylphosphate were available, the molar proportions of thymidine : CEP of 2 : 1 were maintained. The reactants were thoroughly mixed and the solution concentrated to an oil *in vacuo* at 40°. Anhydrous pyridine (10 ml.) was then added and the solution again concentrated to an oil *in vacuo*. This procedure was repeated one more time and the residue was dissolved in 10 ml. of anhydrous pyridine to which was added 620 mg.

of DCC. The flask was shaken until all DCC had dissolved, then securely stoppered and left for 18 hours at room temperature.

The reaction was terminated by the addition of 5 ml. of distilled water and, after 30 minutes at room temperature, 10 ml. of concentrated ammonium hydroxide (specific gravity 0.880) was added and the solution heated at 60° for one hour. The resulting mixture was concentrated to dryness *in vacuo* at 40° and the residue extracted with 10 ml. of distilled water. Removal of the precipitated DCU was effected by filtration at the water pump. The DCU was washed carefully on the filter with several small volumes of distilled water, the washings being combined with the original filtrate.

At this stage a sample of the filtrate was taken for descending chromatography according to the method described elsewhere (see Section 2.2.3 (b)) to assess the relative proportions of the thymidine, thymidine 3'-monophosphate and thymidine 5'-monophosphate present. An approximate assessment of the thymidine 5'-monophosphate produced could be obtained by elution of all the ultraviolet light absorbing spots by capillary flow in water and measuring their respective extinctions at 267 mμ (pH 2); the percentage of TMP-5' relative to the total extinction could thus be estimated.

The combined filtrate and washings (see above) was then run on to a column of Dowex-1-Cl⁻ (30 x 2 cm., dia.) and subjected to gradient elution. Removal of unreacted nucleoside was effected by washing repeatedly with distilled water until the extinction at 267 mμ in the effluent approached zero. TMP was then recovered from the column by repeated washings with

0.05 N hydrochloric acid. Some radioactivity was retained at the top of the column after this treatment and this was discarded since it probably contained a mixture of radioactive orthophosphate, polyphosphate and thymidine polyphosphates.

Approximately 60% of the TMP in the reaction products was TMP-5' and the remainder was accounted for as TMP-3'. TMP-3' is not phosphorylated in the chemical preparation of TTP as described below and may therefore be regarded as an undesirable contaminant. Similarly, in experiments where (^{32}P) TMP itself was used as substrate for the thymidylate kinases, steps were taken to remove contaminants and the TMP-5' was separated from TMP-3' as follows: The bulk of the effluent from the Dowex-1-Cl$^-$ column (see above) was diluted to 500 ml. and adsorbed on a second column of Dowex-1-Cl$^-$ (20 x 1 cm., dia.) and the column washed with water to remove traces of ultraviolet light absorbing unadsorbed material. The TMP-5' and TMP-3' were eluted from the column by gradient elution with 1000 ml. of distilled water in the mixing vessel and 0.06 N hydrochloric acid in the reservoir, collecting fractions of 10 ml. with an elution rate of 0.5 ml. per minute. The gradient so obtained was non-linear and similar to that described by Hurlbert, Schmitz, Brumm and Potter (1954). Two radioactive peaks possessing extinction at 267 mμ were recovered, the first of which contained the TMP-5' and the second contained the TMP-3'. Complete seperation of these two peaks was achieved by this procedure as shown in Fig. 13. Fractions from both peaks were seperately combined and evaporated to dryness *in vacuo* at 25°. The residues were each extracted with 5 ml. of distilled water and the

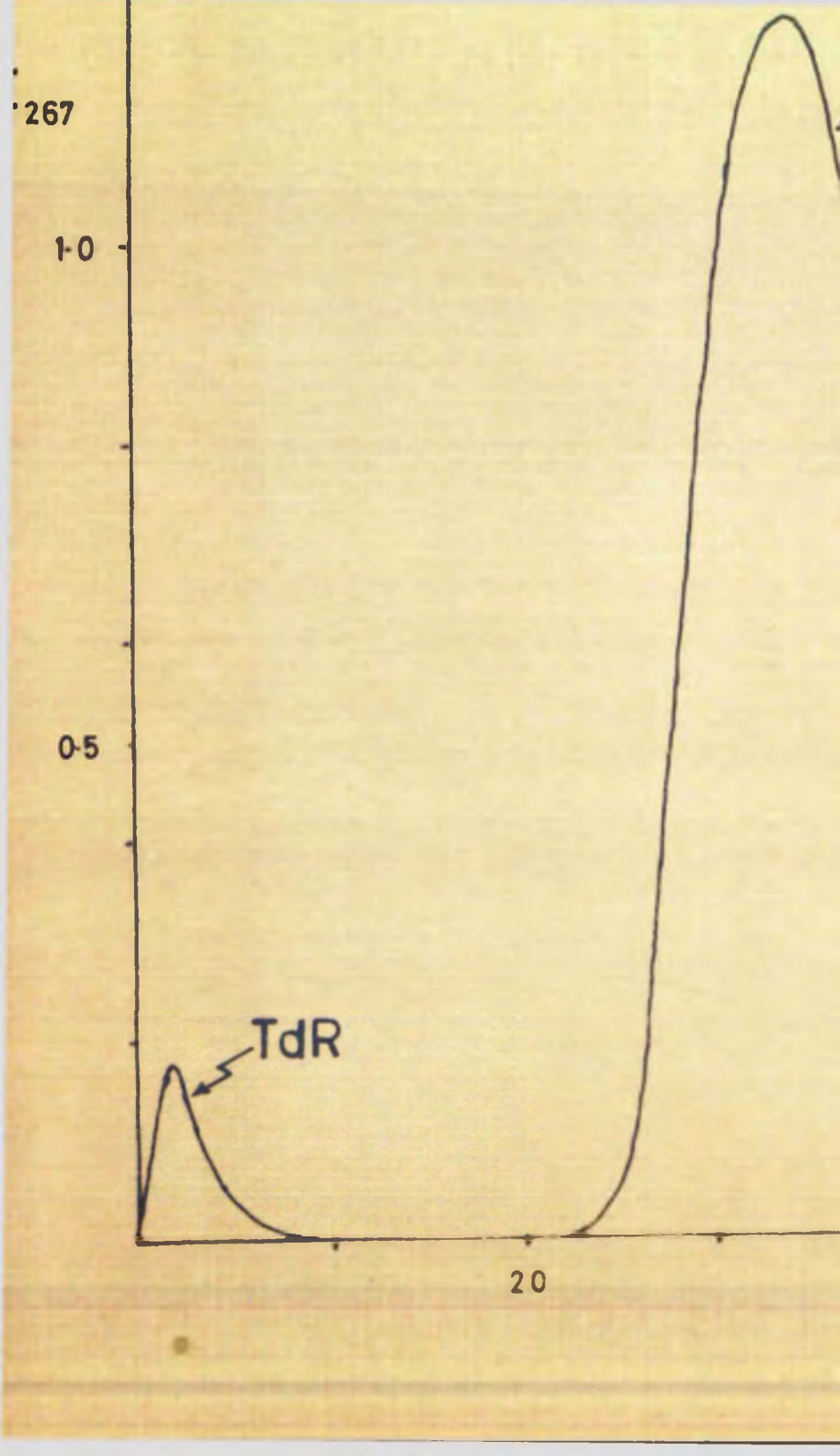

1·0
0·5
TdR
20

Fig. 13.

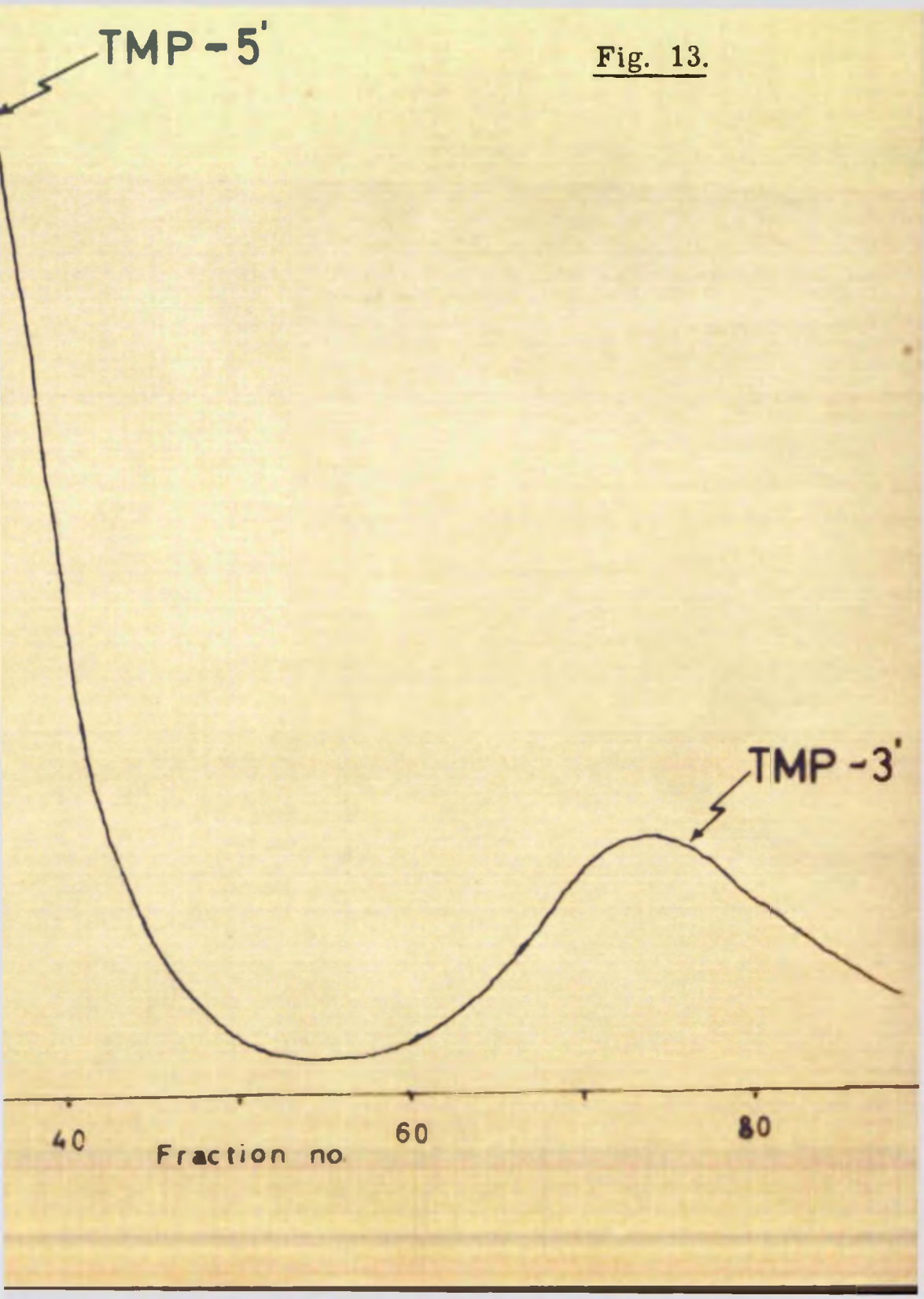


Fig. 13. Separation of the reaction products in the chemical synthesis of (^{32}P) -labelled thymidine 5'-monophosphates from (^{32}P) 2-cyanoethylphosphate and thymidine.

The nucleotide mixture was adsorbed on a column of Dowex-1-Cl⁻ (20 x 1 cm., dia.) and elution was effected by applying to the column a non-linear gradient with 750 ml. distilled water in the mixing vessel and 0.06 N hydrochloric acid in the reservoir. Fractions of 8 ml. were collected at a column flow rate of 1 ml. per minute. The eluted fractions were scanned spectrophotometrically at 267 mμ. Three peaks were obtained representing TdR, TMP-5' and TMP-3', in that order. Fractions 25 - 50 were combined and found to contain 12.8 μmoles of TMP-5' while fractions 57 - 107 contained 7.6 μmoles of TMP-3'; the recovery of total nucleotide material was 96 per cent.

resulting solutions passed through columns of Dowex-50-Na^+ (5 x 1.5 cm., dia.). The effluents, containing the sodium salt of TMP-5' or TMP-3', were collected and the total amounts of each nucleotide was determined after appropriate dilution by measurement of the extinction at 267 mμ. The yield of TMP-5' was 281 μmoles and that of TMP-3' 111μmoles, representing an overall yield of thymidine monophosphates of 39%. The specific activity was 103 x 10^6 counts per minute per μmole as determined in a Nuclear-Chicago windowless gas-flow counter.

The purity of the preparation was checked by paper chromatography in ammonium isobutyrate solvent (Section 2.2.3 (b)) with subsequent detection of orthophosphate and bound phosphate (Section 2.2.3 (c)) and of the principal regions of radioactivity on appropriate lanes cut from the chromatograms (Section 2.2.3 (d)).

Other methods for the elimination of contamination with the 3'-isomer were investigated and, in some phosphorylations, thymidine was replaced by 3'-O-acetyl-thymidine prepared by the method of Michelson and Todd (1953). The potential advantage of employing a nucleoside with blocked 3'-hydroxyl group was that nucleophilic attack by the phosphorylating agent could be expected to result in phosphorylation of the 5'-hydroxyl group only. It was found, however, that this modification led to a proliferation of by-products, many of which were of uncertain composition and led simultaneously to a depression in the total yield of TMP-5'. The method was therefore not used for routine preparations.

(b) Preparation of thymidine 5'-triphosphate labelled with (^{32}P) in the proximal position ((^{32}P-α) TTP) and labelled with (^{32}P) in the two terminal positions ((^{32}P-β, γ) TTP)

The method employed was essentially that described by Smith and Khorana (1958) with some minor modifications. Thymidine 5'-triphosphate proximally labelled with (^{32}P) was prepared by condensation of (^{32}P) TMP with unlabelled orthophosphoric acid in presence of DCC (see Fig. 12, Stage III). The following reaction mixture was prepared:

per 100 μmoles of (^{32}P) TMP (free acid form),

1.8 ml. of tri-n-butylamine,

6.0 ml. of pyridine,

0.2 ml. of 85% orthophosphoric acid,

3.0 gm. of dicyclohexylcarbodiimide (DCC).

The reactants were dissolved in the order shown and the contents of the flask thoroughly mixed. The flask was then securely stoppered and allowed to stand at room temperature for 48 hours.

After a few hours, a thick precipitate of DCU formed and the reaction mixture gradually solidified. The reaction was finally terminated by the addition of one volume of distilled water, the contents of the flask were agitated, breaking up the solidified reaction products, and then allowed to stand for one hour at 0°. The DCU sediment was removed by filtration through a Buchner funnel and the residue was carefully washed on the filter with distilled water which was subsequently added to the bulk of the filtrate. The solution was extracted with several small volumes of ether to remove

pyridine. Traces of nucleotide material were, in turn, recovered from the combined ether phase by washing twice with small volumes of distilled water and adding the washings so obtained to the main aqueous phase. The ether extracts were then discarded and the aqueous solution was concentrated to dryness *in vacuo* at 25°.

The residue was dissolved in distilled water and the solution passed through a column of Dowex-50-Na^+ (10 x 2 cm., dia.) to remove tri-n-butylamine and traces of pyridine and, at the same time, to convert the mixture of deoxyribonucleotides from the pyridinium to the sodium salts. The resin was repeatedly washed with distilled water until the ultraviolet light absorption of the effluent had reached a low level and most of the radioactivity had been recovered. On the basis of the extinction at 267 mμ the recovery of thymidine nucleotides was of the order of 95 - 98%. The water washings and the original effluent were combined and diluted to 1000 ml. with distilled water to give a solution of pH 6 - 7. This solution was run slowly on to a column of Dowex-1-Cl^- (15 x 2 cm., dia.) thus adsorbing the nucleotide material present. When the adsorption was complete, the nucleotides were divested of any deoxyribonucleoside contamination by washing the column bed repeatedly with distilled water until the extinction at 267 mμ in the effluent approached zero.

Resolution of the nucleotide mixture was effected by gradient elution with hydrochloric acid and lithium chloride. A non-linear gradient (Hurlbert, Schmitz, Brumm and Potter, 1954) was employed with a single mixing vessel containing 0.01 N hydrochloric acid where the volume of hydrochloric acid was

determined by the total amount of material to be separated. Thus, for amounts up to 100 μmoles of nucleotides, the volume of HCl was 1000 ml. for amounts up to 500 μmoles 1500 ml. and for amounts up to 1 mmole a volume of 2000 ml. was used. The reservoir contained 0.3 M lithium chloride in 0.01 N hydrochloric acid. The elution rate was adjusted to 1.5 - 2.0 ml. per minute and fractions of 20 ml. were collected. The fractions so obtained were scanned spectrophotometrically at 267 mμ and the extinction plotted against the fraction number; alternatively, radioactivity was plotted against fraction number. The final elution pattern revealed three definite and well separated peaks corresponding to thymidine 5'-monophosphate thymidine 5'-diphosphate and thymidine 5'-triphosphate in that order as illustrated in Fig. 14. All the three peaks were shown to be associated with radioactivity. The final peak containing 60 - 80% of the total radioactivity represented the (^{32}P-α) TTP and all fractions from this peak were combined and the amount of triphosphate estimated, after appropriate dilution, by the extinction at 267 mμ.

Contamination of the triphosphate with inorganic polyphosphates necessitated further purification. The solution containing the TTP was therefore treated with 30 ml. of a suspension of acid-washed animal charcoal (see Section 2.2.6 (a)) and the mixture shaken occasionally and stored for 18 hours in the cold to complete the adsorption. The charcoal was subsequently packed into a column (10 x 1 cm., dia.) and the column bed washed liberally with distilled water until the effluent was free from chloride ions. To remove contamination due to inorganic ortho- and pyrophosphate, washing

Fig 14.

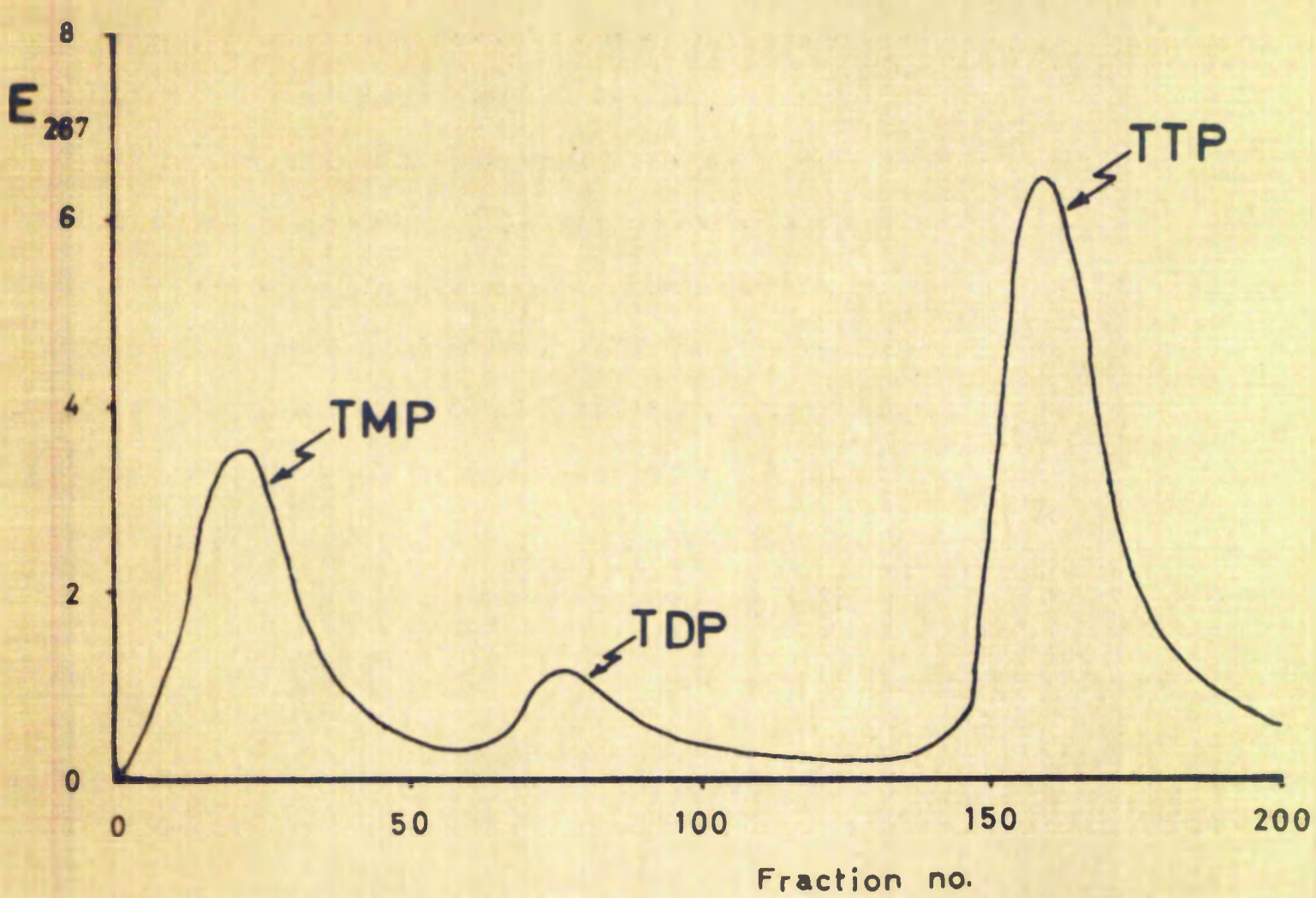


Fig. 14. Separation of reaction products in the chemical synthesis of (^{32}P) -labelled thymidine 5'-triphosphate from (^{32}P) thymidine 5'-monophosphate and un-labelled orthophosphate.

The nucleotide mixture was adsorbed on a column of Dowex-1-Cl^{-} (15 x 2 cm., dia.) and elution was effected by applying to the column a non-linear gradient with 2000 ml. 0.01 N HCl in the mixing vessel and 0.3 N lithium chloride in 0.01 N HCl in the reservoir. Fractions of 20 ml. were collected at a flow rate of 2ml. per minute. Three peaks representing TMP, TDP and TTP, in that order, were obtained. Fractions 145 - 210, representing the third peak, were combined and found to contain 240 μmoles (^{32}P) TTP, the over-all yield being 60 per cent.

was commenced with 0.01 M sodium bicarbonate and this treatment was continued for about 10 column volumes or until ultraviolet light absorbing material began to appear in the effluent. At this stage the elution with bicarbonate was terminated and the column allowed to drain. The charcoal bed was then treated with two separate column volumes of distilled water and allowed to drain each time thus removing traces of bicarbonate. TTP was finally recovered from the column by elution with 0.14 M ammonium hydroxide in 70% ethanol and the elution continued until the extinction in the effluent at 267 mμ fell to a low value. A recovery of 90 - 95% of the adsorbed TTP was achieved by the application of 15 - 20 column volumes of eluant.

The combined ethanol - ammonia eluate thus obtained was concentrated to dryness *in vacuo* at 25° to remove the ethanol and excess ammonia and the residue of TTP, now in the form of the ammonium salt, was converted to the sodium salt by extraction of the residue with a small volume of water and passing this extract through a column of Dowex-50-Na^+ (5 x 2 cm., dia.). The (^{32}P-α) TTP (sodium salt) was then either lyophylised directly and stored at -50° or made up to a suitable concentration and stored in aqueous solution at -10°. Starting with 200 μmoles of (^{32}P) TMP, the final yield was 120 μmoles and the specific activity of the product was 60 x 10^6 counts per minute per μmole when measured in a windowless gas-flow detector (Section 2.2.3 (f)).

A sample taken for paper chromatography (Section 2.2.3 (b)) revealed a single ultraviolet light absorbing spot corresponding to TTP and subsequent scanning of the chromatogram showed that all detectable radioactivity was

associated with the triphosphate region (Section 2.2.3 (d)).

(^{32}P-β, γ) TTP was prepared by a similar procedure using unlabelled TMP and (^{32}P) - labelled orthophosphate as starting materials. In this case the product was obtained in a final yield of 70 µmoles with a specific activity of 18 x 10^6 counts per minute per µmole when the reaction mixture contained 100 µmoles of TMP and 5 mc of carrier-free (^{32}P)- orthophosphate.

(c) Preparation of unlabelled thymidine 5'-diphosphate (TDP)

Thymidine 5'-diphosphate was prepared from thymidine 5'-triphosphate by partial acid hydrolysis as follows:- 200 µmoles of TTP (trisodium salt) and 0.8 ml. of 5 N hydrochloric acid in a total volume of 20 ml. were incubated at 50° for 45 minutes with continuous shaking. The reaction mixture was then cooled rapidly and the pH adjusted to 7.5 with 10 N sodium hydroxide. Samples of this solution were submitted to chromatography on paper in the ammonium isobutyrate system (Section 2.2.3 (b)) to obtain a measure of the extent of hydrolysis and the bulk of the material was diluted with distilled water to 1000 ml., i.e., less than 0.01 N with respect to chloride ions. The neutralised solution was then slowly applied to a column of Dowex-1-Cl° (20 x 2 cm., dia.) to adsorb the nucleotide material present. The resin bed was washed repeatedly with distilled water to remove any unadsorbed nucleoside and the washing was continued until the extinction at 267 mµ in the effluent fell to a low level (E_{267} less than 0.01) The thymidine nucleotides were then eluted by non-linear gradient elution with 2000 ml. of 0.01 N hydrochloric acid in the reservoir. Elution was carried out at the rate of 1 ml. per minute and fractions of 20 ml. were

collected. The progress of the elution was followed spectrophotometrically at 267 mμ and the contents of the tubes from the second peak (tubes 75 to 100) were combined for the isolation of thymidine 5'-diphosphate (Fig. 15).

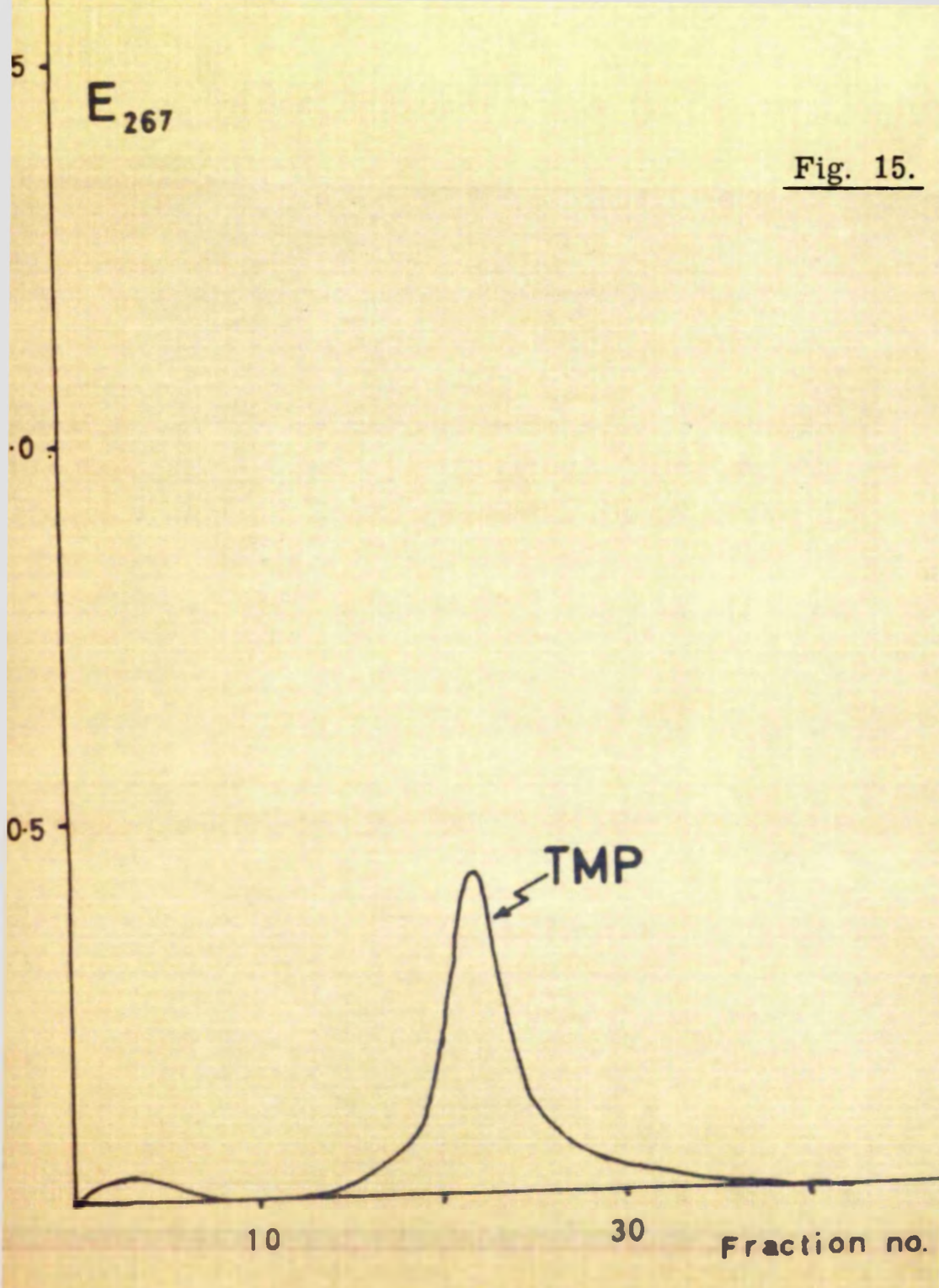
The TDP so produced was contaminated with inorganic ortho- and pyrophosphate and the combined TDP fraction was treated with 15 ml. of a suspension of acid washed charcoal (see Section 2.2.6 (a)). Lithium chloride and inorganic ortho- and pyrophosphate were removed in the manner described for the preparation of (^{32}P-α) TTP above whereupon the TDP was recovered from the charcoal by eluting the column with 0.14 N ammonium hydroxide in 70% ethanol. This eluate was concentrated to a small volume in vacuo at 25° and the concentrate passed through a column of Dowex-50-Na^+ to convert the TDP to the sodium salt.

Paper chromatography of the product (Sections 2.2.3 (b) and (c)) revealed a single ultraviolet light absorbing region corresponding to TDP and the overall yield was 40 μmoles or 20%.

(d) Preparation of thymidine 5'-diphosphate labelled with (^{32}P) in the proximal position ((^{32}P-α) TDP) and labelled with (^{32}P) in the terminal position ((^{32}P-β) TDP)

(^{32}P-α) TDP was prepared from (^{32}P-α) TTP by the procedure described above, the product in this case being slightly contaminated with inorganic pyrophosphate. No other nucleotides could be detected in the preparation and with a starting material of specific activity of 80 x 10^6 counts per minute per μmole, that of the product was 12 x 10^6 counts per minute per

Fig. 15.



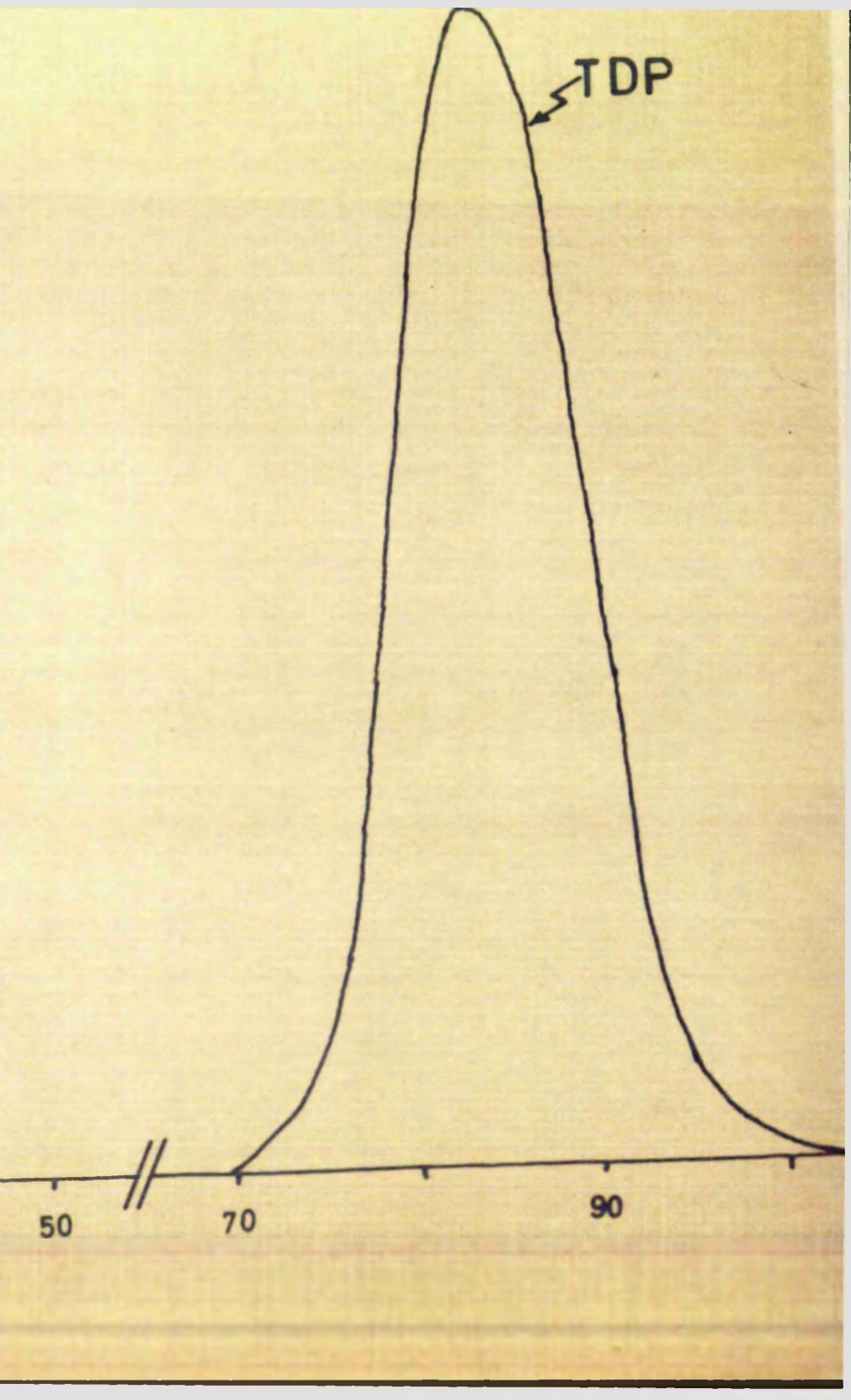

TDP
50
70
90

Fig. 15. The separation of reaction products in the preparation of thymidine 5'-diphosphate from thymidine 5'-triphosphate by partial acid hydrolysis.

The nucleotide mixture was adsorbed on a column of Dowex-1-Cl⁻ (20 x 1 cm., dia.) and elution was effected by applying to the column a non-linear gradient with 2000 ml. of 0.01 N hydrochloric acid in the mixing vessel and 0.2 N lithium chloride in 0.01 N hydrochloric acid. Fractions of 20 ml. were collected at a flow rate of 1.5 ml. per minute. The elution was discontinued after the two peaks containing TMP and TDP, in that order, had been collected. Fractions 72 - 97, representing the second peak, were combined and found to contain 30.2 μmoles of TDP, the over-all yield being 16 per cent.

µmole. The overall yield of (^{32}P-α) TDP was 18%.

(^{32}P-β) TDP was prepared from (^{32}P-β, γ) TTP by partial hydrolysis with dilute hydrochloric acid as already described. A sample of the product taken for chromatography in the ammonium isobutyrate system (Section 2.8.3 (b)) revealed one major ultraviolet light absorbing region corresponding to TDP: however, subsequent analysis (Sections 2.8.3 (c) and (d)) showed that about 25% of the total radioactivity was present in the form of (^{32}P)-orthophosphate presumably due to partial degradation of the TDP in the course of isolation and during storage. With a (^{32}P-β, γ) TTP preparation of specific activity 18 x 10^6 counts per minute per µmole as starting material, the (^{32}P-β) TDP produced had a specific activity of 10 x 10^6 counts per minute per µmole when determined in a windowless gas-flow counter. The overall yield was 20%.

On some occasions, when only small amounts of starting material were available, a simplified isolation procedure was adopted in the preparation of (^{32}P)-labelled thymidine diphosphates. This procedure entailed separation of 1 - 8 µmoles of total nucleotide material obtained by the usual partial hydrolysis of the appropriate labelled TTP. The neutralised hydrolysis products were applied as streaks on Whatman 3MM paper chromatograms which were subsequently developed in the ammonium isobutyrate system (Section 2.8.3 (b)). It was found that while TDP could be obtained free from other nucleotide contaminants in this way, the products invariably contained a relatively high proportion of radioactive inorganic pyrophosphate and the TDP so prepared could only be used as substrate for the thymidylate kinases in preliminary or qualitative experiments.

2.2.3 General analytical methods

(a) Estimation of protein

The estimation of protein was in all cases carried out by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin (Armour Pharmaceutical Company Ltd., Eastbourne, England) as standard. The colour intensity was determined using a Unicam SP-600 spectrophotometer at 750 mμ.

(b) Chromatography on paper

For separation and identification of deoxyribonucleotides and nucleotides obtained as reaction products in enzyme conversions the procedure described by Keir and Smellie (1959) was used with certain modifications. A similar method of paper chromatography was employed at various stages in the chemical preparations of thymidine nucleotide substrates.

Deproteinised neutral solutions containing nucleotide material were applied to Whatman No. 1 chromatographic paper as small spots using as markers the appropriate nucleosides or nucleotides. The solvent system used was a single phase containing: 100 ml. isobutyric acid, 4.2 ml. ammonium hydroxide (specific gravity 0.880), 1.6 ml. of 0.1 M ethylenediaminetetra-acetate (EDTA) and 55.8 ml. distilled water. This solvent was first described by Krebs and Hems (1953) but was modified by Keir and Smellie (1959) to give a pH of 4.6. The paper was developed as a descending chromatogram at 23° for times varying from 18 to 30 hours depending on the composition of the mixture to be separated. It was found that while 18 hours sufficed

to bring about separation of deoxyribonucleosides from deoxyribonucleotides, irrigation times of up to 30 hours were necessary to achieve reproducible resolution of deoxyribonucleoside di- and triphosphates. After drying the paper in air at room temperature, the spots were located by virtue of the quenching of fluorescence by purine and pyrimidine derivatives under a low-pressure mercury lamp emitting at 2537 Å. This procedure provided satisfactory resolution of mixtures where the total concentration of nucleosides and nucleotides did not fall below 0.05 µmoles or exceed 2 µmoles. Table 1 gives R_f values of all relevant nucleosides and nucleotides as determined using the above procedure.

(c) Location of free or bound phosphate on paper chromatograms

It was frequently desirable to be able to demonstrate the production or presence of inorganic ortho- or pyrophosphate or ester-linked phosphate in mixtures submitted to paper chromatography. In such cases the following procedure was adopted: the dry, developed chromatogram was dipped in a solution prepared by dissolving 1 gm. of ammonium molybdate in 8 ml. of distilled water to which was added 3 ml. 70% perchloric acid, 3 ml. concentrated hydrochloric acid and 86 ml. of acetone. The paper was then allowed to dry in a stream of air after which the areas representing inorganic phosphates were visible as light green coloured spots. Exposure of paper so treated to ultraviolet light from a low-pressure mercury lamp emitting at 2537 Å for 30 minutes revealed all phosphorus containing sections as blue spots on a white background. It was thus possible to distinguish between spots containing inorganic phosphates and those containing ester-

Table 1.

Substance	R_f
AMP	0.52
ADP	0.40
ATP	0.32
CdR	0.67
dCMP	0.53
dCDP	0.40
dCTP	0.30
UdR	0.45
dUMP	0.33
dUTP	0.18
TdR	0.63
TMP-5'	0.44
TMP-3'	0.49
TDP	0.30
TTP	0.26
inorganic phosphate	0.40

Table 1. R_f values of nucleosides and nucleotides as determined by descending chromatography with isobutyric acid: concentrated ammonium hydroxide (specific gravity 0.880) : 0.1 M EDTA:distilled water (100:4.2:1.6:55.8) as solvent. Irrigation was for 18 hours at 23°.

linked phosphate. The colours produced by this reaction were somewhat transient but could be made to reappear by exposing the paper to ammonia.

(d) Location of radioactivity of paper chromatograms

Mixtures of radioactive nucleosides and nucleotides were separated on paper chromatograms as described above (Section 2.2.3 (b)). Non-radioactive marker spots of AMP, ADP, ATP and the appropriate deoxyribonucleosides and deoxyribonucleotides were also applied. After irrigation and drying, the channel bearing the radioactive mixture was cut from the paper as a strip, 1.5 inches wide, and attached to a strip of unused chromatographic paper of the same width. The paper strip was then scanned for radioactivity using a Nuclear-Chicago 2 pi Actigraph system with a windowless counting chamber. In order to relate the chromatogram strip to the chart from the recorder, marker spots of material containing the appropriate radioactive isotope were applied to the chromatogram strip at a point 1 cm. beyond the origin. Areas bearing radioactivity were identified by comparison with non-radioactive marker spots on the original chromatogram. At maximum sensitivity, the Actigraph system could detect approximately 1500 counts per minute (or 0.002 μc) of (^{3}H) in a spot 1 inch in diameter, the corresponding value for (^{32}P) being 200 counts per minute (or 0.0005 μc).

(e) Spectrophotometric measurements

For general spectrophotometric measurements in the ultraviolet region, it was found convenient to use either the Unicam Model SP-500 or the Beckman Model DU spectrophotometers. In general, measurements at pH 1 - 2 gave

the most consistently reproducible results and, unless otherwise stated, this condition applies throughout the present investigation. In some enzyme assays where relatively large amounts of unlabelled deoxyribonucleotides were included in the reaction mixtures, the identity of the products was checked by determination of the absorption spectra with the use of the Cary Recording Quartz Spectrophotometer Model 11. The adsorption spectra of TMP, dCMP, dUMP and AMP as determined with the use of the above instrument are shown in Fig. 16. The spectral constants; molar extinction coefficient (ϵ_{max}). λ_{max}, λ_{min} and the ratio E_{280}/E_{260} were obtained directly or calculated from the ultraviolet absorption spectra of the appropriate nucleotide. Table 2 shows the values for ϵ_{max} obtained experimentally in comparison with those reported in the literature for TMP (Fox and Shugar, 1952; MacNutt, 1952; Beaven, Holiday and Johnson, 1955), dCMP (MacNutt, 1952; Boch, Ling, Morell and Lipton, 1956) and dUMP (Fox and Shugar, 1952). Since the molar extinction coefficient is a fundamental property of the pyrimidine deoxyribose moiety this value was assumed to be a constant for all members of the same homologous series, e.g., thymidine, TMP, TDP and TTP.

(f) Techniques used for determination of radioactivity

The radioactive isotopes employed in the present investigation were (^{32}P) and (^{3}H) and the emitted pulses were recorded with the use of a Nuclear-Chicago Model C-110B windowless gas-flow counter. When substrates bearing (^{32}P) were involved it was sometimes convenient to modify the counting chamber by the introduction of a thin end window. The efficiency of the detector system with (^{32}P) was near to 50% and this efficiency was only

Fig. 16.

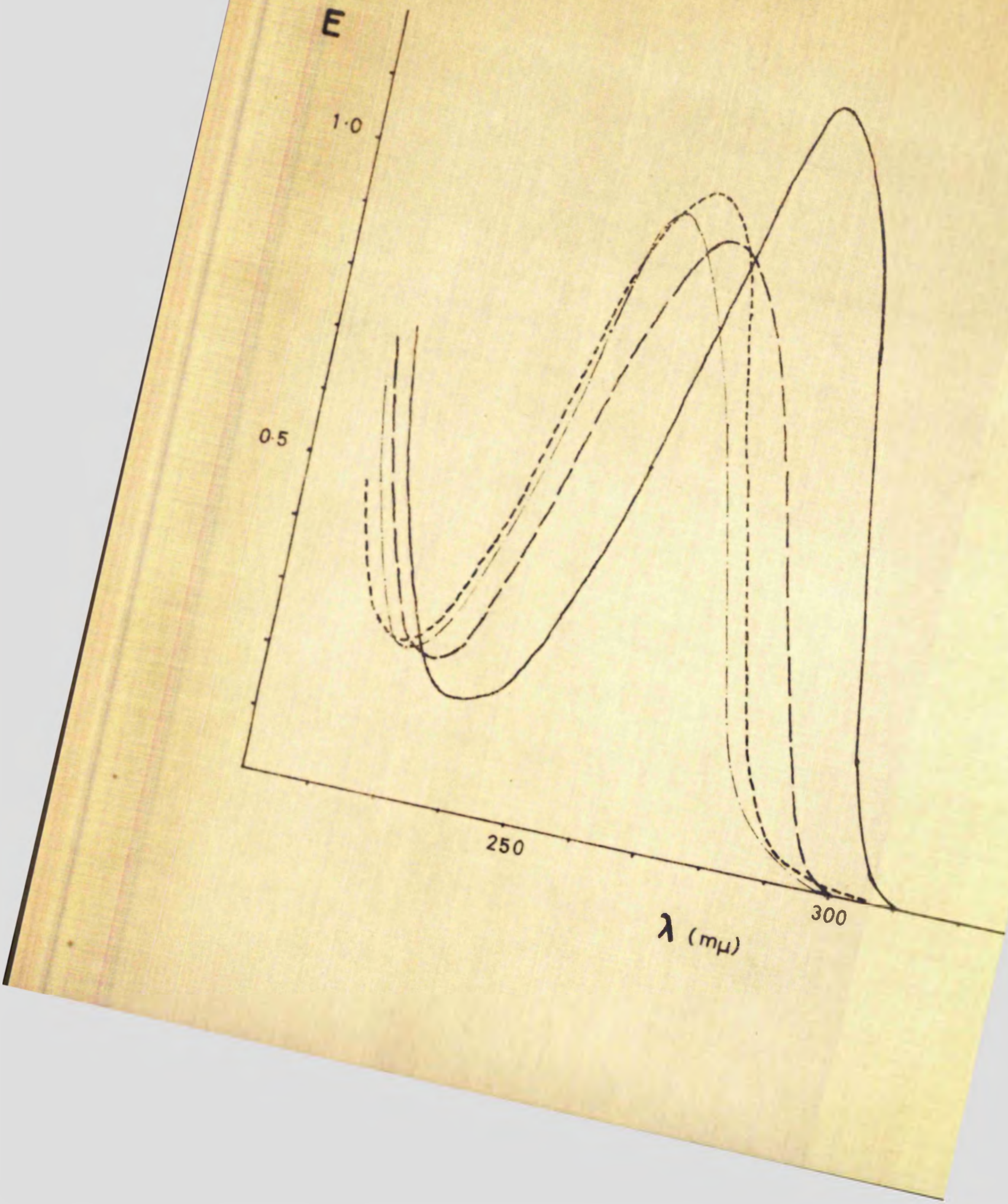


Fig. 16. Ultraviolet spectra of nucleoside 5'-monophosphates at pH 2.

The spectra were determined using a Cary Model 11 recording spectrophotometer and refer to solutions of TMP (TMP-Ca $\cdot$ 3 H_2O), dCMP, dUMP (dUMP-Na_2 $\cdot$ 3 H_2O) and AMP (AMP $\cdot$ H_2O) of the concentrations 0.991 x 10^{-4} M, 0.925 x 10^{-4} M, 1.032 x 10^{-4} M and 0.662 x 10^{-4} M, respectively.

TMP _____ _____

dCMP _____

dUMP - - - - - - - -

AMP _____ . _____

Table 2.

Substance	λ_{max}	λ_{min}	$\epsilon_m \times 10^{-3}$	$\frac{E_{280}}{E_{260}}$	$\epsilon_m \times 10^{-3}$ from the literature
TMP	267 mu	234 mu	9.63	0.715	9.6
dCMP	278	239	12.85	2.05	13.0
dUMP	262	229	10,00	0.36	10.0
AMP	258	229	14.95	0.224	15.1

Table 2. Spectral constants of nucleotides applicable in the ultraviolet region and at pH 2.

The constants were calculated or derived from the spectra recorded in Fig. 15 and the molar extinction coefficients (ϵ_{max}) so obtained were compared with those reported in the literature (Fox and Shugar, 1951; Beaven, Holiday and Johnson, 1955).

fractionally reduced with the introduction of the end window.

The samples (0.1 ml.) to be counted were plated on circular stainless steel planchettes and the moisture allowed to evaporate under an infra-red lamp. Such samples frequently contained relatively high concentrations of hydrochloric acid and aluminium planchettes could therefore not be used; stainless steel planchettes appeared to be resistant to concentrations of hydrochloric acid up to 0.5 N. It was found that the efficiency of the counter in detecting the powerful β-radiation from (^{32}P) samples remained unimpaired by variations in the plating technique and it was therefore not necessary to ensure an even spread of the sample. Tritium-containing materials, being weak β-emitters, are especially susceptible to self-absorption and corrections must normally be introduced in order to obtain the true counting rate. However, if the sample can be regarded as being of infinite thickness or of infinite thinness such corrections may be disregarded. In the present experiments care was taken to spread the sample evenly over the surface of the planchette with the aid of 5 - 10 drops of absolute alcohol and evaporating the fluid slowly under an infra-red lamp. All samples to be counted were protein-free and invariably contained only minute amounts of solid material; films formed by the above procedure were invisible approaching infinite thinness. The validity of the technique was checked by counting progressive dilutions from the same sample and showing that the number of counts per minute was inversely proportional to the dilution. The efficiency of the windowless gas-flow detector for (^{3}H) was 30%.

The background radioactivity was 20 - 25 counts per minute as determined with a windowless counting chamber while in the end window system used for (^{32}P) only, the background radioactivity was reduced to 14 - 17 counts per minute. Such background radioactivity was estimated by plating 0.1 ml. of distilled water and 5 drops of ethanol on stainless steel planchettes and introducing the resulting dried planchette into the appropriate counting chamber.

2.2.4. Procedures used for the assay of kinase activities

(a) Assay of kinases catalysing the formation of thymidine 5'-triphosphate from thymidine

The thymidine and thymidylate kinases were assayed by a modification of the method described by Weissman, Smellie and Paul (1960). Unless otherwise indicated, the reaction mixtures contained the following components in a total volume of 3.0 ml.: 15 µmoles of $MgCl_2$, 15 µmoles adenosine 5'-triphosphate, 300 µmoles of tris buffer, pH 7.9, 0.3 µmoles of 2-mercaptoethanol and either 3 µc (^{3}H) thymidine of specific activity 360 mc per mmole or (^{32}P) thymidine 5'-monophosphate, (^{32}P-α) thymidine 5'-diphosphate or (^{32}P-β) thymidine 5'-diphosphate in the quantities indicated in the legends to the figures and tables. Extracts from Landschutz ascites tumour cells (protein concentration 3 - 6 mg. per ml.) or extracts from rat liver (protein concentration 5 - 7 mg. per ml.) were occasionally used directly as the source of enzymes in which case a volume representing 4.5 mg. of protein was included in the reaction mixture described above. Normally,

the enzyme solutions were prepared from a stock of lyophylised cell-free extract preparations obtained as described earlier (Section 2.2.1.). Solutions containing 6 - 8 mg. of protein per ml. were prepared by extracting 40 mg. of the lyophylised enzyme preparation with 3 ml. of distilled water and removing the undissolved material by centrifugation at 2000 x g for 20 minutes at 0°. Portions of the enzyme solution so obtained, containing 4.5 mg. of protein, were incubated with the above reaction mixture for varying times at 37° in a shaking water-bath.

The reaction was terminated by adding 1.5 ml. of ice-cold 2.1 N perchloric acid and all subsequent manipulations were carried out in the cold to prevent non-enzymic hydrolysis of the reaction products. The protein precipitate was removed by centrifugation, washed three times with 0.5 ml. of 0.5 N perchloric acid and the supernatant fluid and the washings were combined. The solution was adjusted to pH 7.8 with 7 N potassium hydroxide, allowed to stand for 6 hours in the cold and then centrifuged to remove the precipitate of potassium perchlorate. The supernatant fluid was decanted and diluted to 7.0 ml. with distilled water and after samples had been reserved for measurement of radioactivity and for paper chromatography (Section 2.2.3 (b)), part of the remainder was applied to columns of ECTEOLA cellulose, prepared as described elsewhere (Section 2.2.6 (d)), for the separation of thymidine derivatives.

The procedure adopted was as follows: Portions of the supernatant fluids from the reaction mixtures (3.5 ml.) were run on to columns of ECTEOLA cellulose (10 x 1.5 cm., dia.) and the columns were eluted

successively with 100 ml. of distilled water, 50 ml. of 0.01 N hydrochloric acid, 50 ml. of 0.02 N hydrochloric acid, 100 ml. of 0.04 N hydrochloric acid and 70 ml. of 0.1 N hydrochloric acid. Thymidine was removed in the water fraction, TMP in the 0.02 N HCl, TDP in the 0.04 N HCl and TTP in the 0.1 N HCl fraction. The effluent was collected in fractions of 10 ml. with a maximum flow rate of 0.5 ml. per minute and 0.1 ml. portions from each fraction were plated on stainless steel planchettes. The time for 400 disintegrations was determined in a Nuclear-Chicago windowless gas-flow counter (see Section 8.2.3 (f)) and the total counts per minute in each portion of the eluate were calculated as a percentage of the total counts per minute, recovered from the column. The pattern of elution of thymidine, TMP, TDP and TTP is shown in Fig. 17.

The assay procedure invariably resulted in losses of radioactivity particularly when (^{3}H) TdR was used as substrate. In such cases it was found that 25 - 30% of the total counts per minute present initially were lost in course of the manipulations leading to the neutralised supernatant fluid and that a further 10 - 15% of the total radioactivity present prior to adsorption on ECTEOLA cellulose could not be recovered from the columns. Direct losses to the environment by randomisation and by radiochemical decomposition have been implicated as a major source of removal of tritium label in biological systems (Evans and Stanford, 1963a, b; Goutier, 1964). In addition to effects of this nature, it became evident that serious losses of radioactivity occurred in conjunction with the precipitation and removal of $KClO_4$ from the reaction media. Washing of the precipitate with distilled

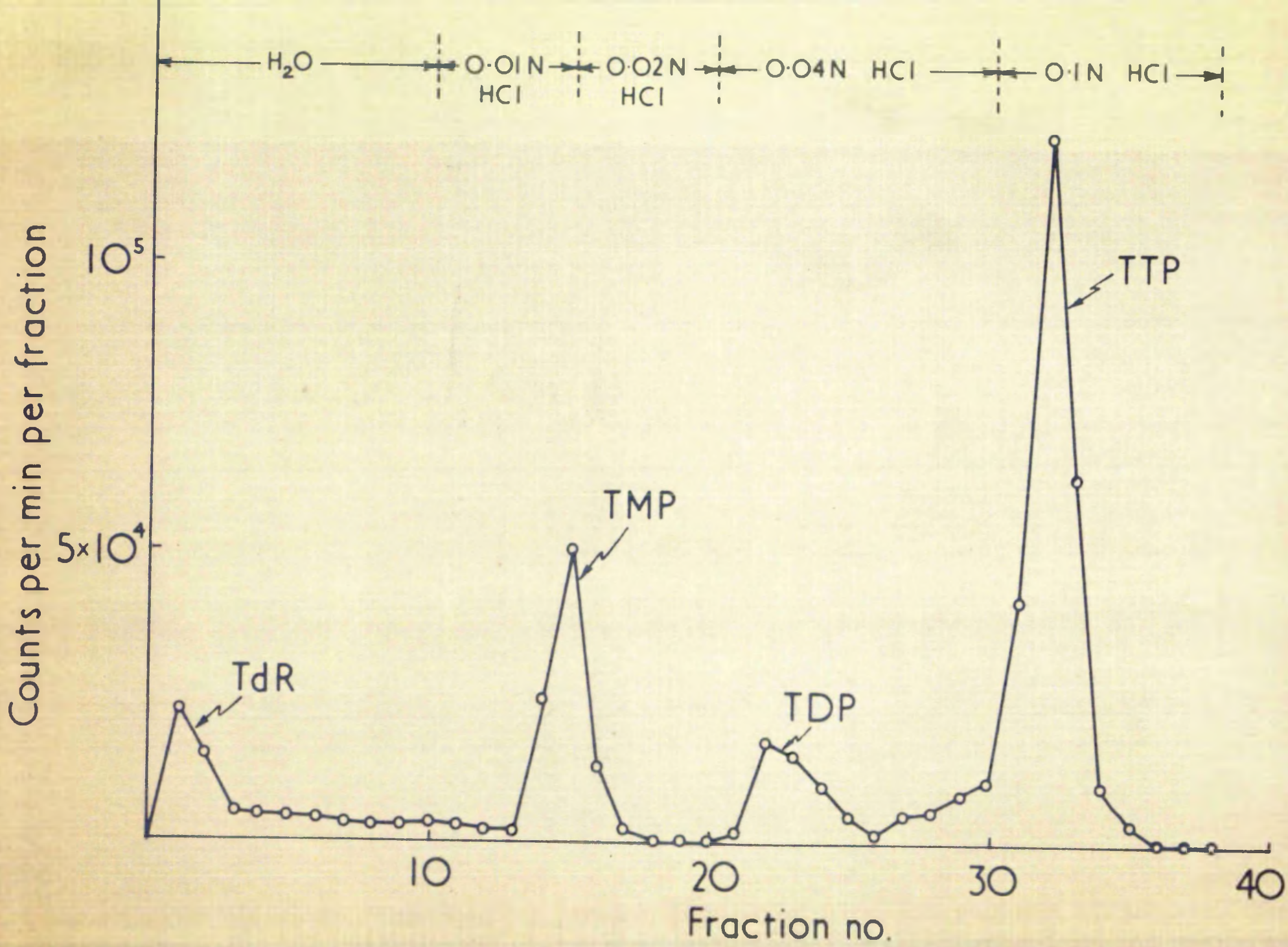

H2O
0·01N HCl
0·02N HCl
0·04N HCl
0·1N HCl
Counts per min per fraction
10^5
5×10^4
TdR
TMP
TDP
TTP
10
20
30
40
Fraction no.

Fig. 17.

Fig. 17. Chromatographic separation of (^{3}H) -labelled thymidine, TMP, TDP and TTP on columns of EOTEOLA cellulose (10 x 1.5 cm., dia.) by stepwise elution with increasing concentrations of hydrochloric acid.

water was impracticable because of the relatively high solubility of $KClO_4$ in water (0.75 gm. % at 0°) and subsequent interference from high salt concentrations with chromatography on ECTEOLA cellulose. When substrates bearing a (^{32}P) label were employed, the recovery of radioactivity was consistently high, 90 - 95% of the total counts per minute present initially being recovered prior to adsorption on ECTEOLA cellulose while the loss of radioactivity subsequently incurred by adsorption and elution from such columns never exceeded 5 - 10%. Calculations and results as presented in Section 8.3. were based on the assumption that losses of radioactivity derived in equal proportion from each of the radioactive reaction products.

In spite of the reservations listed above and the relatively long time required for the completion of a single assay (5 - 7 days), the present procedure has the advantage of giving a high degree of resolution, particularly in the clear seperation of TDP and TTP, and it also provides a means for the precise measurement of all the four reaction products.

(b) Determination of specific activities

In some experiments with (^{32}P)-labelled substrates, the specific activities of the TMP, TDP and TTP present in the reaction mixtures were determined. Because of the minute absolute amounts of these compounds normally present in reaction mixtures, a large excess of TMP, TDP and TTP was added in certain experiments to provide sufficient nucleotide material for accurate spectrophotometric measurement of the amount present. Direct spectrophotometric measurement of the quantities of thymidine nucleotides in the fractions recovered from the ECTEOLA cellulose columns were impracticabl

on account of the large quantities of adenosine nucleotides present in the reaction products.

Portions of the eluates from the columns of ECTEOLA cellulose corresponding to TMP, TDP and TTP were separately combined in 150 ml. flasks and 5.0 N hydrochloric acid was added in the quantity required to make the solution 0.2 N. Flasks containing TMP were heated at 100° for 20 minutes, those containing TDP or TTP were heated at 100° for 60 minutes after which time the mixtures were cooled under the tap and the pH was adjusted to 7.5 with 10 N sodium hydroxide. A series of preliminary experiments had shown that under these conditions of controlled acid hydrolysis, TMP remained intact while TDP and TTP were quantitatively degraded to thymidine monophosphate and adenosine phosphates were hydrolysed to give a mixture of AMP and adenine. Each of the fractions were then diluted to a finite volume and a portion, usually one-half of the total volume, was diluted with distilled water to give a sodium chloride concentration of less than 0.01 M. The solutions so obtained were applied to columns of Dowex-1-Cl^- (12 x 2 cm., dia.) for subsequent separation of TMP from AMP and adenine (Gray et al., 1960). The purine derivatives were removed from the columns by repeated washing with distilled water followed by 50 ml. portions of 0.01 N hydrochloric acid until the extinction of the effluent at 257 mμ approached zero. TMP was finally recovered from the columns by eluting with 10 ml. portions of 0.05 N hydrochloric acid, the eluates were each diluted to 50 ml. and the nucleotide concentrations were determined spectrophotometrically at 267 mμ. Complete resolution of the thymidine nucleotides from adenosine

nucleotides was achieved by this procedure and the recovery of TMP was quantitative.

2.2.5. Buffer solutions

(a) Tris buffer

Tris buffer was prepared by adjusting the pH of a 1.0 M solution of 2-amino-2(hydroxymethyl)-propane-1,3-diol to the desired value with concentrated hydrochloric acid. Tris buffers of lower ionic strengths were prepared by dilution of the appropriate concentrated buffer with distilled water.

(b) Phosphate buffer

Phosphate buffers were prepared by adding 0.1 M KH_2PO_4 to 0.1 M $Na_2HPO_4 \cdot 2 H_2O$ or by adding 0.01 M KH_2PO_4 to 0.01 M $Na_2HPO_4 \cdot 2 H_2O$ until the desired pH was attained.

2.2.6. Preparation of adsorbents for column chromatography

(a) Preparation of charcoal adsorbent

Animal charcoal was obtained commercially (British Charcoals and McDonalds Ltd., Grangemouth) and purified as follows: The crude 20 - 60 grist charcoal was ground and sieved to 40 - 60 mesh, suspended in two volumes of 5 M hydrochloric acid and allowed to stand overnight. The fluid was decanted and the charcoal was resuspended in distilled water and

washed several times until the supernatant had attained a pH of 7.

Coloured and ultraviolet light absorbing substances were then removed by washing the charcoal liberally in absolute alcohol. The most convenient procedure entailed washing the material embedded in a large Buchner funnel fitted with a Whatman No. 1 filter paper in one litre portions with resuspension of the charcoal in ethanol between each filtration. This treatment, which required large volumes of ethanol, was continued until all colouration was absent from the filtrate and the ultraviolet light absorption approached zero.

The charcoal was then resuspended in distilled water and washed several times to remove the ethanol whereupon the adsorbent was packed into a column (5 cm., dia.,. Further treatment was by washing with 10 - 12 column volumes of N hydrochloric acid followed by distilled water until the effluent attained pH 7. Several column volumes of 0.01 N sodium bicarbonate were then applied to the column bed and washing was continued with this solvent until no ultraviolet light absorbing material could be detected in the effluent. After washing with distilled water to pH 7, the nucleotide solvent (0.14 N ammonium hydroxide in 70% ethanol) was applied to the charcoal bed until the extinction at 220, 260 and 290 mμ of the effluent approached zero. The solvent was removed by a final washing with distilled water, the column was unpacked and the charcoal suspended and stored in 0.1 N hydrochloric acid.

The charcoal was regenerated after use by further washing with the ethanol-ammonia solvent until all the residual adsorbed material had been

removed. Further treatment was by washing with distilled water to pH 7, treatment with 10 column volumes of N hydrochloric acid and the charcoal was finally washed with distilled water until the pH of the effluent returned to neutrality.

(b) Pretreatment of Dowex-1-Cl^- for column chromatography

Dowex AG1 - X8 (chloride form) of 100 - 200 mesh was suspended in N hydrochloric acid for 18 hours and the resin so activated packed into a column fitted with a sinter glass filter. The resin bed was washed liberally with distilled water until the effluent was free from chloride ions. A small plug of glass wool was finally inserted 2 cm. above the surface of the resin bed to prevent any disturbance of the surface in the course of elution. The resin could be regenerated after use by further washing with 0.5 M lithium chloride in 0.1 N hydrochloric acid until all residual adsorbed material had been removed followed by washing with water until the pH of the effluent returned to 7.

(c) Preparation of columns of Dowex-50-Na^+

Dowex AG50 W - X8 (hydrogen form) of 100 - 200 mesh was suspended in N sodium hydroxide for 18 hours and the resin so transformed was packed into a column. Continuous washing of the resin bed with distilled water was performed until the effluent had attained pH 7. After use, the resin could be regenerated by treatment with three column volumes of N hydrochloric acid followed by washing with distilled water to pH 7, and reconversion to the Na^+ form by the application of 10 column volumes of N sodium hydroxide followed

by washing with distilled water until the effluent returned to neutrality.

(d) Preparation of ECTEOLA cellulose for column chromatography

ECTEOLA cellulose (Kodak Ltd., London or Serva Entwiklungslabor, Heidelberg, West Germany) was prepared for use by preliminary steeping in twice its own volume of distilled water followed by vigorous agitation with a Vibro-mix Model E1 (AG für Chemie-Apparatebau, Männedorf, Zurich, Switzerland) for periods of 30 minutes. Between each treatment the adsorbent was allowed to settle and the fluid was decanted to remove the smaller particles. This procedure was repeated until the supernatant fluid was water-clear (6 - 8 treatments). The suspension of ECTEOLA cellulose was then transferred to columns and the adsorbent bed washed twice with 50 ml. portions of distilled water followed by 50 ml. portions of 0.5 N hydrochloric acid until the effluent was free from material absorbing ultraviolet light in the region 257 - 267 mμ. The columns were finally washed with distilled water until the pH of the effluent returned to 7. To ensure that the surface of the adsorbent bed remained undisturbed a plug of glass wool was inserted 2 cm. above the surface and the solvent was never allowed to drain below this mark. Regeneration of the ECTEOLA cellulose was carried out by washing the adsorbent bed with four 50 ml. portions of 0.5 N hydrochloric acid or until the eluate was free from radioactive material followed by washing with distilled water until the effluent reached pH 7. It was observed that the adsorbent was slowly oxidised when used over long periods and the column contents were discarded after three adsorption - regeneration cycles.

2.2.7. Materials

TdR, UdR, CdR and ATP were purchased from The Sigma Chemical Corporation, TMP, dCMP and dUMP were purchased from Schwarz Bioresearch Inc. and from California Corporation for Biochemical Research and TTP and dCTP were obtained from Pabst Laboratories. Additional samples of ATP were purchased from British Drug Houses Ltd. (^{3}H) TdR of specific activity 360 mc per mmole and (^{3}H) CdR of specific activity 1100 mc per mmole were purchased from Schwarz Bioresearch Inc. (^{32}P)-cyanoethylphosphate of specific activity not less than 50 mc per mmole was either purchased from The Radiochemical Centre, Amersham or prepared by the method of Toner (1961) using carrier-free (^{32}P) from the same source. DCC was purchased from The Aldrich Chemical Company Inc., Milwaukee, and 2-cyanoethanol was obtained from British Drug Houses Ltd.

2.3. Results

2.3.1. Optimal conditions for the formation *in vitro* of TTP from thymidine by cell-free extracts of Landschutz ascites tumour cells.

Several preliminary experiments were performed in order to determine which were the most favourable conditions for the phosphorylation of thymidine to thymidine 5'-triphosphate under the influence of enzymes from cell-free extracts of Landschutz ascites tumour cells. Such experiments have invariably shown that the activities of the thymidine and thymidylate kinases were particularly low and that the over-all conversion of TdR to TTP by the crude extracts was an inefficient process. One important consequence of this observation was that at no stage of the present investigation did it become feasible to study the formation of TTP *in vitro* with the use of unlabelled substrates. Thus refuge had to be taken in the use of radioactive substrates, variously labelled with (^{3}H) and (^{32}P), and thereby restricting the permissible interpretation of experimental results. The loss of analytical accuracy was particularly serious in the case of substrates labelled with (^{3}H) and the procedures based on the use of this isotope have been regarded throughout the present investigation as little more than a semi-quantitative measure of the reactions under study. The powerful β-radiation emitted by (^{32}P), on the other hand, allows the easy detection and precise measurement of material containing this isotope, and interpretations based on data from experiments with (^{32}P)-labelled substrates have been regarded with confidence as the closest approach to

quantitative evaluation obtainable.

In the assembly of a reaction mixture for the study of the formation of TTP *in vitro* from its nucleoside precursor, due regard was paid to the known requirements for phosphokinase activity such as a phosphoryl group donor, a divalent cation and suitable stabilising and activating factors. Moreover, it was realised that little reliable information could be derived from the ensuing enzyme experiments in the absence of a suitable method for determination and detection of reaction products. In the present investigation, the manipulation of the reaction products was greatly facilitated by having available a method for the complete separation of all the presumed products of the reaction (TdR, TMP, TDP and TTP) by chromatography on columns of ECTEOLA cellulose (see Fig. 17) in what was essentially a single operation. The failure of complete resolution of TDP and TTP has been a major objection to many previous assay procedures based on paper chromatography (Bollum and Potter, 1959; Bianchi, Butler, Crathorn and Shooter, 1961). The recent development of rapid assays as reported by Ives, Morse and Potter (1963) and by Furlong (1963) employing DEAE cellulose strips for the separation of thymine nucleotides likewise suffers from inability to resolve the higher phosphates.

Experiments performed to ascertain the most favourable pH for the transformation of (^{3}H) TdR to TTP showed that the reaction exhibited a relatively narrow pH -optimum giving maximal activity over the range pH 7.8 to 8.1 when the reaction was studied in a medium 5×10^{-3} M with respect to $MgCl_2$ and ATP. The proportion of the total radioactivity appearing in TTP

declined sharply below pH 7.7 and above pH 8.2 so that the percentage of the total radioactivity recovered in TTP when the reaction was performed at pH 7.5 was approximately one-third of the value obtained at pH 7.9. In such experiments (as shown in Fig. 18), only the proportion of the total radioactivity present in fractions containing TdR or TTP was recorded, no account being taken at this stage of the radioactivity present in possible intermediates. The assay could, therefore, be expected to reflect the efficiency of the thymidine and thymidylate kinase system of crude extracts of Landschutz ascites cells in catalysing the conversion of (^{3}H) TdR to (^{3}H) TTP. Tris was found to be a suitable buffer in which to conduct such experiments and at a concentration of 0.1 M provided a rigid and accurate control of the pH in the range 7 - 9.

Experiments in which the conversion of (^{3}H) TdR to (^{3}H) TTP was studied as a function of the Mg^{++} concentration at a constant ATP concentration of 5×10^{-3} M, showed that the proportion of the total radioactivity recovered in TTP after a 60 minute incubation period was maximal in the range 5×10^{-3} to 15×10^{-3} M of $MgCl_2$ while no radioactive TTP could be detected in the absence of Mg^{++} (see Fig. 19). Subsequent experiments designed to study the effect of variable concentrations of ATP as phosphoryl group donor conducted in the presence of 5×10^{-3} M $MgCl_2$, similarly revealed that the proportion of (^{3}H) TdR phosphorylated to TTP over a 60 minute period was at its maximum when the reaction medium was from 5×10^{-3} to 15×10^{-3} M with respect to ATP (see Fig. 20). Such experiments also showed that radioactive TTP could not be detected among the products when ATP was omitted from the

Fig. 18. The effect of pH on the formation of TTP from (^{3}H) TdR by enzymes from cell-free extracts of Landschutz ascites tumour cells.

The following reaction mixtures were prepared, each in a total volume of 3.0 ml.: 0.3 µmoles 2-mercaptoethanol, 15 µmoles ATP, 15 µmoles $MgCl_2$, 3 µc (^{3}H) TdR (specific activity 360 µc per µmole; 100 µc per ml.), enzyme solution containing 4.1 mg. protein and 300 µmoles of tris buffer of the appropriate pH. Incubation was for 60 minutes at 37°. Of a total volume of deproteinised, neutralised assay solution of 7.0 ml., 5.0 ml. were adsorbed on a column of EGTEOLA cellulose (10 x 1.5 cm., dia.) and eluted as described in the text (Section 2.2.4 (a)). The proportion of the total radioactivity present in each of the products, TdR, TMP, TDP and TTP was determined. However, on the diagram is shown only the proportion of the total radioactivity being recovered in the TTP fraction. The source of enzymes was the crude cell-free extract from Landschutz ascites carcinoma.

Fig. 19.

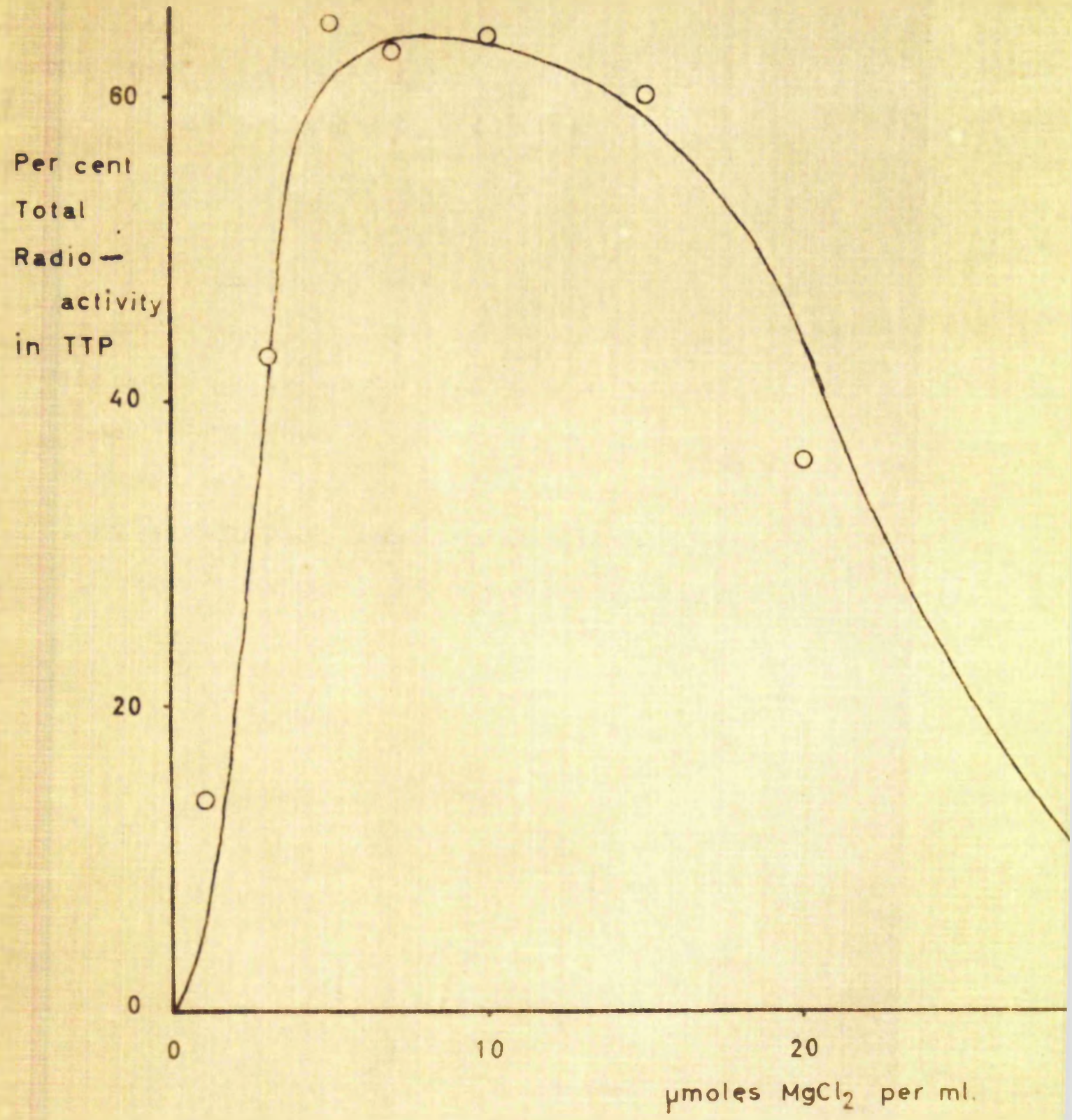

60
Per cent
Total
Radio—
activity
in TTP
40
20
0
0
10
20
μmoles $MgCl_2$ per ml.

Fig. 19. Effect of Mg^{++} concentration on the formation of TTP from (^3H) TdR by enzymes from cell-free extracts of Landschutz ascites tumour cells.

The reaction mixtures contained the components given in the legend to Fig. 13 in a total volume of 3.0 ml. except that they contained $MgCl_2$ concentrations varying from zero to 40 μmoles per ml., 300 μmoles tris buffer, pH 7.9, and 3.7 mg. protein from a cell-free extract of Landschutz ascites carcinoma. Incubation was for 60 minutes at 37° and further treatment was as described in Fig. 18.

Fig. 20

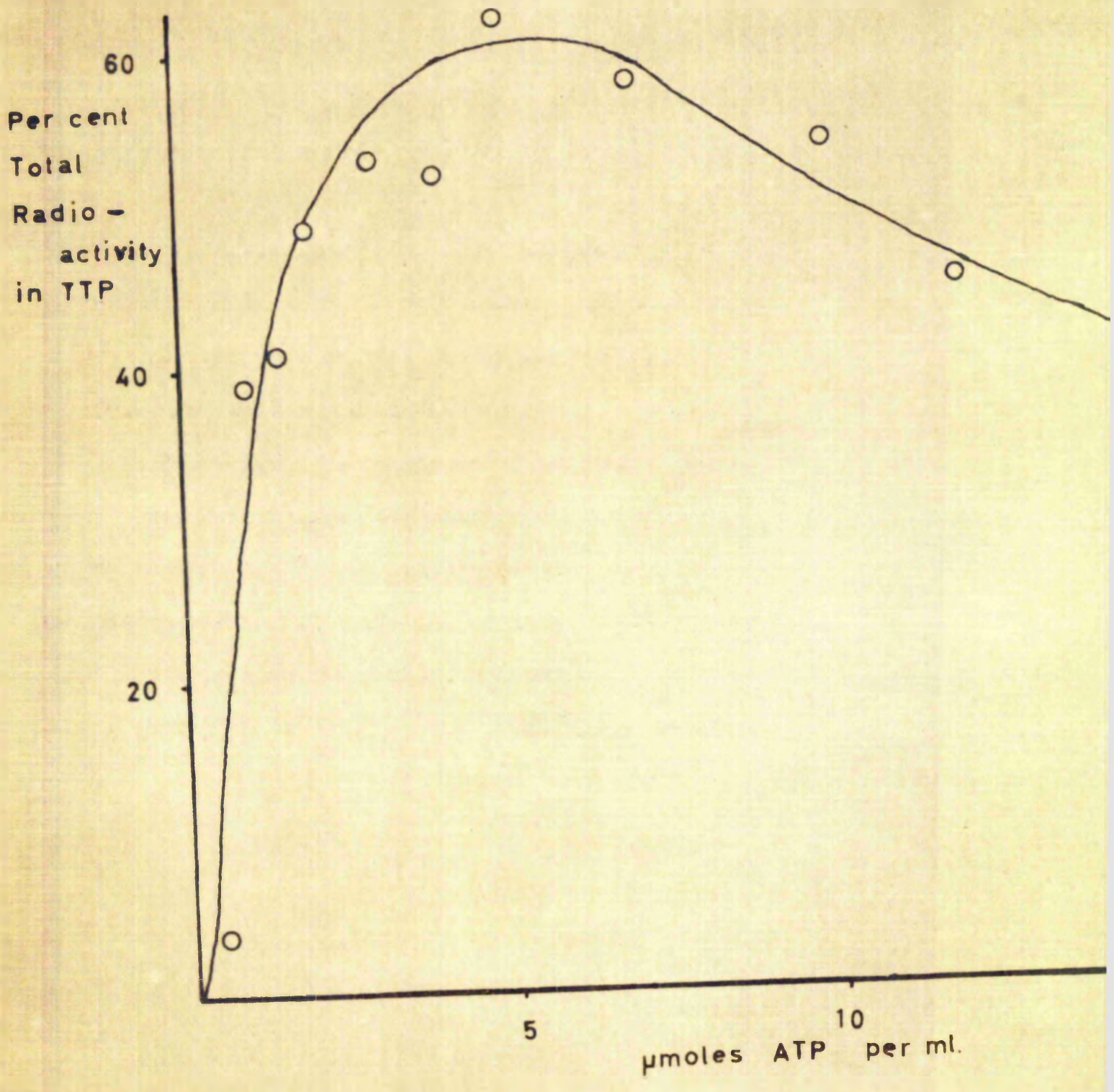

60
Per cent
Total
Radio –
activity
in TTP
40
20
5
10
μmoles ATP per ml.

Fig. 20. Effect of ATP concentration on the formation of TTP from (^{3}H) TdR by enzymes from cell-free extracts of Landschutz ascites tumour cells.

Reaction mixtures contained ATP in concentrations varying from zero to 30 µmoles per ml. with other components, including 300 µmoles tris buffer, pH 7.9, in a total volume of 3.0 ml. as described for Fig. 18. The reaction mixtures also contained a volume of enzyme solution corresponding to 4.5 mg. of protein. Incubation was for 60 minutes at 37° and further treatment was as described for Fig. 18. The source of enzymes was the lyophylised cell-free extracts of Landschutz ascites carcinoma.

reaction medium. While the proportion of the total radioactivity recovered in TTP rose sharply in response to an increase in the ATP concentration from zero to 5×10^{-3} M, at which point more than 60 per cent of the total radioactivity was present as TTP, the proportion fell only slightly with ATP concentrations increasing up to, and beyond 30×10^{-3} M. The procedures involving (^{3}H) -labelled substrates did not allow precise kinetic interpretation of results so obtained. Nevertheless, the data suggested that the phosphorylation of TdR to TTP with the crude cell-free extract operated at a maximal rate in the presence of ATP and Mg^{++} in the ratio 1 : 1. Such a ratio would be expected to apply if the true co-substrate for the thymidine and thymidylate kinases was an ATP - Mg^{++} chelate. The presence in cell-free extracts of the Landschutz ascites cells of phosphomonoesterases capable of degrading thymidine polyphosphates has been indicated previously (Gray et al., 1960) and since it is now well established that such enzymes show specific requirements for divalent cations, it was to be expected that an increase in the Mg^{++} concentration of the reaction medium would cause a stimulation of phosphatase activity. An Mg^{++} concentration in excess of that required to maintain an ATP - Mg^{++} chelate would, therefore, lead to a net stimulation of the catabolic activity relative to the combined kinase activity and a situation such as that shown in Fig. 19 at the higher Mg^{++} concentrations could thus partly be accounted for by the availability of un-chelated Mg^{++} ions to the nucleotide phosphatases. Another consequence of the presence of phosphatases was that all measurements of anabolic reaction rates reflected net (or apparent) kinase activities and not the absolute

potentialities of the thymidine and thymidylate kinase system.

In the majority of investigations of this type it has been customary to augment the reaction mixture with a system which promotes the regeneration of ATP and thereby ensures maintenance of a constant phosphoryl group donor concentration. On dynamic grounds, it seems clear that the primary effect of introducing a regenerating system of this type would be to give the system a bias towards phosphorylation of a phosphoryl group acceptor. 3-Phosphoglycerate has been favoured as a "regenerating agent" in experiments with nucleoside and nucleotide kinases and Potter (1963) has shown that the presence of this agent enhanced three-fold the thymidine and thymidylate kinase activity of cell-free extracts from Novikoff hepatoma. In the present investigation, the effect of the ATPase in the crude ascites extracts on the phosphorylation of thymidine nucleotides was minimised by the use of an ATP - Mg^{++} chelate in vast excess of the stoichiometric requirements of the thymidine nucleotide substrates. The ratio of ATP - Mg^{++} chelate concentration to phosphoryl group acceptor concentration was 2000 : 1 in the case of (^{3}H) -labelled substrates and 200 : 1 in experiments with (^{32}P) -labelled substrates. Moreover, several preliminary experiments had shown that over 50 per cent of the ATP so added remained intact at the end of incubation periods up to one hour.

A number of other preliminary experiments had indicated that the ability of the crude ascites extracts to produce TTP from TdR was quickly lost over a period of a few hours even when a rigid temperature control ($0-2°$) was applied and it became necessary, therefore, to supplement the reaction

mixtures with a stabilising factor in order to minimise possible progressive enzyme inactivation in the course of enzyme experiments at 37°. 2-Mercaptoethanol, as a well established protector of enzyme sulphydryl groups, was considered a suitable candidate to fill this function and a series of experiments showed that the presence of 2-mercaptoethanol (0.1×10^{-3} M) caused a slight stimulation of the ability of the extract to convert (^{3}H) TdR to (^{3}H) TTP. Higher concentrations of this compound, however, depressed the proportion of total radioactivity recovered in TTP and serious inhibition of the thymidine and thymidylate kinase system was observed in the presence of 2-mercaptoethanol concentrations exceeding 5×10^{-3} M, this effect being presumably due to chelation of Mg^{++} ions.

However, it was early recognised that the phosphorylation of TdR to TTP was a composite reaction involving the participation of certain intermediate thymidine nucleotides. It became clear also that interpretations regarding the efficiency of the phosphorylation of TdR to TTP based only on the measurement of the formation of TTP from its nucleoside precursor were relatively superficial unless account was also taken of the intervening reaction steps.

The factors which regulate the formation of TTP *in vitro* may have wider significance in relation to the synthesis of DNA and a series of studies were initiated in order to elucidate the mechanism of the formation of TTP from TdR with particular regard to the sequence in which each intermediate intervened in the reaction.

2.3.8. The mechanism of formation of TTP from thymidine by enzymes from cell-free extracts of Landschutz ascites tumour cells.

As a preliminary to any study on the mechanism of the formation of TTP from TdR by crude extracts of the tumour cells, it was necessary to ascertain that the assay procedure used for the determination of the thymidine and thymidylate kinases (Section 2.2.4 (a)) was valid under the conditions indicated in the preceding section. The results shown in Fig. 21 demonstrate that the formation of TMP, TDP and TTP from (^{3}H) TdR was a linear function of the protein concentration of the crude extracts of Landschutz ascites carcinoma. A set of data obtained with enzymes derived from a redissolved lyophylised preparation from such extracts, likewise, showed that the production of reaction products was directly proportional to the enzyme concentration. In the experiment shown (in which incubation was for 60 minutes at 37°), it appeared that the proportion of the total radioactivity recovered in TTP rose at a slightly faster rate than did radioactive TMP and TDP so that at a protein concentration of 1.65 mg. per ml., the TTP contained 60 per cent of the total radioactivity while TDP contained 11 per cent and TMP 19 per cent. The radioactivity remaining associated with TdR, similarly was seen to decrease as an inverse function of the enzyme concentration. While these experiments clearly indicated that at no point within the protein concentration range of zero to 1.65 mg. per ml. did the enzyme concentrations exceed the substrate concentrations, no information could be gleaned from such data as to the sequence in which the various products appeared in the reaction.

Studies on the time course of the formation of TTP from (^{3}H) TdR such

Fig 21.

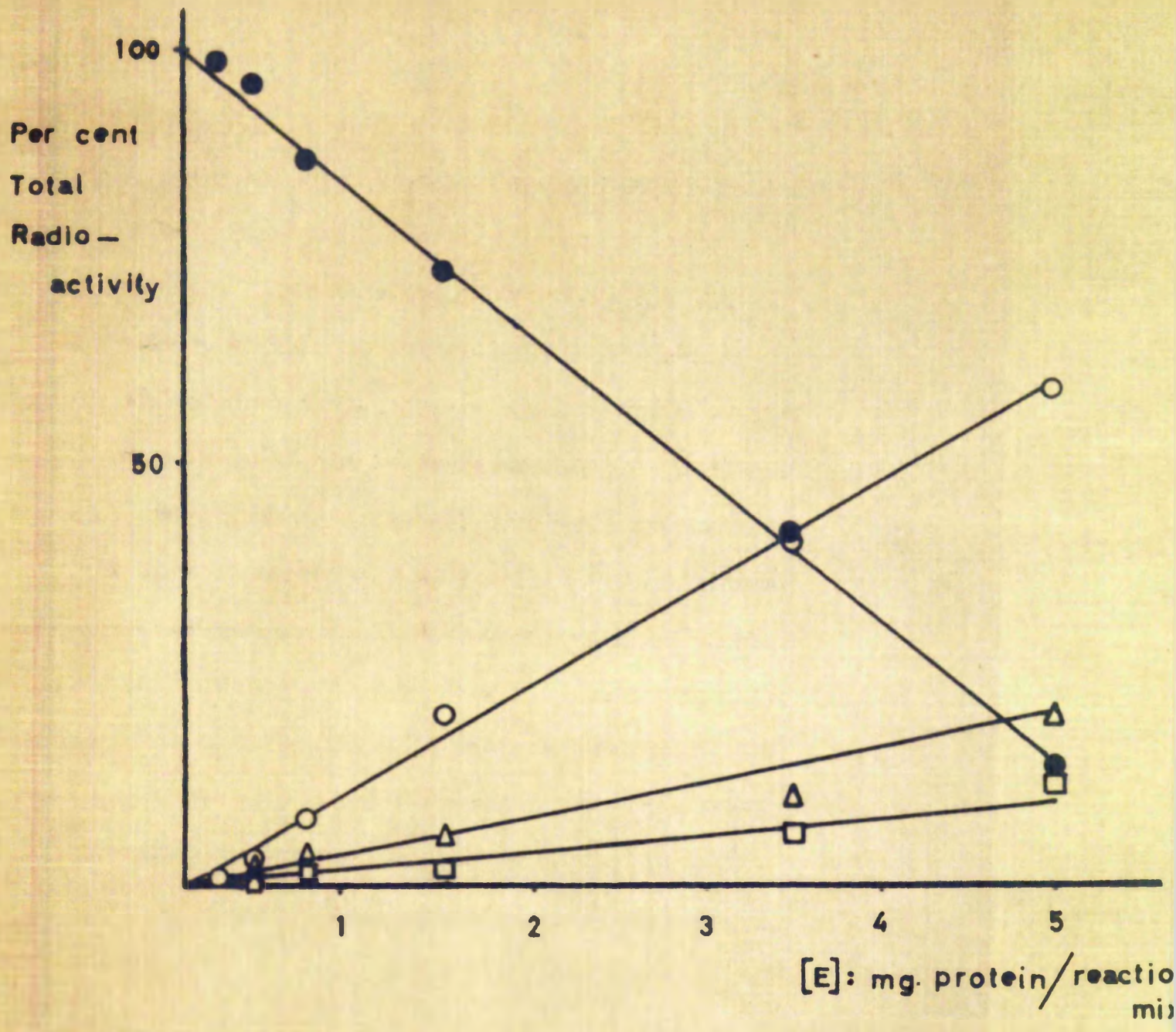

100
Per cent
Total
Radio—
activity
50
1
2
3
4
5
[E]: mg. protein/reactio
mi

Fig. 21. The effect of enzyme concentration on the formation of TMP, TDP and TTP from (^{3}H) TdR with enzymes from cell-free extracts of Landschutz ascites tumour cells.

The reaction mixtures contained the following components in a total volume of 3.0 ml.: 300 µmoles tris buffer, pH 7.9, 0.3 µmoles 2-mercaptoethanol, 15 µmoles $MgCl_2$, 15 µmoles ATP, 3 µc (^{3}H) TdR and enzyme solution corresponding to the quantities of protein given on the diagram. Incubation was for 60 minutes at 37° and further treatment was as described in the text (Section 2.2.4 (a)) and in the legend to Fig. 18 except that the percentage of total recovered radioactivity present in each of the products, TdR, TMP, TDP and TTP was plotted against the protein concentration of the reaction mixtures. The source of enzyme was a solution prepared from the lyophylised cell-free extracts of Landschutz ascites carcinoma.

TdR ● ; TMP △ ; TDP □ ; TTP ○.

as those illustrated in Fig. 22 have invariably shown that labelled TMP and TDP appear in the reaction products very shortly after the start of the reaction. The proportion of the total radioactivity in TMP rose very sharply from the start of the incubation and reached a plateau at about 15 minutes which was maintained until about 60 minutes. Thereafter, there was a further slight increase in the total radioactivity in TMP. The radioactivity in TDP also began to rise from time zero and rapidly reached a steady low level that never exceeded 20 per cent of the total radioactivity. Labelled TTP also appeared early in the course of the reaction, the proportion rose to a maximum value of the order of 60 per cent of the total radioactivity in 15 to 20 minutes, remained reasonably constant up to 60 minutes, and then declined slightly, presumably as a result of dephosphorylation of the TTP. On no occasion was labelled TTP detected earlier than labelled TDP.

It became clear from experiments of this type that the crucial events related to the sequential formation of the reaction intermediates occurred early in the reaction and experiments in which the early time intervals were examined in more detail (see Fig. 23) showed that while small amounts of radioactive TMP and TDP were produced within the first minute, no radioactivity could be detected in the TTP until after 3 minutes.

These results were consistent with the view that TMP and TDP are intermediates in the formation of TTP from TdR, but, in the absence of more discriminating kinetic information about the relative rates of the different reactions, could not be taken as a definite indication of such a reaction sequence.

Fig. 22.

Time course of the phosphorylation of ^{3}H-TdR.

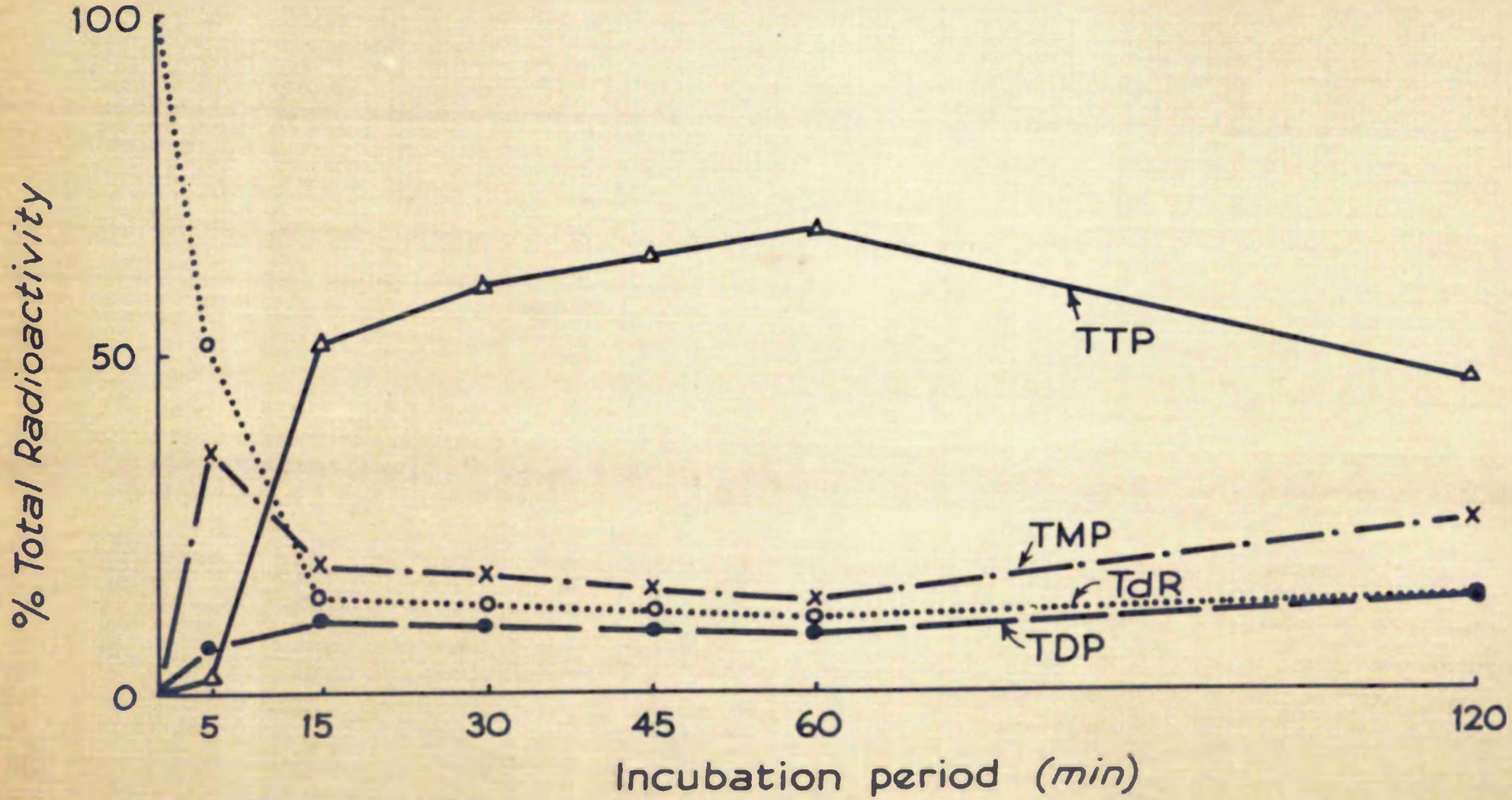


Fig. 22. The time course of phosphorylation of (^{3}H) TdR by cell-free extracts of Landschutz ascites tumour cells.

The reaction mixtures contained the following components in a total volume of 3.0 ml.: 300 µmoles tris buffer, pH 8.0, 0.3 µmoles 2-mercaptoethol, 15 µmoles $MgCl_2$, 15 µmoles ATP, 3 µc (^{3}H) TdR and enzyme solution containing 4.1 mg. of protein. The reaction mixtures were incubated for the intervals indicated on the diagram in a shaking water-bath at 37°. Further treatment was as described in the text (Section 2.8.4 (a)). The source of the enzymes was the crude cell-free extract of Landschutz ascites carcinoma.

TdR .....○.....

TMP —·—×—·—

TDP ——●——

TTP ——△——

Fig 23.

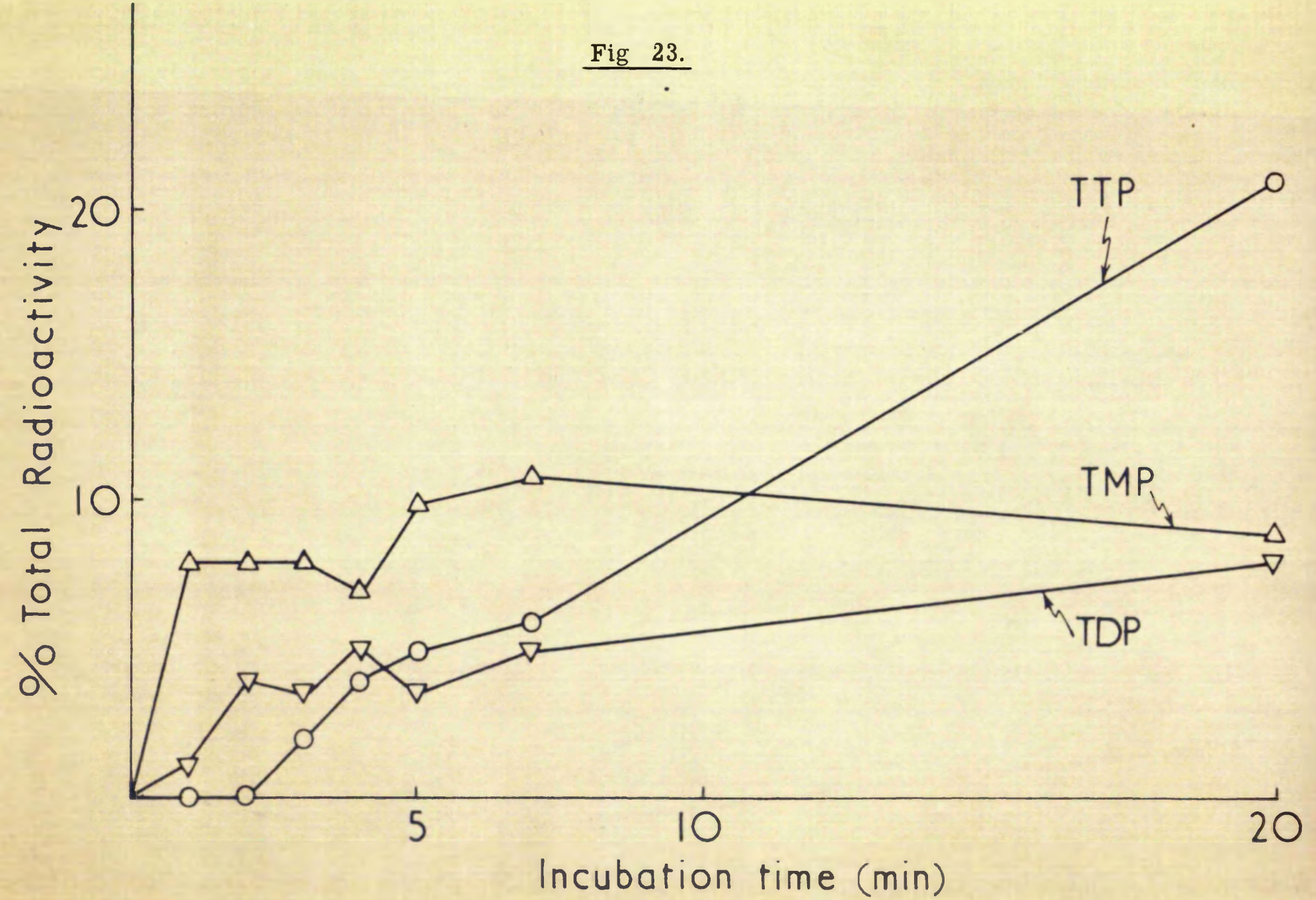

% Total Radioactivity
20
10
TTP
TMP
TDP
5
10
20
Incubation time (min)

Fig. 23. The initial time course of phosphorylation of (^{3}H) TdR by cell-free extracts of Landschutz ascites tumour cells.

The composition of the reaction mixtures was the same as that given in the legend to Fig. 22 except that each reaction mixture contained enzyme solution corresponding to 4.5 mg. protein. Further treatment was as given for Fig. 22 and in the text (Section 2.2.4 (a)). The source of enzymes was the crude cell-free extracts of Landschutz ascites carcinoma. Different enzyme preparations were used for the experiments recorded in Figures 22 and 23.

TMP Δ; TDP ∇; TTP o.

It was apparent that while the initial reaction in the formation *in vitro* of TTP from TdR was the relatively well established phosphorylation of TdR to TMP (Ives, Morse and Potter, 1963; Belts, 1962; Sköld, 1960), little information was available on the precise sequence of the subsequent anabolic events and the crucial point at issue would seem to be the part played by TDP in the process. A series of experiments was, therefore, carried out in order to determine whether the crude enzyme system could catalyse the formation of TTP directly from TDP.

When (^{32}P - α) TDP was used as substrate, it was found that TDP was extensively converted to TTP. Thus, more than 60 per cent of the total radioactivity was recovered as TTP at the end of a one hour incubation (see Fig. 24). Moreover, the addition of a 20-fold excess of unlabelled TMP did not significantly alter the small proportion of radioactivity found in the TMP; subsequent investigation indicated that the radioactivity of the TMP fraction was due to non-enzymic degradation of the TDP during storage and incubation.

The basic assumption in all experiments with (^{32}P) -labelled substrates was that any (^{32}P) orthophosphate present or formed did not exchange with co-valently linked phosphate by other than metabolic means. With this assumption in mind, the object of the present experiment was to discover whether the addition of unlabelled TMP led to any dilution in the radioactivity appearing in TTP. Two possible pathways for the transfer of radioactivity from TDP to TTP could be visualised under the present circumstances, one of which was the direct transformation of TDP to TTP (a). The alternative was a preliminary degradation of TDP to TMP followed by pyrophosphorylation of

Fig 24.

Phosphorylation of α-^{32}P-TDP to TTP

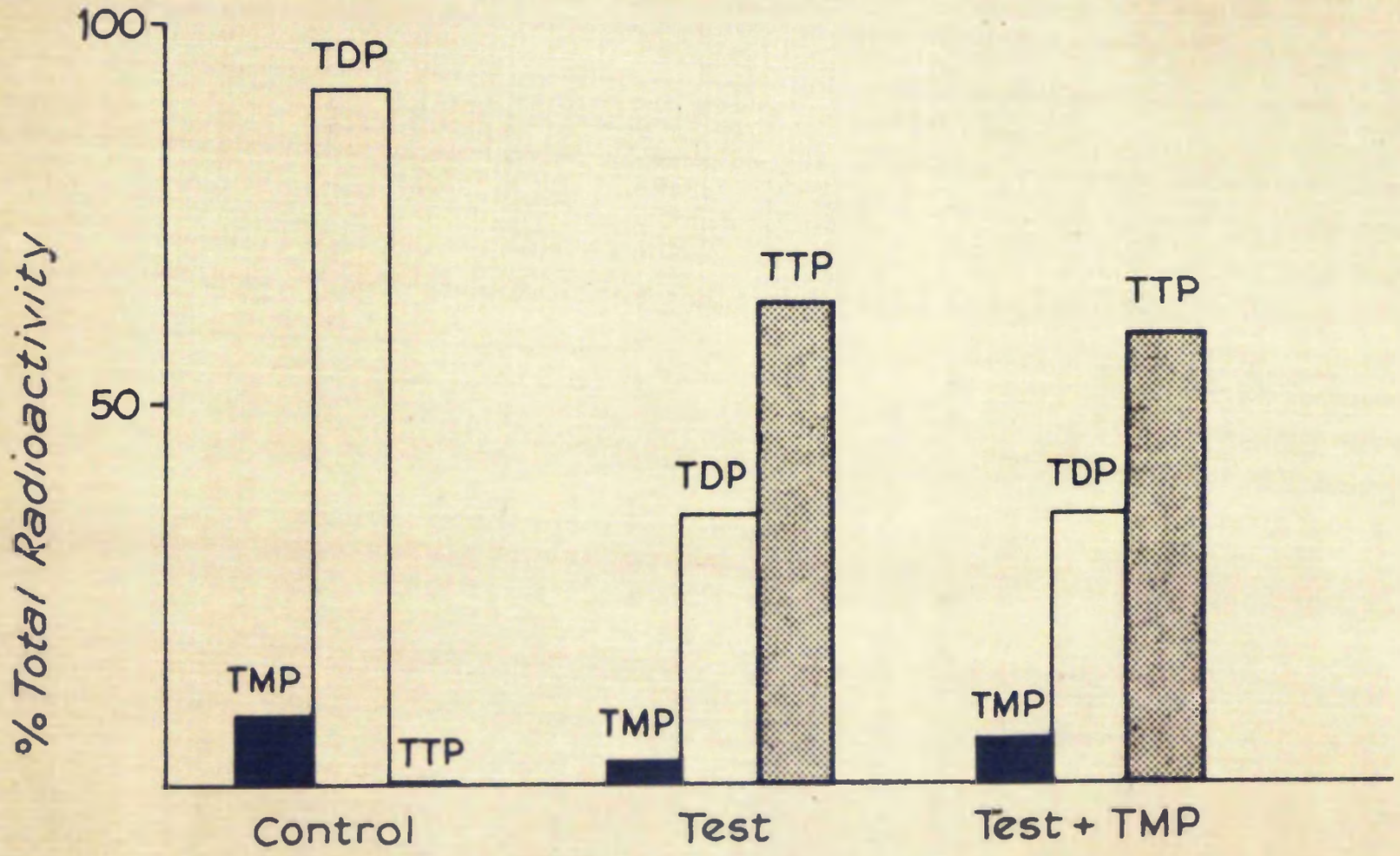
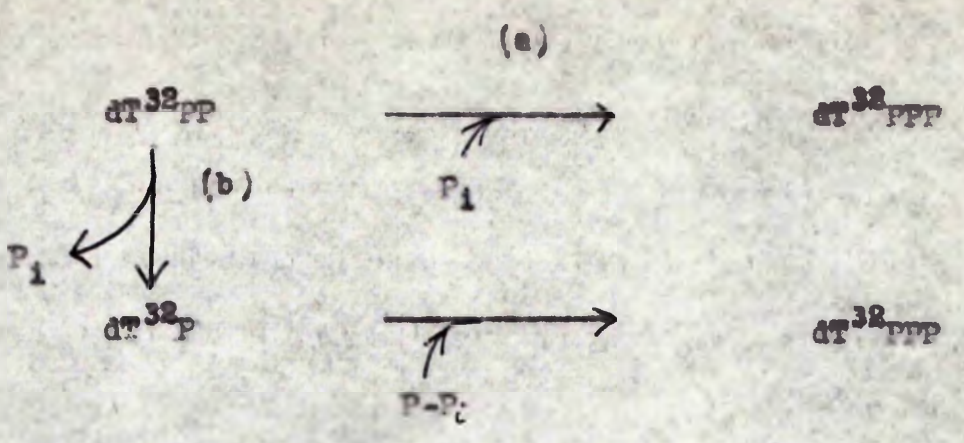


Fig. 24. The phosphorylation of (^{32}P - α) TDP by cell-free extracts of Landschutz ascites tumour cells in the presence and absence of added unlabelled TMP.

The reaction mixtures contained the following components in a total volume of 3.0 ml.: 300 μmoles tris buffer, pH 8.0, 0.3 μmoles 2-mercaptoethanol, 15 μmoles $MgCl_2$, 15 μmoles ATP, 24 mμmoles (^{32}P - α) TDP of specific activity 3.5×10^6 counts per minute per μmole and, where indicated, 500 mμmoles TMP in addition to a volume of enzyme solution corresponding to 4.5 mg. of protein. Incubation was for 60 minutes at 37° and further treatment was as indicated in the text (Section 2.2.4 (a)). The experiment designated "control" represents an incubation in which enzyme solution was omitted from the reaction mixture. The source of enzymes was a solution prepared from the lyophylised cell-free extracts of Landschutz ascites carcinoma.

TMP to give TTP (b) thus:



An experiment in which (^{32}P - a) TDP was incubated with enzyme could, therefore, not be expected to differentiate between the two alternative pathways for producing TTP. However, in the presence of an excess of unlabelled TMP, the operation of alternative (b) would inevitably result in a dilution of the radioactively labelled material entering the monophosphate pool so that, in subsequent pyrophosphorylation, a relatively smaller proportion of the TTP would be labelled. No dilution in the radioactivity recovered in TTP could be expected if the reaction proceeded according to alternative (a). Thus, while the experiment shown in Fig. 34 seems to favour the direct formation of TTP from TDP, the application of steady-state tracer kinetics was inadmissible since the criterion of measurement was the proportion of total radioactivity present in TMP, TDP or TTP. Other experiments were, therefore, carried out in which the specific activities of the TTP formed in the absence and the presence of a large excess of unlabelled TMP were determined. As recorded in Table 3, such data was generally consistent with the earlier conclusion in that only a relatively small reduction in the specific activity of the TTP resulted from the addition of

Table 3.

Reaction mixture	Percentage of total radioactivity recovered	Fraction of total nucleotide content recovered	PERCENTAGE of total radioactivity recovered in:-			Specific activity of thymidine nucleotides counts/min./umole		
			TMP	TDP	TTP	TMP	TDP	TTP
Complete - enzyme	98	*	10	90	-	-	-	-
Complete +2.5 µmoles TMP - enzyme	93	95	10	90	-	2.24×10^3	2.14×10^6	-
Complete + 1.25 µmoles TMP - enzyme	94	91	9	91	-	4.3×10^3	2.2×10^6	-
Complete	90	*	9	32	59	*	2.4×10^6	3.6×10^6
Complete + 2.5 µmoles TMP	92	94	9	31	60	2.27×10^3	1.6×10^6	1.4×10^6
Complete + 1.25 µmoles TMP	89	92	10	27	63	4.21×10^3	1.4×10^6	1.7×10^6

* not determined

Table 3. The formation of thymidine 5'-triphosphate from (^{32}P - α) TDP in presence of added, unlabelled TMP.

The complete reaction mixtures contained the following components in a total volume of 3.0 ml.: 300 μmoles tris buffer, pH 8.0, 0.3 μmoles 2-mercaptoethanol, 15 μmoles $MgCl_2$, 15 μmoles ATP, 85 mμmoles (^{32}P - α) TDP of specific activity 2.41×10^6 counts per minute per μmole and enzyme solution containing 4.5 mg. of protein. Some reaction mixtures included 2.5 μmoles TMP or 1.25 μmoles TMP representing an excess of 100 or 50, respectively, vis a vis the absolute quantity of TDP. Incubation was for 60 minutes in a shaking water-bath at 37°. Deproteinised, neutralised reaction mixtures were diluted to 7.0 ml., 5.0 ml. of which were fractionated on columns of ECTEOLA cellulose (10 x 1.5 cm., dia.), 0.1 ml. portions of each 10 ml. eluate fraction being taken for counting. Eluate fractions containing either TMP, TDP or TTP were hydrolysed and the adenosine nucleotides removed as described in the text (Section 2.8.4 (b)) and the total extinction at 267 mμ determined in the recovered reaction products. The enzyme source was the lyophylised cell-free extracts of Landschutz ascites carcinoma.

100-fold or 50-fold excess of unlabelled TMP to the reaction mixtures. The minor fluctuations in specific activities that were observed could be accounted for by the considerable error involved in measuring the extremely small amounts of TTP present and no differences were observed such as would have been necessary had the TTP been formed from the TDP via a large pool of TMP. Moreover, some of the observed reduction in the specific activity of the TTP in the presence of unlabelled TMP could be due to phosphorylation of such unlabelled material.

These experiments clearly demonstrated the ability of the enzyme preparations to form TTP from TDP and provided no evidence for the intermediate formation of TMP. Furthermore, they provided no evidence for the formation of any higher phosphorylated derivatives of thymidine than TTP in contrast to the experience of Bianchi, Butler, Cruthorn and Shooter (1961). This was shown by washing the columns of ECTEOLA cellulose repeatedly with 0.5 N hydrochloric acid after all the TTP had been eluted (see Section 2.2.4 (a)).

Further confirmation of these conclusions was furnished by experiments on the time course of phosphorylation of (^{32}P - β) TDP as shown in Fig. 25. It became clear that the TDP was very rapidly and extensively converted to TTP more than 60 per cent of the total radioactivity being recovered in the TTP within 5 minutes. About 20 per cent of the total radioactivity at all time intervals was accounted for as inorganic orthophosphate, but this proved to be due to contamination of the TDP preparation (see Section 2.2.2 (d)) and did not vary significantly during the course of the incubation.

Fig. 25.

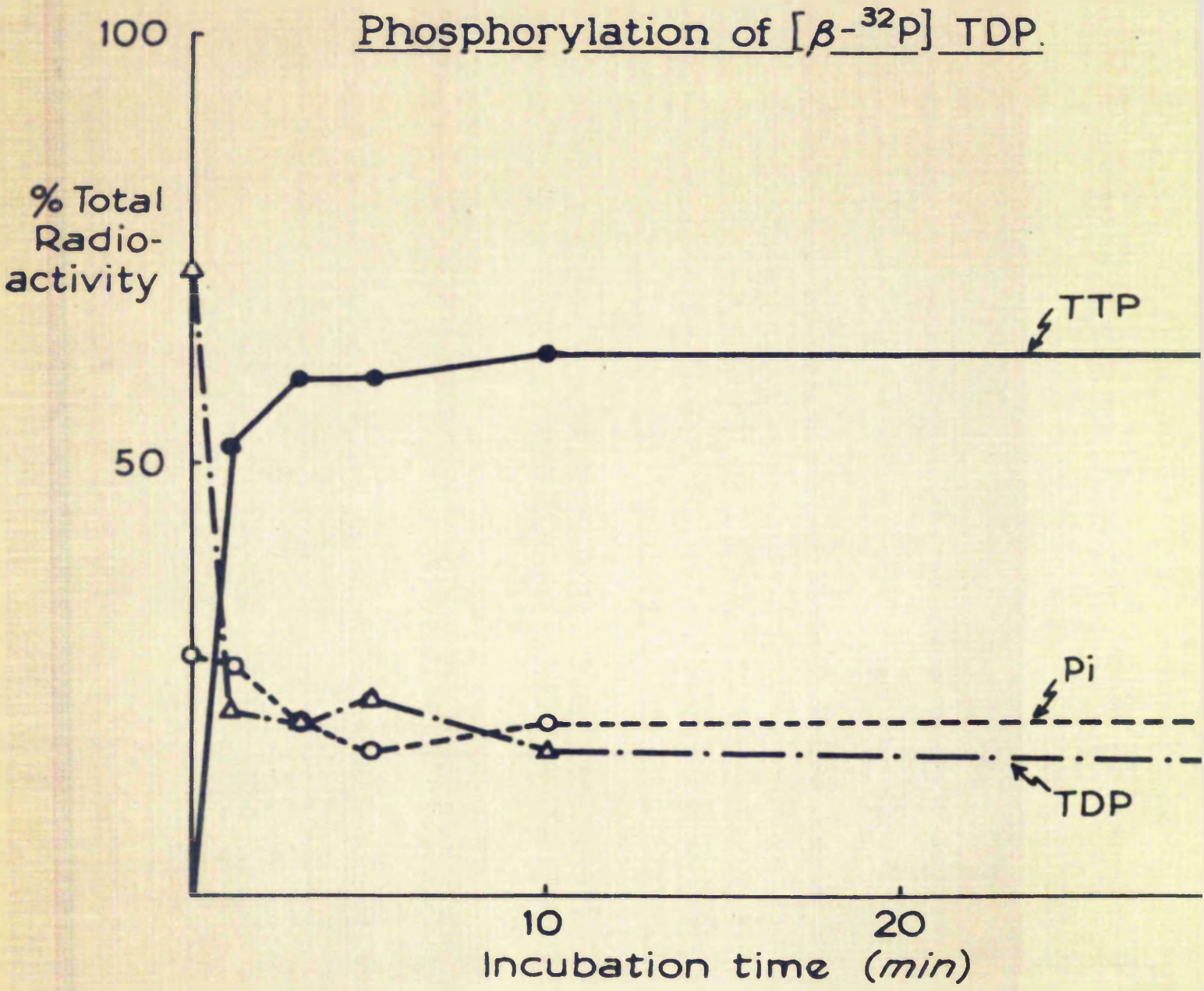

Phosphorylation of [β-32P] TDP.
100
% Total Radio-activity
50
TTP
Pi
TDP
10
20
Incubation time (min)

Fig. 25. The time course of phosphorylation of (^{32}P - β) TDP by cell-free extracts of Landschutz ascites tumour cells.

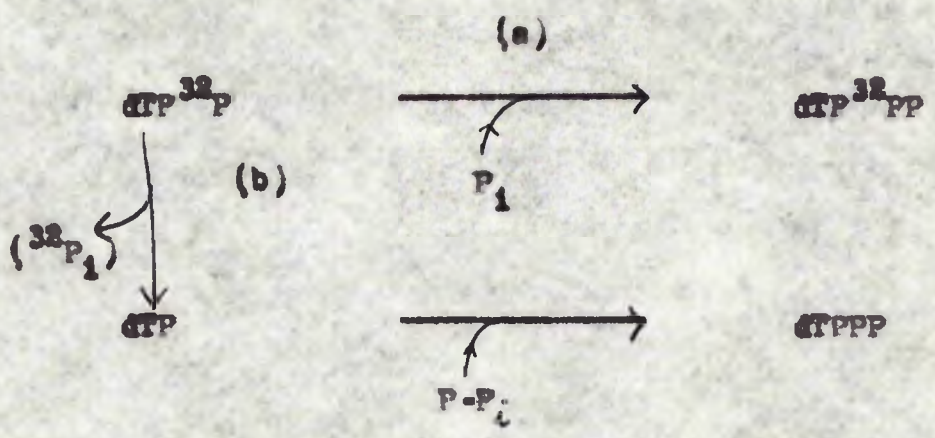
The reaction mixtures contained the following components in a total volume of 3.0 ml.: 300 μmoles tris buffer, pH 7.9, 0.3 μmoles 2-mercaptoethanol, 15 μmoles $MgCl_2$, 15 μmoles ATP, 0.172 μmoles (^{32}P - β) TDP of specific activity 1 x 10^6 counts per minute per μmole and a volume of enzyme solution corresponding to 4.5 mg. of protein. Incubation was for the time intervals indicated on the diagram in a shaking water-bath at 37°. Further treatment was as described in the text (Section 2.2.4 (a)). The source of enzymes was a solution prepared from the lyophylised cell-free extracts of Landschutz ascites carcinoma.

TDP —·—△—·—

TTP ———●———

Inorganic orthophosphate (P_i) - - - - -○- - - - -

Since the TDP used in these experiments was labelled with (^{32}P) in the β-phosphate group, any degradation of the TDP, by for example phosphatase action, would have given rise to unlabelled TMP and (^{32}P) orthophosphate so that labelled TTP could only have been formed from such TMP if the (^{32}P) orthophosphate had been utilised in the phosphorylation of the TMP or had taken part in an enzyme catalysed exchange with the phosphate group of TMP. The two main alternatives for the formation of TTP from TDP in such a system may be indicated as follows:



In order to exclude the possibility that (^{32}P) orthophosphate might be involved in exchange reactions with covalently linked phosphate groups under the prevailing experimental conditions, experiments were carried out in which unlabelled TDP together with an amount of carrier-free (^{32}P) orthophosphate corresponding to the amount of (^{32}P) in the (^{32}P - β) TDP was substituted for the (^{32}P - β) TDP. No labelled TTP was formed under these conditions, nor was there any evidence in any of these experiments for the formation, however transient, of higher phosphorylated derivatives of thymidine than TTP.

From these two types of experiment, it became clear that the crude preparations of enzyme contained a kinase for the phosphorylation of TDP to

TTP and that this reaction occurred very rapidly without there being any evidence for the intermediate formation of TMP or of higher phosphates than TTP.

Further studies were focussed on the remaining step in the phosphorylation of thymidine to TTP. It seemed likely that if the initial step consisted of phosphorylation of TdR to TMP and the ultimate step was the phosphorylation of TDP to TTP, there should also exist an enzyme in the crude enzyme preparations capable of catalysing the formation of TDP, with TMP as its substrate.

Studies on the time course of phosphorylation of (^{32}P) TMP were, consequently, carried out and the results of such an experiment is shown in Fig. 26. It is clear from this that at very early time intervals labelled TDP appears among the reaction products before there is any evidence of labelled TTP, thus the proportion of total radioactivity in TDP increased from time zero while no labelled TTP could be detected until after 5 minutes, thereafter labelled TDP reached a plateau at about 15 per cent of the total recovered radioactivity. That the radioactivity in the TDP never rose to a high level may be due to the rapid rate at which TDP is converted to TTP as shown in Fig. 25. The general pattern of phosphorylation of (^{32}P) TMP was consistent with the picture revealed by the experiments recorded in Figures 21 and 23 where the initial substrate was (^{3}H) TdR. While the interpretation of these experiments were subject to the reservations stated above (p.99), the data seems to suggest the possibility that the rate of formation of TDP may be rate limiting in TTP synthesis.

Fig. 26. The time course of phosphorylation of (^{32}P) TMP by cell-free extracts of Landschutz ascites tumour cells.

The reaction mixtures contained the following components in an total volume of 1.0 ml.: 100 µmoles tris buffer, pH 7.9, 0.1 µmoles 2-mercaptoethanol, 5 µmoles $MgCl_2$, 5 µmoles ATP, 5.5 mumoles (^{32}P) TMP of specific activity 86 x 10^6 counts per minute per µmole and enzyme solution containing 1.5 mg. of protein. Incubation was for the times indicated on the diagram in a shaking water-bath at 37°. Further treatment was as described in the text (Section 2.8.4 (a)). The source of enzymes was a solution prepared from lyophylised cell-free extracts of Landschutz ascites carcinoma.

TMP △ ; TDP ▽ ; TTP o.

In subsequent experiments, attempts were made to measure the specific activities of the TMP, TDP and TTP at various time intervals during the conversion of (^{32}P) TMP to TTP. In these experiments, large excesses of unlabelled TDP and TTP were added to the reaction mixtures in order to provide sufficient quantities of TDP and TTP in the reaction products for accurate spectrophotometric measurements. The results of such an experiment, in which the reaction mixtures included a 10-fold excess of TDP and TTP relative to the absolute TMP concentration, are shown in Fig. 27. The high total thymidine nucleotide concentration thus contained in the reaction mixture, however, resulted in serious inhibition of the reaction so that only about 2 per cent of the (^{32}P) TMP was phosphorylated within time intervals up to one hour. Nevertheless, it was shown that the specific activity in the TDP rose to an early maximum value before any significant increase in the specific activity of the TTP was observed, this observation being therefore consistent with the reaction sequence TMP $\rightleftharpoons$ TDP $\rightleftharpoons$ TTP. The significance of these data was, however, minimised by two important factors. Firstly, the fact that the extent of the total reaction was so small made precise measurements of reaction products difficult and, secondly, it seemed clear that the ideal conditions as visualised by Zilversmit, Entenman and Fishler (1943) for steady-state precursor - product relationships did not obtain in the isolated reaction system studied here. In the present case, it seems reasonable to suggest that the enzyme system was particularly sensitive to accumulation of reaction products and that the efficiency of the process of TTP synthesis thus depends, in part, on the efficient

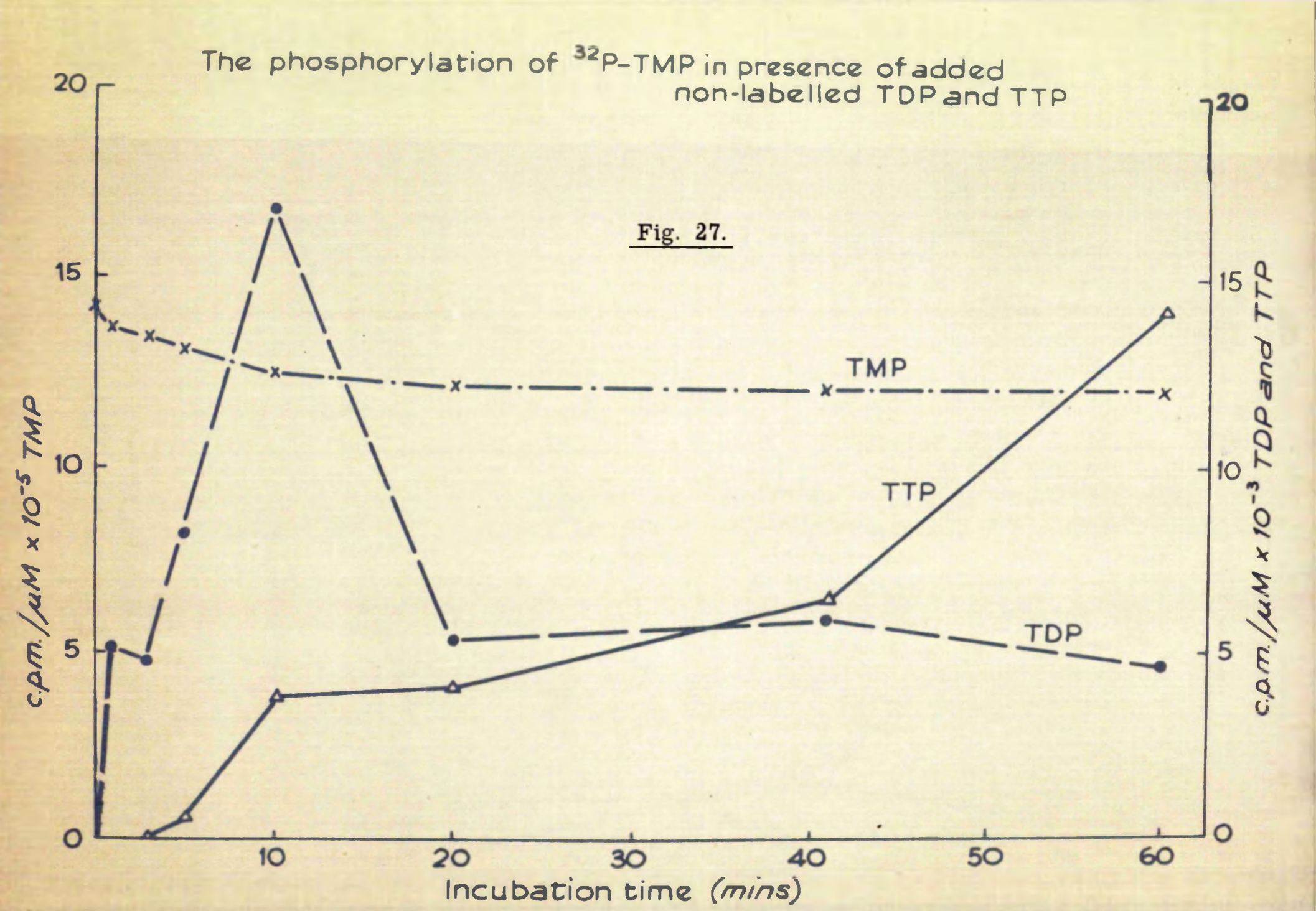

The phosphorylation of ^{32}P-TMP in presence of added non-labelled TDP and TTP
TMP
TTP
TDP
c.p.m./μM x 10^{-5} TMP
c.p.m./μM x 10^{-3} TDP and TTP
Incubation time (mins)
0
5
10
15
20
30
40
50
60

Fig. 27.

Fig. 27. The time course of phosphorylation of (^{32}P) TMP in presence of added, unlebelled TDP and TTP by cell-free extracts of Landschutz ascites tumour cells.

The reaction mixtures contained the following components in a total volume of 3.0 ml.: 300 µmoles tris buffer, pH 8.0, 0.3 µmoles 2-mercaptoethanol, 15 µmoles $MgCl_2$, 15 µmoles ATP, 50 mumoles (^{32}P) TMP of specific activity 1.5×10^6 counts per minute per µmole, 0.75 µmoles TDP, 0.75 µmoles TTP and enzyme solution containing 4.5 mg. of protein. Incubation was for the time intervals indicated on the diagram in a shaking water-bath at 37°. Neutralised, deproteinised assay solutions were diluted to 7.0 ml., 3.5 ml. of which were fractionated on columns of EGTEOLA cellulose, 0.1 ml. portions of each 10 ml. eluate fraction being plated on stainless steel planchettes and counted. Eluate fractions containing either TMP, TDP or TTP were hydrolysed and the adenosine nucleotides removed as described in the text (section 2.2.4 (b)), and the total extinction at 267 mµ measured in the recovered reaction products. The source of enzymes was a solution prepared from lyophylised cell-free extracts of Landschutz ascites carcinoma.

TMP —·—×—·—

TDP — — ● — —

TTP ——→△←——

utilisation of TTP. Such a situation may be visualised *in vivo* when the cell is actively synthesising DNA.

Thus, circumstantially, all the evidence presented in the present Chapter supports the hypothesis of Weissman, Smellie and Paul (1960) that the formation of TTP from thymidine proceeds in a stepwise manner according to the reaction sequence:

$$TdR \rightleftharpoons TMP \rightleftharpoons TDP \rightleftharpoons TTP$$

However, these experiments failed to establish beyond all doubt the participation of an enzyme capable of catalysing specifically the phosphorylation of TMP in the synthesis of TTP and attempts to resolve this uncertainty is the subject of the investigation reported in Chapter III.

C H A P T E R III

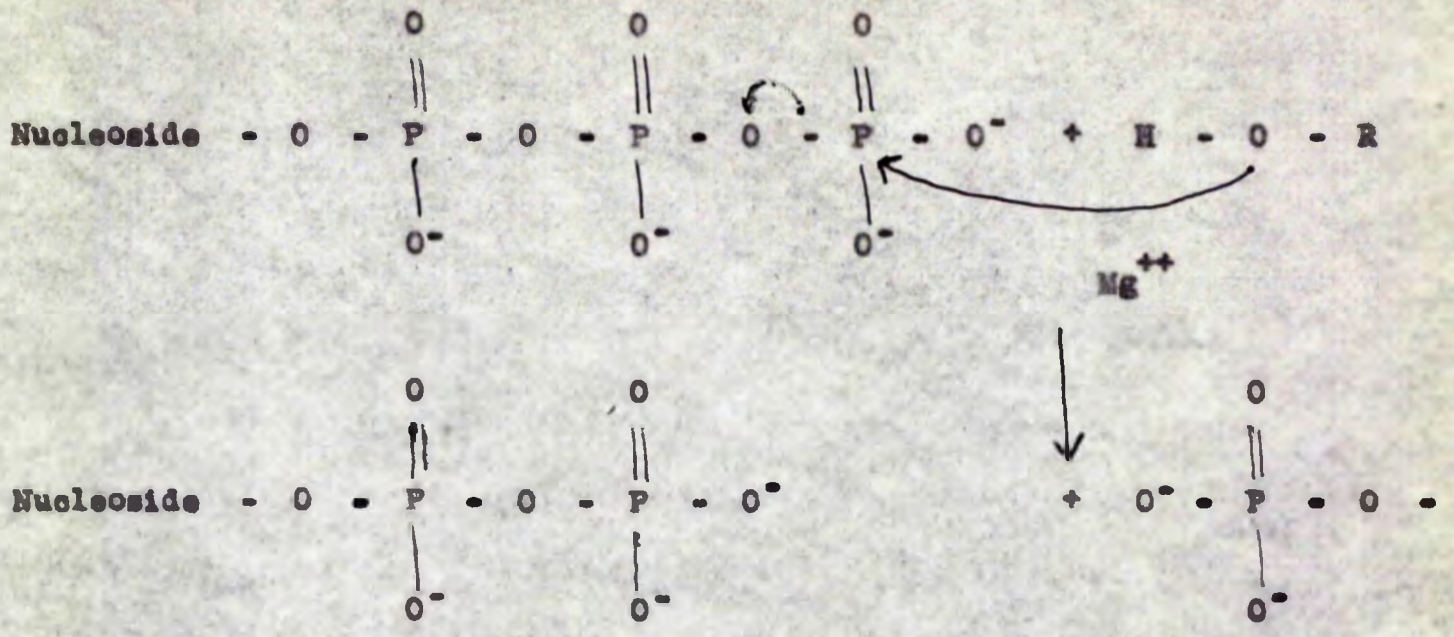
"PRELIMINARY ENZYMOLOGICAL STUDIES ON THE THYMIDINE AND THYMIDYLATE KINASES OF LANDSCHUTZ ASCITES TUMOUR CELLS."

3.1. Introduction

General properties of nucleoside and nucleotide phosphokinases

The phosphorylation of nucleosides and nucleotides to the corresponding nucleoside 5'-triphosphates (see Figs. 10 and 11) has been presumed to occur under the influence of a series of phosphate transferring enzymes, termed phosphokinases, in reactions which involve the transfer of the terminal phosphoryl group of a nucleoside triphosphate to the acceptor molecule (see Section 1.2.(d)). Such kinases belong to a class of enzymes which catalyse coupled reactions involving the transfer of energy and, as such, they are normally considered markedly exergonic; the catalysed reactions, therefore, have equilibria strongly in favour of the production of phosphorylated products. A large number of such enzyme activities has been described, but in the absence of discriminating kinetic data, it is frequently difficult to decide whether the reported enzymes represent unique molecular species or different forms of the same enzyme.

Critical scrutiny of the available data shows that only very few of the reported nucleoside and nucleotide kinases satisfy the rigid set of criteria demanded of specific enzymes while information on a number of other kinases is no more than the evidence of their existence. A few well known kinases, notably myokinase (see p. 25), have been intensively studied and it now seems probable that the reaction mechanism involves a nucleophilic attack by the acceptor hydroxyl group on the terminal phosphorus atom of the nucleoside triphosphate in the following manner:-



That the reaction catalysed by myokinase involves the transfer of a terminal phosphoryl group, and not a phosphate group, from ATP to AMP was demonstrated by Cohn (1956) with the use of ^{18}O-labelled substrates and there is currently reason to think that phosphoryl group transfer from the donor is a general feature of kinase catalysed reactions. Most kinase catalysed reactions appear to possess pH optima of 7 - 9, a region where nucleotides can be considered to exist in their completely dissociated forms. One further important feature of enzymic phosphoryl group transfer reactions seems to be their dependence on the presence of di-valent cations, usually Mg^{++} or Mn^{++}. The generally accepted explanation for the Mg^{++} dependence of many kinase reactions is that the ion forms a chelate with the terminal phosphate groups of the nucleoside triphosphate and, in so doing, screens a number of negative charges on phosphate oxygens of both donor molecules and acceptor molecules (Lowenstein, 1958). However, there is little information as to the precise details of the mechanism or the sequence of

events leading through chelation, binding of substrates at the active sites and the transfer of the phosphoryl group; but of the kinases so far studied sufficient evidence is available to justify the conclusion of Noda (1962) that: "Undoubtedly the mechanism of phosphoryl transfer in all the so-called high-energy phosphate-transferring enzymes is similar, and as the picture is pieced together for one enzyme the principles will become generally applicable

Since the kinases catalyse coupled reactions it seems reasonable to suppose the existence of two binding sites, one for each nucleotide substrate, and, in the case of myokinase, it has in fact been found that AMP and ATP bind specifically at adjacent sites on the enzyme surface (Kuby, Mahowald and Noltmann, 1962). One consequence of this finding is that the kinases exhibit double substrate specificity, i.e., specificity towards donor and specificity towards acceptor molecules. The majority of purified kinases prefer ATP as the phosphoryl group donor, but several systems have been described where other nucleoside 5'-triphosphates can substitute for ATP.

Nucleoside kinases are thought to catalyse the phosphorylation of a nucleoside to the corresponding nucleoside 5'-monophosphate. Such reactions are normally regarded as part of the preformed pathways in the formation of nucleic acid precursors. Kinase catalysed reactions involving all the ribonucleosides and deoxyribonucleosides which occur as major components of the nucleic acids are known to take place. However, only in the case of adenosine kinase (Caputto, 1951; Kornberg and Pricer, 1951) and uridine kinase (Skold, 1960) have these activities been shown to be due to specific enzymes. With both types of preparation strict stoichiometry was observed

and ATP was the preferred phosphoryl group donor.

Enzymes which catalyse the transfer of a phosphoryl group from nucleoside 5'-triphosphates to nucleoside 5'-monophosphates are widely distributed and include enzymes exhibiting a wide range of substrate specificities. At the extreme end of the scale, myokinase requires the presence of ATP and AMP while, at the other end, the ATP-nucleoside monophosphate kinase from calf liver (Strominger, Heppel and Maxwell, 1959) requires ATP as phosphate donor but can utilise AMP, GMP, CMP and UMP as acceptors. Similarly, the nucleoside triphosphate-AMP kinase from the same source (Heppel, Strominger and Maxwell, 1959) is specific for AMP but the nucleoside triphosphate could be ITP, ATP, GTP, CTP or UTP. Several enzymes which phosphorylate deoxyribonucleoside 5'-monophosphates have also been reported, but only very few exhibit definable specificity. In particular it seems uncertain whether the substrate specificity of such kinases extends to distinction between deoxyribosyl and ribosyl moieties of the same base. This uncertainty stems from the properties of an ATP-dGMP kinase discovered in extracts of Azotobacter vinelandii (Maley and Ochoa, 1958) which, after purification, was shown to catalyse the phosphorylation of GMP at 60 per cent the rate of dGMP while being inactive on other nucleoside monophosphates. Similarly, a dGMP kinase purified from E.coli (Bessman, 1963) appears to phosphorylate GMP at the same rate as dGMP while ATP is required as the donor of the phosphoryl group.

Further phosphorylation of the nucleoside 5'-diphosphates produced by the above reactions has been attributed to the action of nucleoside di-

phosphokinases. Such enzymes are known to have a wide distribution, but the reactions which they catalyse are not well characterised and a specific nucleoside diphosphokinase has yet to be discovered. Thus, the enzyme from rabbit muscle (Berg and Joklik, 1954) appeared to require ATP while those derived from yeast (Berg and Joklik, 1954) and from rat liver mitochondria (Chiga and Plaut, 1959, 1962) could utilise ITP, ATP, GTP, CTP and UTP; all preparations could use any ribonucleoside 5'-diphosphate as the phosphoryl group acceptor. Little evidence is available on the existence of enzymes capable of phosphorylating deoxyribonucleoside 5'-diphosphates but it seems probable that such reactions do occur as part of the de novo pathway for the formation of dGTP, dATP, dCTP and TTP. Evidence is presented elsewhere (see Fig. 25) that an ATP-TDP kinase occurs in Landschutz ascites tumour cells.

In the preceding Chapter, a number of experiments have been described which strongly support the hypothesis (Weissman, Smellie and Paul, 1960) that the formation of TTP from TdR proceeds by stepwise phosphorylation through TMP and TDP. It seems plausible, therefore, to suggest that the reaction is catalysed by a series of at least three phosphokinases, each responsible for on step in the sequence:

$$\text{TdR} \xrightarrow{a} \text{TMP} \xrightarrow{b} \text{TDP} \xrightarrow{c} \text{TTP}$$

For the sake of brevity, it is proposed to give the names TdR kinase, TMP kinase and TDP kinase to the enzymes catalysing reactions a, b and c, respectively. In the present Chapter, an attempt is made to characterise and resolve the relevant kinase activities from extracts of Landschutz ascites tumour cells.

3.2. Experimental

3.2.1. General methods

The investigations recorded in Chapters 2 and 3 overlap sufficiently for a number of the experimental procedures given in Section 2.2. to be applicable to problems concerned with the enzymology of the thymidine and thymidylate kinases. Such methods include the preparation of substrates (Section 2.2.2.), the measurement of thymidine and thymidylate kinase activities (Section 2.2.4) and various analytical methods to which the appropriate references are made. Only those experimental methods which have been modified in any fundamental way to satisfy new requirements or new analytical procedures are described in detail below. Some additional techniques are also given in the legends to the figures and the tables.

3.2.2. Biological material

(a) Extracts from Landschutz ascites tumour cells

The source of enzymes for the majority of the experiments described hereafter has been an accumulated pool of lyophylised cell-free extracts from Landschutz ascites carcinoma prepared as described earlier (Section 2.2.1.). This pool of lyophylised material was derived from about 400 tumour bearing mice of the departmental colony, ascites fluid being withdrawn from 30 mice per preparation, and the resulting lyophylised powder carefully mixed with earlier preparations so as to give a relatively homogeneous stock from which material could be withdrawn for the ensuing enzyme experiments. Evidence

for the homogeneity of the enzyme source was repeatedly obtained by determining the initial activity of the relevant enzymes. In such experiments, the initial activity showed insignificant variation over a period of months. Further information on the stability of the thymidine and thymidylate kinases is given elsewhere (Section 3.3.3.).

(b) Extracts from normal rat liver

Male albino rats, weighing from 180 to 220 g., from the departmental colony were employed. The rats were anaesthetized with ether and the liver excised after severing the hepatic artery, and allowing the blood to drain. Immediately after removal, the liver was chilled on crushed ice, cut into small pieces with scissors, weighed and homogenised in 8 volumes of ice-cold 0.01 M tris buffer, pH 7.5, in a cooled Potter-type homogeniser. After the initial suspension of the tissue, about 5 passes of the homogeniser pestle was found to give an extract relatively free from whole cells without disrupting many nuclei, as judged by microscopic examination of wet smears, stained with 1 per cent crystal violet in 0.1 M citric acid. The resulting homogenate was centrifuged in a Servall Type SS-4 Superspeed centrifuge at 27 000 x g for 1 hour at 0°, and the practically clear supernatant fluid decanted. This extract had a protein concentration of 5 - 7 mg. per ml. and was either used directly in enzyme experiments or lyophylised and stored at -50°.

3.2.3. Procedures used for the assay of enzymes

(a) Thymidine kinase

While the assay procedure described earlier (Section 2.2.4 (a))

provided a precise means for the determination of each of the products (TdR, TMP, TDP and TTP) involved in the formation of TTP from TdR, this procedure was found to be unsuitable for the purpose of assessing TdR kinase activity in the course of enzyme fractionation experiments. The rapid assay which was developed for TdR kinase followed in principle the earlier procedure but involved less rigorous separation of the reaction products. This assay was based on the determination of the total radioactivity found in all phosphorylated reaction products when the substrate was (^{3}H) TdR.

The following reaction mixture was prepared:- 100 µmoles of tris buffer, pH 8.0, 5 µmoles ATP, 5 µmoles of $MgCl_2$, 0.1 µmoles of 2-mercapto-ethanol, 1 µc (^{3}H) TdR of specific activity 360 mc per mmole and enzyme solution containing various quantities of protein as given in the legends to the figures and the tables. The total volume of the reaction mixture was 1.0 ml. Enzyme solutions, when prepared from the stock of lyophylised cell-free crude extracts, were made up in distilled water to give a protein concentration of 6 - 8 mg. per ml. Incubation was for various times in a shaking water bath at 37°. The reactions were terminated by heating for 3 minutes at 100° and all subsequent manipulations were carried out in the cold. Coagulated protein was removed by centrifugation and washed twice with 0.5 ml. distilled water each time, adding the washings to the supernatant fluid. The solution was then diluted to 3.0 ml. with distilled water. A portion of this solution (2.0 ml.) was adsorbed on a column of EDTEOLA cellulose (10 x 1.5 cm., dia.) and the TdR and thymidine nucleotides were eluted by applying successively 150 ml. distilled water and 100 ml. 0.2 M

hydrochloric acid. The two eluates were diluted separately to finite volumes, 0.1 ml. portions of which were plated on stainless steel planchettes and counted in a Nuclear-Chicago windowless gas-flow counter. The results were expressed in terms of the percentage of the total radioactivity recovered in all phosphorylated thymidine derivatives (TMP + TDP + TTP) or as the number of mumoles of TdR phosphorylated.

Preliminary experiments had shown that the water fraction contained TdR only while the HCl fraction contained the TMP, TDP and TTP. Thus the method provided a direct means of measuring the total phosphorylation of TdR.

In order to assess the recovery of total radioactivity, blanks in which enzyme solution was omitted were included in each series of experiments. Such blank determinations showed a consistent recovery of total radioactivity of 80 - 90 per cent. In experiments where the specific activity of TdR kinase was sought, appropriate corrections were made for the blank values. A further check on the validity of the assay procedure was by paper chromatography. The method adopted involved taking a portion (0.5 ml.) from the assay solution prior to the separation on ECTEOLA cellulose, concentrating the reaction products by lyophylisation and separating the thymidine nucleotides by chromatography on paper as described earlier (Section 2.2.3 (b)). The fully developed chromatogram was then scanned for radioactivity by means of the Actigraph scanning device (Section 2.2.3 (d)).

Several preliminary experiments had shown that TMP was relatively resistant to non-enzymic hydrolysis (see also Section 2.2.4 (b)) and placing reaction mixtures in which (^{3}H) TdR was replaced by 5 µmoles of TMP in a

boiling water-bath for periods up to 10 minutes did not result in the production of sufficient TdR to be detectable by paper chromatography. It would, therefore, appear that the pattern of total phosphorylation of (^{3}H) TdR remained undisturbed whether the TdR kinase was inactivated by heat or by the use of perchloric acid (Section 2.2.4 (a)).

(b) Radioactivity assay for TMP kinase

On a few occasions when it was necessary to obtain exact data on the relative amounts of each of the reaction products, the original kinase assay (Section 2.2.4 (a)) was employed. On most other occasions, however, this procedure proved too time consuming and a rapid assay was developed based on a principle similar to that underlying the rapid assay for TdR kinase (see above). Thus the rapid assay measured the total phosphorylation of (^{32}P) TMP by determination of the total radioactivity found in the phosphorylated derivatives (TDP + TTP).

The following reaction mixture was prepared in a total volume of 1.0 ml.:- 100 µmoles tris buffer, pH 7.6, 5 µmoles ATP, 5 µmoles $MgCl_2$ and (^{32}P) TMP and enzyme solution in the quantities given in the legends to the figures and the tables. In experiments where the TMP kinase activity of the lyophylised crude ascites extract stock was measured, the dry material was dissolved in distilled water or in 0.01 M phosphate buffer, pH 7.6, to give solutions containing 6 - 8 mg. protein per ml. Incubation was performed for various times in a shaking water-bath at 37°. The reactions were terminated by heating at 100° for 3 minutes and subsequent manipulations were carried out in the cold. The precipitated protein was removed by centri-

fugation and washed twice with 0.5 ml. portions of 0.01 M phosphate buffer, pH 7.6, the washings and supernatant fluid were combined and the solution diluted to 3.0 ml. A portion of this solution (1.0 ml.) was adsorbed on a column of ECTEOLA cellulose (10 x 1.5 cm., dia.) and the thymidine nucleotides eluted by applying successively 150 ml. of 0.015 N hydrochloric acid and 100 ml. of 0.2 N hydrochloric acid. TMP was recovered in the 0.015 N HCl fracti and TDP and TTP were eluted in the 0.2 N HCl fraction. The two fractions were separately diluted to finite volumes, 0.1 ml. portions of which were plated on stainless steel planchettes and counted in a Nuclear-Chicago gas-flow counter fitted with a thin end window. The results were expressed in terms of the percentage of the total radioactivity recovered in thymidine polyphosphates (TDP + TTP) or as the number of mumoles of TMP phosphorylated. Control incubations in which enzyme solution had been omitted showed that 90 - 95 per cent of the total radioactivity present initially was consistently recovered.

Preliminary elution experiments had shown that complete resolution of TMP and thymidine polyphosphates could be effected by this procedure which, therefore, provided a measure of the total phosphorylation of TMP. Other experiments had indicated that the two reaction products, TDP and TTP, were considerably more thermolabile than TMP and that inactivation of the enzyme system by heating at 100° was likely to result in some hydrolysis of these products. However, it was found that heating of the reaction mixture at 100° for 2 minutes led to only marginal degradation of TDP and TTP while the treatment sufficed to coagulate the protein and inactivate the enzyme system.

Additional data on the proportion of the reaction products due to the action of TMP kinase and the TDP kinase was obtained by paper chromatography. In such experiments, a portion (1.5 ml.) of the deproteinised assay solution at the stage prior to adsorption on ECTEOLA cellulose was lyophylised, redissolved in 0.1 ml. of distilled water and a 0.05 ml. portion of this solution was submitted to descending chromatography on paper as described earlier (Section 8.2.3 (b)). The positions of the radioactive areas on the chromatogram were ascertained by the use of the Nuclear-Chicago Actigraph system (Section 8.2.3 (d)). Moreover, since (^{32}P) -labelled TMP was employed as substrate in such experiments and as the efficiency of the windowless gas-flow chamber for this isotope was relatively high, it proved possible to obtain semi-quantitative estimates of the three nucleotides TMP, TDP and TTP by scanning appropriate fully developed chromatogram strips. Such estimates were obtained by weighing relevant peaks cut from the recorder chart. The validity and reproducibility of this procedure was checked by cutting relevant radioactive areas from the chromatogram strip, eluting the nucleotides by capillary flow in distilled water and counting portions of the eluates on stainless steel planchettes in a Nuclear Chicago windowless gas-flow counter. Although the kinase activity as measured by the above procedure primarily reflects TMP kinase activity, it was possible to obtain a measure of the TDP kinase activity by suitable correlation of the data from the two types of product fractionation outlined above. TDP kinase activity was not measured in enzyme fractions in which TMP kinase activity was absent.

(c) Spectrophotometric assay for TMP kinase

A spectrophotometric assay for TMP kinase was developed on the basis of procedures described for the measurement of adenosine kinase (Kornberg and Pricer, 1951) and myokinase (Lieberman, Kornberg and Simms, 1955) activities. This method measured the rate of formation of nucleoside diphosphates by coupling their formation to the oxidation of NADH. In the presence of excess lactic dehydrogenase, pyruvate kinase, phospho(enol)-pyruvate and NADH, ADP was quantitatively converted to ATP with the simultaneo stoichiometric oxidation of NADH and the following sequence of reactions can be visualised:-

$$\text{TMP} + \text{ATP} \longrightarrow \text{TDP} + \text{ADP}$$

$$\text{Phospho(enol)pyruvate} + \text{ADP} \longrightarrow \text{pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} \longrightarrow \text{lactate} + \text{NAD}^{+}$$

Thus, if TMP kinase can be considered the rate limiting factor in the reaction the disappearance of NADH as measured by a decrease in extinction at 340 mμ should be a direct measure of the TMP kinase activity. However, on account of the nonspecific nucleoside diphosphokinases present in pyruvate kinase preparations (Klenow and Lichter, 1957), both ADP and TDP were converted to their respective triphosphates. Thus 2 moles of NADH were oxidised for every mole of TMP phosphorylated.

A reaction mixture containing the following components in a total volume of 2.29 ml. was prepared in a 10 m.m. quartz cuvette:- 0.1 μmoles TMP, 10 μmoles ATP, 10 μmoles $MgCl_2$, 0.25 μmoles NADH, 0.5 μmoles phospho(enol)pyruvate, 200 μmoles tris buffer, pH 7.6, 20 μg. lactic

dehydrogenase, 20 µg. pyruvate kinase and enzyme solution. The reaction was initiated by the addition of TMP and the rate of NADH oxidation followed by the decrease of extinction at 340 mµ in a Beckman Model DU spectrophotometer fitted with a chart recorder. All experiments were conducted at 30°. The average decrease in extinction over a 10 minute period was determined in each experiment. A time curve of the spectrophotometric procedure for two different enzyme concentrations is shown in Fig. 23. It became apparent, however, that the TMP kinase preparations frequently contained phosphatases acting on ATP thus distorting the pattern of NADH oxidation due to TMP kinase and in such cases the observed reaction rate was corrected by subtracting the values obtained in reactions where TMP had been omitted (see also Section 3.2.3 (e)). The results were calculated as µmoles TMP phosphorylated per 25 minutes per ml. enzyme solution or per mg. protein.

The assay could not be used for measuring TMP kinase in the crude ascites extract because of endogenous phosphatases and NADH oxidases and it was not used for other than highly purified TMP kinase preparations. In a series of determinations with 50, 100, 200 and 400 µg. of protein from the SC_8AS 4.7 fraction (see Section 3.3.5 (e)) the respective corrected ΔE_{340} values of 0.062, 0.060, 0.065 and 0.063 per minute per mg. protein were obtained thus reflecting a linear relationship between reaction rate and enzyme concentration.

The spectrophotometric assay was occasionally also used for the quantitative measurement of nucleoside diphosphates. When the assay was employed for this purpose, the TMP kinase was omitted from the reaction

Fig. 28.

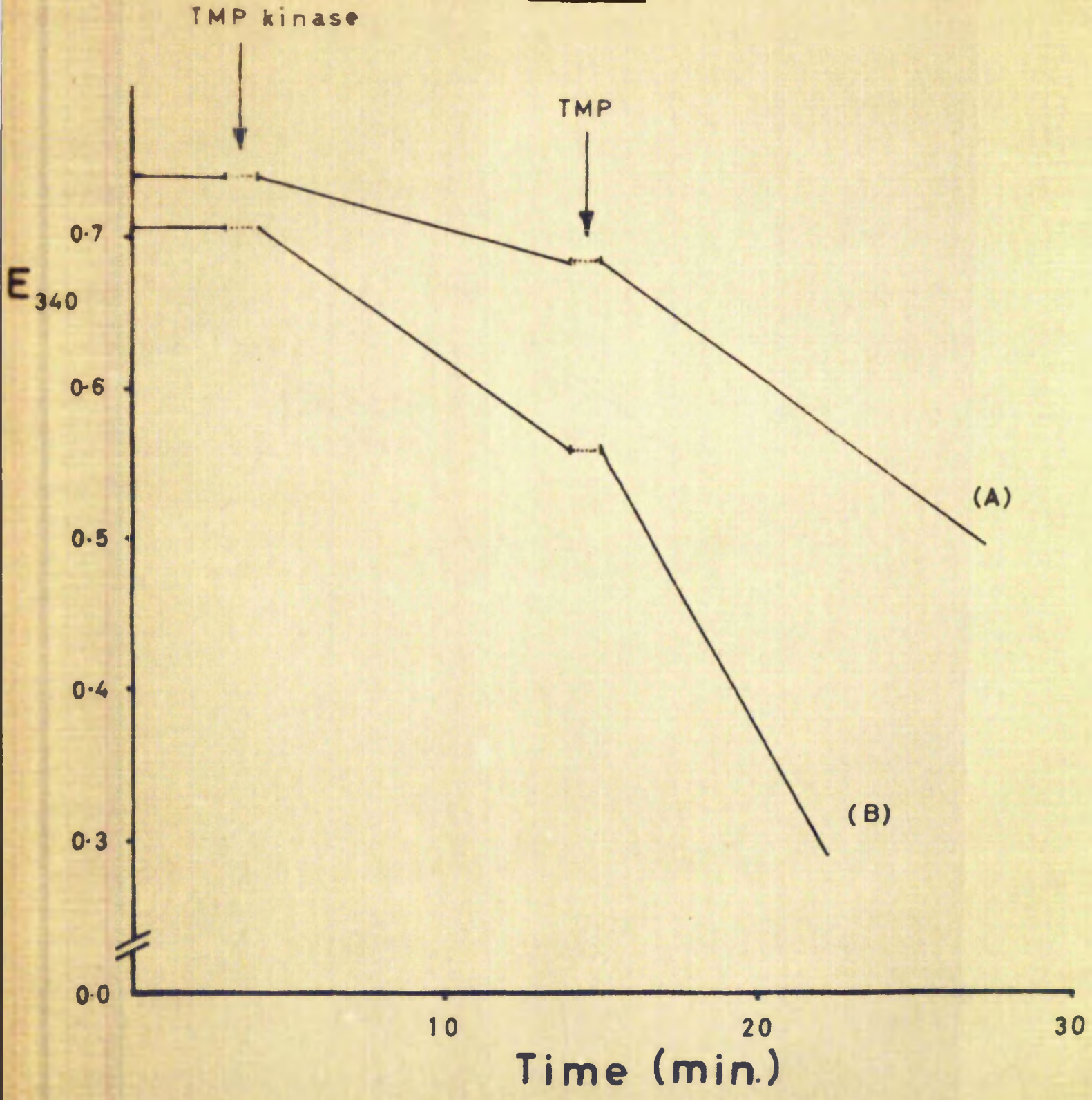


Fig. 38. Time course of TMP kinase action as followed spectrophotometrically at two concentrations of enzyme.

The reaction mixture contained the components given in the text (Section 3.2.3 (a)) in a total volume of 2.89 ml. The arrows indicate the addition of TMP kinase and TMP, respectively, and the oxidation of NADH was followed spectrophotometrically by the decrease in the extinction at 340 mμ and at 30°. The drop in optical density observed upon addition of TMP kinase was due to the ATPase contaminant in the enzyme preparation employed, the source of enzyme being the SG$_\gamma$ AS 4.7 Fraction (see Section 3.3.5, Method I). The corrected TMP kinase reaction rates ($\frac{\Delta E_{340}}{\Delta t}$) were 0.009 per minute for 0.1 ml. enzyme solution (curve A) and 0.0175 per minute for 0.2 ml. enzyme solution (curve B). In this situation, the TMP kinase activity was given as:

$$1.145 \left[\left(\frac{\frac{\Delta E_{340}}{\Delta t} \times 10^3}{\epsilon_{340}} \right)_{TMP} - \left(\frac{\frac{\Delta E_{340}}{\Delta t} \times 10^3}{\epsilon_{340}} \right)_{ATP} \right] = \text{μmoles TMP phosphorylated}$$

where the suffixes ATP and TMP refer to the reaction rate observed after the addition of TMP kinase and TMP respectively. 1 Unit of TMP kinase activity was defined as 1 μmole of TMP phosphorylated per 25 minutes.

mixture and the oxidation of NADH was followed at 340 mμ until the reaction had subsided and the extinction reached a low, constant value. The total amount of NADH oxidised was corrected for the oxidation observed in reaction mixtures where the nucleoside diphosphates were absent.

In all spectrophotometric assays, highly purified ATP was required in order to reduce the blank values to a minimum. The ATP employed in the present experiments (Sigma Chemical Corporation) was shown spectrophotometrically to contain less than 1 per cent ADP. Pyruvate kinase, lactic dehydrogenase, phospho(enol)pyruvate and NADH were obtained from Biochemica "Boehringer", Mannheim. The Molar extinction coefficient of the NADH supplied was shown to be 6.2×10^3 cm.^{2} per mole when measured at 340 mμ.

(d) Assay of deoxycytidine and deoxycytidylate kinases

The deoxycytidine and deoxycytidylate kinases were assayed by a procedure similar to that described elsewhere (Section 2.2.4 (a)) for the thymidi and thymidylate kinases. In practice, it proved difficult to secure sufficient resolution of the deoxycytidine nucleotides by elution from columns of ECTEOLA cellulose and a procedure was developed which incorporated deamination of the deoxycytidine nucleotides to the corresponding deoxyuridine nucleotides prior to the separation of reaction products on ECTEOLA cellulose. The deamination method was based on the procedure described by Bixmie (1959).

The reaction mixtures contained the following components in a total volume of 3.0 ml. :- 300 μmoles tris buffer, pH 7.9, 15 μmoles $MgCl_2$, 15 μmoles ATP, 0.3 μmoles 2-mercaptoethanol, 3 μc (^{3}H) CdR of specific activity

1100 mc per mmole and enzyme solution in the quantities given in the legends to the tables. The enzyme solutions were derived from lyophylised extracts of ascites carcinoma or from rat liver and contained 6 - 8 mg. protein per ml. The reaction mixtures were incubated for various times in a shaking water-bath at 37°.

The reactions were terminated by the addition of 1.5 ml. of 2.1 N perchloric acid and the precipitated protein removed by centrifugation at 0°. The residue was washed three times with 0.5 ml. portions of 0.5 N perchloric acid and the washings and the supernatant fluid combined. The solution was brought to pH 7.5 with 7 N potassium hydroxide, allowed to stand overnight in the cold, centrifuged to remove the precipitate of potassium perchlorate and diluted to 7.0 ml. with distilled water. A portion (1.0 ml.) was reserve for estimation of total radioactivity and paper chromatography, and of the remainder, 5.0 ml. was employed for the conversion of deoxycytidine nucleotides to the deoxyuridine derivatives as follows:- After concentration by freeze-drying in a 15 ml. conical centrifuge tube, 350 µl. 2N $NaNO_2$ and 70 µl. glacial acetic acid was added to the residue and the contents of the tube thoroughly mixed. The reaction mixture was incubated in a shaking water-bath for 5 hours at 40°. A considerable amount of the potassium perchlorate prese in the lyophylised residue failed to dissolve in the reaction medium, but control experiments showed that this material was without effect on the deamination process. The precipitate was removed by centrifugation at 0° at the end of the treatment, washed twice with 0.5 ml. portions of distilled water and the washings and the supernatant fluid were combined. The solution

was adjusted to pH 7.5 with 7 N potassium hydroxide and finally diluted to 3.0 ml. A portion (1.0 ml.) of this solution was run on to a column of ECTEOLA cellulose (10 x 1.5 cm., dia.) and the deoxyuridine nucleotides recovered by successively eluting the column with 100 ml. of distilled water, 100 ml. of 0.02 N hydrochloric acid, 50 ml. of 0.035 N hydrochloric acid, 100 ml. of 0.05 N hydrochloric acid and 70 ml. of 0.2 N hydrochloric acid. UdR appeared in the water fraction, dUMP in the 0.02 N HCl fraction, dUDP in the 0.05 N HCl fraction and dUTP in the 0.2 N HCl fraction. The column flow rate was 20 ml. per hour and portions of 10 ml. were collected. Samples (0.1 ml.) were removed from each portion, plated on stainless steel planchettes and counted in a Nuclear-Chicago windowless gas-flow counter. The pattern of elution of UdR, dUMP, dUDP and dUTP is shown in Fig. 29. The results were calculated as the percentage of the total radioactivity recovered in each of the reaction products.

A series of control experiments with unlabelled CdR, dCMP and dCTP had shown that the deamination was quantitative under the conditions described above. Such control experiments were conducted by the use of paper chromatography (Section 2.2.3 (b)) in the ammonium isobutyrate system in which the R_f values of the nucleotides of the deoxycytidine series and deoxyuridine series differed sufficiently to indicate the progress of the reaction (see Table 1). That the deamination was quantitative was also shown by running the deamination experiments in a Cary Recording spectrophotometer with measurements at regular time intervals of the ratio E_{280}/E_{260} and recording the changes in the ultraviolet spectra of the reaction mixtures.

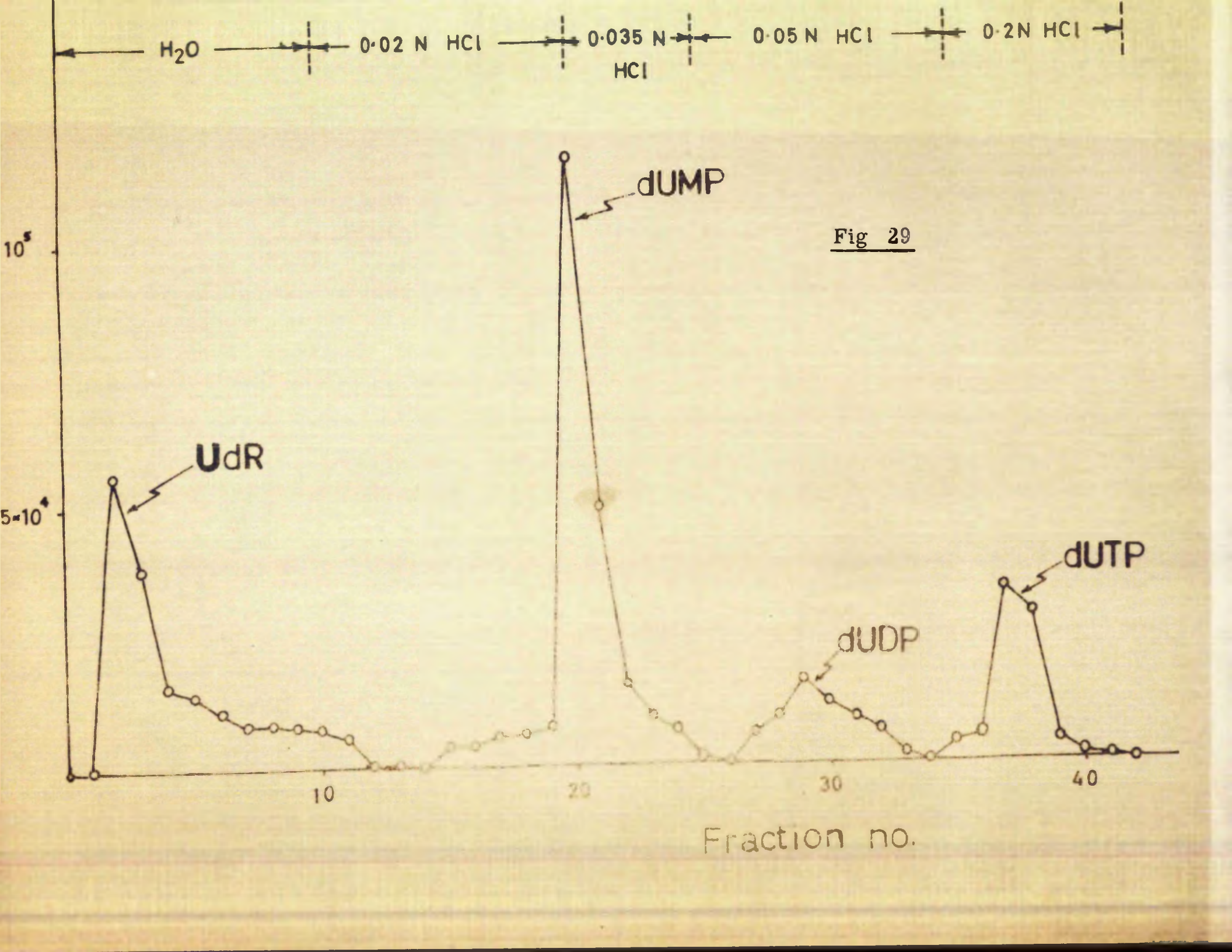

H2O
0·02 N HCl
0·035 N HCl
0·05 N HCl
0·2N HCl
dUMP
UdR
dUTP
dUDP
10⁵
5·10⁴
10
20
30
40
Fraction no.

Fig 29

Fig. 29. Chromatographic separation of (^{3}H) -labelled UdR, dUMP, dUDP and dUTP on columns of WHATMAN cellulose (10 x 1.5 cm., dia.) by stepwise elution with increasing concentrations of hydrochloric acid.

The (^{3}H) -labelled deoxyuridine derivatives were obtained from the corresponding (^{3}H) -labelled deoxycytidine derivatives by deamination with nitrous acid as described in the text (Section 3.2.3 (d)).

In general, it was found that the deoxycytidine nucleotides were relatively resistant to non-enzymic deamination and complete transformation to deoxyuridine derivatives required incubation at 40° for 4 - 5 hours in presence of a large excess of acidified $NaNO_2$, while the deamination of CdR was complete within 1 hour under similar reaction conditions.

(e) Phosphatase assays

Although phosphatase activities were not routinely determined, it was occasionally necessary to have access to the absolute values of kinase activities in which case the apparent kinase activity was corrected for the competitive hydrolysis of reaction products due to endogenous phosphatases. Thus, no attempt was made to measure optimal phosphatase activities and the only values that were considered were those obtainable in an environment dictated by the parameters of optimal kinase activity.

An assay for phosphatases acting on TDP or TTP was developed on the basis of the radioactivity assay for TMP kinase (Section 3.2.3 (b)) in which (^{32}P) -labelled TDP or TTP replaced (^{32}P) TMP and ATP was omitted. Reaction mixtures were prepared containing the following components in a total volume of 1.0 ml.:- 100 µmoles tris buffer, pH 7.6, 5 µmoles $MgCl_2$, 20 mµmoles (^{32}P - a) TDP or (^{32}P - a) TTP of specific activity 10^7 counts per minute per µmole and enzyme solution in the quantities given in the legends to the tables. Incubation was in shaking water-bath for 25 minutes at 37°. The reactions were stopped by heating at 100° for 2 minutes and the coagulated protein removed by centrifugation at 0°. Each protein sediment was washed twice with 0.5 ml. portions of 0.01 M phosphate buffer, pH 7.6, the washings and super-

natant fluid combined and the solution diluted to 3.0 ml. A portion (1.5 ml. from each solution was lyophylised and the resulting residue dissolved 0.1 ml. of distilled water, 0.02 ml. of which being taken for chromatography on paper (Section 2.2.3 (b)). The percentage of total radioactivity present in each of the products was determined by scanning the appropriate chromatogram strips in the Actigraph windowless gas-flow detector (Section 2.2.3 (d)) and subsequently cutting and weighing the relevant peaks from the recorder chart. The validity of the results so obtained was checked by cutting out radioactive sections from the chromatogram strip and eluting the nucleotide material by capillary flow in distilled water and diluting each eluate to 2.0 ml., 0.1 ml. portions of which were plated on stainless steel planchettes and counted in a Nuclear-Chicago windowless gas-flow counter. The results were calculated as mumoles TDP or TTP hydrolysed per 25 minutes per mg. protein. Controls were included in which enzyme was omitted in order to correct for non-enzymic hydrolysis of the substrates in the course of incubation and heat inactivation

Phosphatases acting on ATP (ATPases) were frequently found in the crude ascites extracts and at several stages of the purification of TMP kinase. ATPase was measured by means of the spectrophotometric procedure described above (Section 3.2.3 (c)) in which TMP was omitted. The reaction mixtures (2.29 ml.) contained the following components:- 10 µmoles ATP, 10 µmoles $MgCl_2$, 200 µmoles tris buffer, pH 7.6, 0.15 µmoles phospho(enol)pyruvate, 0.25 µmoles NADH, 20 µg. pyruvate kinase, 20 µg. lactic dehydrogenase and enzyme in the quantities given in the legends to the figures. The reaction mixtures were prepared in 10 mm. quartz cuvettes and the rate of NADH

oxidation followed spectrophotometrically at 240 mμ and 30°. In order to obtain reproducible results, pyruvate kinase was added only after temperature equilibration (3 minutes). The subsequent drop in E_{340} was adjudged to be due to small amounts of ADP present in the ATP employed. Enzyme solution was added after the E_{340} had attained a constant value (Fig. 30). The average decrease in the extinction at 340 mμ over a 10 minute period using a tungsten light source was determined and the results were calculated as μmoles ADP formed per 25 minutes per mg. protein.

3.2.4. Enzyme fractionation techniques

(a) Fractionation with dilute acetic acid

Fractionation of crude ascites extracts or extracts from lyophylised material from the same source formed an integral part of the majority of the purification procedures outlined in Section 3.3. In experiments where the source of enzymes was the lyophylised crude extract pool (Section 3.2.2 (a)), the material was dissolved in 0.01 M phosphate buffer, pH 7.6 or pH 7.8, for purification of TMP kinase and TdR kinase, respectively, to give solutions containing 6 - 8 mg. protein per ml. The details of the fractionation procedure in which all manipulations were carried out at 0° were as follows:- A volume (10 - 200 ml.) of enzyme solution, from which a sample had been removed to serve as control for the enzyme under study, was transferred to a 50 ml. round-bottomed centrifuge tube or to a beaker of appropriate size. The solution was stirred mechanically in an ice-bath at 0° and the initial pH measured with electrodes previously adjusted to operate over the range pH 4.5 - 7.5. To this solution was then added 1.0 N acetic acid drop by

Fig. 30.

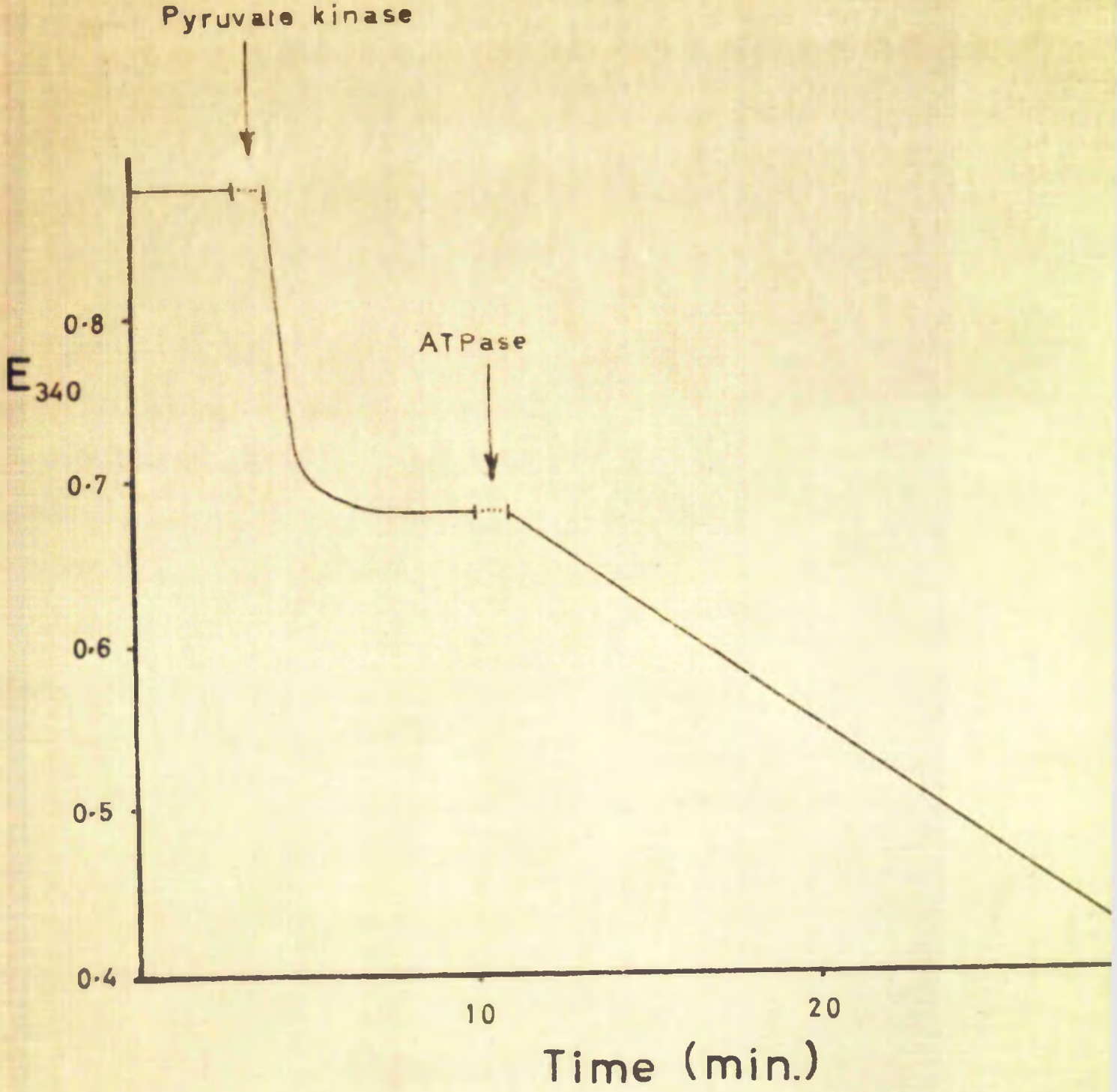


Fig. 30. The time course of ATPase action as followed spectrophotometrically.

The reaction mixtures contained the components given in the text (see Section 3.2.3 (e)) in a total volume of 2.29 ml. and the arrows indicate the addition of pyruvate kinase and ATPase, respectively. The rate of disappearance of NADH was measured in a Beckman DU spectrophotometer at 340 mμ and at 30°. The drop in total extinction observed upon addition of pyruvate kinase is presumably due to the small amount of ADP present as a contaminant of the ATP used. The source of enzyme was the ATPase containing fraction of the effluent from an enzyme fractionation experiment with a column of Sephadex G-100 (see Section 3.3.5, Method II). The ATPase reaction rate $\left(\frac{\Delta E_{340}}{\Delta t}\right)$ was 0.014 per minute and the ATPase activity was given as

$$2.29 \left(\frac{\frac{\Delta E_{340}}{\Delta t} \times 10^3}{\epsilon_{340}}\right)$$

= μmoles ATP dephosphorylated

drop until the desired pH had been attained, the resulting mixture quickly transferred to centrifuge tubes and centrifuged at 2500 rev. per minute for 7 minutes. The supernatant fluid was returned to the pH meter and the pH quickly readjusted to 7.6 with the addition of 1.0 N sodium hydroxide while stirring the solution mechanically in an ice-bath. The sediment, if required, was added a volume of distilled water equivalent to one-half the original volume of the solution and the precipitate redissolved by adjusting the pH to 7.5 as above. Both the neutralised fractions so obtained were diluted to the original volume, assayed for the appropriate enzyme activities and the protein concentrations determined.

In order to avoid serious losses of enzyme activities due to contact with the dilute acetic acid, it was essential to reduce the duration of this treatment to a minimum and the time taken over the whole operation was, consequently, not allowed to exceed 15 minutes (see also Section 3.3.3). Strict control of pH was necessary to achieve reproducible recoveries of TdR kinase and TMP kinase and the validity of the pH meter scale readings was frequently checked in the course of the operation with the use of standard buffers. A narrow combination electrode was used in conjunction with a Beckman Zeromatic pH meter for fractionations involving 10 - 20 ml. of enzyme solution in which case the whole operation was carried out in a 50 ml. centrifuge tube. For fractionations involving 20 - 200 ml. of enzyme solution a Direct Reading pH meter (Model 23A, Electronic Instrument Limited, Surrey) connected to a conventional electrode assembly was found more suitable. Larger volumes than 200 ml. could not conveniently be handled, the upper limit

being dictated by the time required for the completion of the acid fractionation in each case. Thus, in experiments where the original volume of enzyme solution exceeded 200 ml., fractionations were conducted as a series of experiments using small volumes of enzyme solution each time and combining the resulting fractions where appropriate.

(b) Fractionations using C γ -alumina gel

The C γ -alumina gel employed in the present investigation was prepared according to the method of Willstätter and Kraut (1923) and stored in aqueous suspension containing 11 mg. $Al_2O_3 \cdot 3 H_2O$ per ml. for a period of 4 years.

The procedure developed for fractionations using C γ -alumina gel involved measurement of the kinase activity remaining in the supernatant fluid under various conditions after the addition of increasing quantities of C γ -alumina suspension. The enzyme solution was prepared by dissolving the appropriate enzyme in 0.01 M phosphate buffer, pH 7.6, to give a protein concentration of 4 - 8 mg. per ml. and all subsequent manipulations were carried out at 0°. In small scale experiments, portions of the aqueous C γ -alumina suspension ranging from 0.5 ml. to 10 ml. were transferred to 15 ml. conical centrifuge tubes and the gel packed tightly in the tube by centrifugation at 1000 x g for 20 minutes at 0°, the aqueous supernatant fluid being discarded. To the gel was then added 5 ml. of 0.01 M phosphate buffer, pH 7.6, whereupon the resulting suspensions were vigorously stirred, centrifuged at 1000 x g for 10 minutes and the supernatant fluids discarded. Washing in this manner with 0.01 M phosphate buffer was repeated twice more; finally, 10 ml. of buffer was added to the gel and the suspensions left

overnight at 0° in order to ensure that the gel - buffer system had become fully equilibrated. After centrifugation and decantation, a 1.0 ml. portion of enzyme solution was added to each tube and the gel dispersed by stirring the suspensions manually from time to time over a 5 minute period. Thereafter, the mixtures were left at 0° for 30 minutes to complete the adsorption. The gel was then sedimented by centrifugation at 1000 x g for 15 minutes and the supernatant fluid reserved for measurement of protein concentration and for the assay for the appropriate kinase. Equivalent values representing the initial enzyme activity were obtained by incubating portions of untreated enzyme solution. From these two sets of data, the fraction of the appropriate kinase activity adsorbed under the prevailing conditions could be calculated.

(c) <u>The use of gel filtration techniques in enzyme fractionations</u>

The uncharged dextran gels Sephadex G-25 and Sephadex G-100, obtained from AB Pharmacia, Uppsala, were employed in the present investigation either as a substitute for conventional dialysis or for the purpose of enzyme fractionation in which case the dextran operated as a molecular sieve.

Pretreatment of Sephadex G-100 (200 - 400 mesh) was performed by adding the dry dextran powder to excess 0.01 M phosphate buffer, pH 7.6, with stirring thus avoiding the formation of clumps, and the gel - buffer system was allowed to equilibrate over a 24 hour period. While the process of swelling was in progress it was found convenient to remove fine particles by stirring the suspension and then allowing the gel to sediment. When a sharp boundary layer became visible, the supernatant fluid was removed by suction and this treatment was repeated until the supernatant fluid was clear. After

equilibration, the gel suspension was further diluted to a viscosity low enough to allow rapid escape of air bubbles during the packing of columns.

Careful packing of the column was essential in order to obtain reproducible enzyme fractionations. The following procedure was adopted to ensure the preparation of a completely homogeneous bed of dextran particles:- A column (1 cm., dia.), fitted with a small plug of cellulose wadding above the outlet tube, was carefully mounted vertically and filled to one-third of the column height with 0.01 M phosphate buffer, pH 7.6, the outlet being closed. The ensuing packing process was conducted at room temperature to prevent the formation of air bubbles on the inside surface of the glass column. The column was then filled with gel suspension which immediately started to sediment. After a layer of 4 - 5 cm. had formed, the outlet tap was opened so as to allow the escape of a slow stream of buffer and the progress of the packing was followed visually from the appearance of the horizontal bed surface. More gel suspension was added before all previously added gel particles had settled and the process repeated until the column surface had reached a level 2 - 3 cm. above the desired bed height. A small volume of buffer was then added with the escape tap closed and the top layer of the bed agitated with a thin glass rod whereupon the particles were allowed to re-settle. This operation was repeated until a perfectly horizontal bed surface had formed. The bed surface so prepared was protected mechanically by placing a well-fitting disc of glass filter paper in contact with the surface. The gel was then washed with 2 - 3 column volumes of phosphate buffer with a flow rate of 15 - 30 ml. per hour until the column was stable. Because of the

danger of gradual packing of the gel particles with consequent decrease in the flow rate, a constant hydrostatic pressure head was not used, the eluant buffer being added instead in small volumes giving a head of not more than 5 cm. The column parameters, column void volume (V_o) and column imbibed volume (V_i) were determined by measuring the volume of eluant required to move peak concentrations of, respectively, γ-globulin (Mol. wt. 150 000. Armour Pharmaceutical Company, Ltd., Eastbourne) and KCl from the top to the bottom of the column bed. Samples were layered on the bed surface with a pipette after the removal of supernatant buffer, whereupon the escape tap was opened and the sample was allowed to enter the bed. Immediately after the disappearance of the sample through the bed surface, a small volume of buffer was added to wash the surface and when this, in turn, had entered the bed a larger volume of buffer was added and the elution started. In enzyme fractionation experiments, eluate fractions of 2.0 ml. were collected with a flow rate of 15 - 25 ml. per hour. All experiments involving active enzymes were carried out at a temperature of between 0° and 4° with columns previously equilibrated with ice-cold eluant buffer. The columns were regenerated after use by washing with 10 column volumes of eluant buffer.

In spite of the precautions taken, the column beds were frequently observed to contract over a period of weeks and to allow for this change in bed volume, V_o was routinely determined prior to each fractionation experiment. The columns were discarded when the minimum flow rate fell below 10 ml. per hour in which case interference from diffusion effects could be expected to affect markedly the reproducibility of the fractionations.

In some enzyme experiments, a relatively large amount of TMP was added as a stabilising factor for TMP kinase. Assays reflecting absolute values of the TMP kinase activity could, therefore, not be performed directly due to the dilution of (^{32}P) TMP resulting from the presence of the unlabelled nucleotide.

In experiments where TMP was added to enzyme preparations in order to protect TMP kinase, the TMP could subsequently be removed by treatment with Sephadex G-25. Sephadex G-25 (Coarse grade) was pretreated with 0.01 M phosphate buffer, pH 7.6, in the manner described above and, after equilibration, was packed carefully into a column (10 x 1 cm., dia.) observing the usual precautions. The column constants, V_o and V_i, were determined by the use of sheep blood haemoglobin and KCl, respectively, at a flow rate of 50 ml. per hour. The sample from enzyme solutions containing TMP was layered on the bed surface as described above and the protein fraction appearing at V_o after the start of the elution was collected and used for the assay of TMP kinase. The dilution of the enzyme solution resulting from this treatment was observed to be of the order of 1 : 3. The column was regenerated after use by washing with 5 column volumes of eluant buffer.

Several preliminary experiments had shown that TMP had a relatively high distribution coefficient (K_d) under the conditions described above. This was contrary to the observations of Flodin (1962) who found that acidic nucleotides were almost completely excluded from the gel phase when eluted with distilled water or very dilute buffers, an effect which had been attributed to the mutual repultion of fixed negative charges. No such

effect was found in the present experiments even when distilled water was used as eluant. In experiments with columns of Sephadex G-25 using 0.01 M phosphate buffer as eluant, the K_d for TMP was shown to be approximately equal to that of KCl (i.e., about 1.0).

(d) Enzyme fractionations using ammonium sulphate

Fractionation of enzyme solutions with $(NH_4)_2SO_4$ was occasionally performed in which case the experimental procedure was as follows:- enzyme solution (10 ml.), prepared by dissolving lyophylised crude ascites extract in 0.05 M phosphate buffer of the appropriate pH to give a protein concentration of 8 - 10 mg. per ml., was transferred to a precooled 50 ml. centrifuge tube and all subsequent operations were carried out at 0°. To this solution was added, with continuous stirring, the volume of saturated ammonium sulphate required to give 30 per cent ammonium sulphate saturation. The precipitate so formed was allowed to settle for 30 minutes at 0°. After the precipitation was complete, the mixture was centrifuged at 1000 x g for 15 minutes and the supernatant fluid transferred to a clean, precooled 50 ml. centrifuge tube. The sediment was redissolved in 10 ml. of 0.01 M tris buffer, pH 7.7. To the supernatant fluid were added further volumes of saturated ammonium sulphate and the precipitates obtained at 40, 50 and 60 per cent ammonium sulphate saturation recovered and dissolved in 10 ml. of 0.01 M tris buffer. The ammonium sulphate fractions in this diluted form were assayed for the appropriate kinase activity without further pretreatment.

(e) Enzyme fractionations using acetone

Acetone fractionation of enzyme solutions was sometimes employed as an initial operation in the purification of the thymidine and thymidylate kinases. In such experiments, the lyophylised crude ascites extract was dissolved in 0.01 M phosphate buffer of the appropriate pH to give a solution containing 8 - 10 mg. protein per ml. Strict temperature control was required in all experiments using acetone; thus, the fractionations described below were performed in an ethylene glycol bath maintained at -15°. The enzyme solution (10 ml.) was transferred to a precooled 50 ml. centrifuge tube to which was added 1 ml. of acetone, previously chilled to -15°, to prevent freezing of the enzyme solution when introduced into the glycol bath. The tube was placed in the bath at -15° and 4 ml. of acetone was added with stirring, thereby giving a solution containing 30 per cent acetone. The resulting mixture was centrifuged at 1000 x g for 15 minutes at -15° and the supernatant fluid was transferred to a clean, precooled 15 ml. centrifuge tube for further fractionation. The precipitate, if any, was immediately redissolved in ice-cold 0.01 M phosphate buffer, pH 7.6, in order to reduce the acetone concentration to a relatively harmless proportion. The precipitates obtained similarly at 40, 50 and 60 per cent acetone concentration were preserved in the same way. Assays for the appropriate kinases were performed without further pretreatment of the diluted acetone fractions.

3.3. Results

3.3.1. Biological factors affecting the activity of the thymidine and thymidylate kinase system from cell-free extracts of Landschutz ascites tumour cells

An original observation by Gray et al. (1960) that the formation of TTP from TdR by cell-free extracts of ascites cells was seriously inhibited by addition of extracts of non-proliferating mammalian tissues merited re-investigation. The experiments of Gray et al. (1960) had indicated, in particular, that the activity of the thymidine and thymidylate kinase system from extracts of Ehrlich ascites tumour cells was cancelled in the presence of extracts of rat liver. It seemed of interest to re-examine this phenomenon with the use of the relatively powerful tool provided by the kinase assay described earlier (Section 2.2.4 (a)). Such experiments sought to reveal the nature of the inhibitory effect caused by the addition of liver extract to the ascites extract and, specifically, whether the inhibition was manifest at any single stage of the reaction sequence leading from TdR to TTP.

Experiments in which the phosphorylation of (^{3}H) TdR was investigated under the influence of cell-free extracts of normal rat liver (Section 3.2.2 (b)) have invariably shown that the activity of the thymidine and thymidylate kinases in such extracts is very low when compared with cell-free extracts of Landschutz ascites carcinoma. Thus only 5 per cent of the total radioactivity was recovered as phosphorylated thymidine derivatives after a one hour incubation period while, in comparable experiments, ascites extracts catalysed the phosphorylation of over 80 per cent of the (^{3}H) TdR. Moreover,

such experiments indicated that the phosphorylation of (^{3}H) TdR was restricted to the formation of TMP from TdR when rat liver extracts were employed as the source of enzymes, and no evidence was obtained for the production of TDP or TTP. Subsequent experiments in which (^{3}H) TdR was replaced as substrate by (^{32}P) TMP have revealed that the liver extracts were incapable of phosphorylating TMP under experimental conditions which allowed phosphorylation of 60 per cent of the TMP in the presence of ascites extracts.

In another type of experiment, rat liver extract was added to ascites extract and the effect of such mixtures on the phosphorylation of (^{3}H) TdR was studied as a function of the ratio of the concentration of liver protein to ascites protein. Such experiments (Table 4) have shown that the presence of liver extract causes pronounced diminution of the net phosphorylating activity of the ascites extracts, and that the degree of inhibition so produced was dependent on the concentration of liver protein in the reaction mixtures. Thus, over an incubation period of one hour, the proportion of the total radioactivity recovered in (TMP + TDP + TTP) was reduced from 65 per cent to 30 per cent in response to the addition of increasing concentrations of liver protein to reaction mixtures containing a constant concentration of ascites protein. The ratio of liver protein to ascites protein thus added varied from 1 : 4 to 2 : 1. The percentage of the total radioactivity found in all phosphorylated derivatives of thymidine was 65 per cent when the incubations were performed in presence of ascites tumour extracts only. By inspection of the results recorded in Table 4, it can be seen that the

Table 4.

Reaction mixture	Percentage of total radioactivity recovered	Per cent total radioactivity in thymidine nucleotides			
		TdR	TMP	TDP	TTP
Complete - enzyme	95	100	-	-	-
Complete + ascites extract (3.7 mg.)	85	15	10	13	62
Complete + liver extract (3.7 mg.)	93	95	5	-	-
Complete + ascites + liver (3.7mg. + 0.9mg.)	86	26	32	17	25
Complete + ascites + liver (3.7mg. + 1.8mg.)	79	35	27	12	16
Complete + ascites + liver (3.7mg. + 3.7mg.)	95	40	46	16	4
Complete + ascites + liver (3.7mg. + 7.4mg.)	89	72	25	3	-

Table 4. The effect of adding cell-free extracts of normal rat liver on the phosphorylation of (^{3}H) TdR by cell-free extracts of Landschutz ascites tumour cells.

The reaction mixtures contained the following components in a total volume of 3.0 ml.: 300 µmoles tris buffer, pH 8.0, 0.3 µmoles 2-mercaptoethanol, 15 µmoles $MgCl_2$, 15 µmoles ATP, 3 µc (^{3}H) TdR and a volume of enzyme solution corresponding to 3.7 mg. of protein, the enzyme solutions being obtained either from cell-free extracts of normal rat liver (Section 3.2.2 (b)) or by redissolving lyophylised cell-free extracts of Landschutz ascites carcinoma. In inhibition experiments, the reaction mixtures contained 3.7 mg. protein from the ascites extracts to which was added volumes of rat liver extracts corresponding to the quantities of protein recorded in column 2 of the table. Incubation was for 60 minutes in a shaking water-bath at 37° and further treatment was as given in the text (Section 2.2.4 (a)).

percentage of inhibition of the thymidine and thymidylate kinases, as measured by the proportion of the total radioactivity recovered as (TMP + TDP + TTP), appears to vary as a linear function of the liver protein concentration.

It was of some interest also to determine whether a similar situation obtained in the phosphorylation of other deoxyribonucleosides. As the additional substrate in such comparative experiments, deoxycytidine (CdR) was shown to be phosphorylated at only one-third the rate of TdR when the source of enzymes was the cell-free extracts of Landschutz ascites tumour cells. This was in contrast to the observations of Gray et al. (1960) and Manstavinos and Canellakis (1959) using Ehrlich ascites cells and regenerating rat liver. Those studies were, however, performed with (^{32}P) dCMP as substra and the observed discrepancy may thus be due to the rate limiting activity of the CdR kinase under the present experimental conditions. Similarly, the rate of phosphorylation of (^{3}H) CdR, as measured by the proportion of the total radioactivity recovered as (dCMP + dCDP + dCTP), by extracts from rat liver was lower than that observed by Gray et al. (1960) using (^{32}P) dCMP as substrate thus indicating that the reaction catalysed by CdR kinase may be rate limiting in this tissue also. However, such extracts of normal rat liver were shown to phosphorylate 4 - 5 times as much (^{3}H) CdR as (^{3}H) TdR over a one hour incubation period.

Given these differences in the patterns of phosphorylation of TdR and CdR, it seemed of interest to determine to what extent the phosphorylation of CdR by ascites extracts would be affected by the addition of liver extract to

the incubation mixtures. In experiments of this type, the proportion of the total radioactivity recovered as phosphorylated derivatives of (^{3}H) dCR (see Section 3.2.3 (d)) and (^{3}H) TdR were compared after incubation for one hour as recorded in Table 5. Again, addition of extracts of normal rat liver to extracts of Landschutz ascites tumour cells reduced the thymidine and thymidylate kinase activity by about 40 per cent when the ratio of liver protein to ascites protein was 1 : 1. In contrast, the addition of liver extract to ascites tumour extracts had no effect on the phosphorylation of (^{3}H) dCR; indeed, no inhibitory effect could be observed on the rate of formation of deoxycytidine nucleotides even when the liver protein to ascites protein ratio was increased to 3 : 1 while the same treatment all but abolished thymidine kinase activity. It seems possible, therefore, that the liver extracts contain a factor specifically concerned with the inhibition of formation of thymidine polyphosphates.

The observed behaviour of the system thus coincides with the pattern expected if the inhibitory factor operates as a competitive inhibitor for the phosphorylating system and, if taken together with the observations of Gray et al. (1960) that the inhibitory factor of rat liver was non-dialysable and was rendered ineffective in boiled liver extracts, seems to suggest the existence of dephosphorylating enzymes in the liver extract. If the inhibition of the phosphorylation of thymidine stems from this source, the observed effect could be accounted for by the presence in the liver extracts of a series of relatively specific phosphatases sufficiently active to abolish net synthesis of thymidine nucleotides. The relatively high level

Table 5.

Reaction mixture	Percentage of total radioactivity recovered	Per cent total radioactivity recovered in nucleotides.							
		UdR	dUMP	dUDP	dUTP	TdR	TMP	TDP	TTP
(^{3}H) CdR. Complete - enzyme	99	100	-	-	-				
Complete + ascites (3.7mg.)	98	35	44	9	12				
Complete + liver (3.7mg.)	91	80	16	2	2				
Complete + ascites + liver (3.7mg.+ 3.7mg.)	96	45	32	10	13				
(^{3}H) TdR. Complete - enzyme	95					100	-	-	-
Complete + ascites (3.7mg.)	98					17	12	15	56
Complete + liver (3.7mg.)	93					93	7	-	-
Complete + ascites + liver (3.7mg. + 3.7mg.)	92					52	32	10	6

Table 5. Kinase activities in extracts of Landschutz ascites tumour cells and of normal rat liver tissues: phosphorylation of (^{3}H) TdR and (^{3}H) CdR.

The reaction mixtures contained the following components in a total volume of 3.0 ml.: 300 µmoles tris buffer, pH 8.0, 0.3 µmoles 2-mercaptoethanol, 15 µmoles $MgCl_2$, 15 µmoles ATP, 3 µc (^{3}H) TdR of specific activity 360 mc per mmole or 3 µc (^{3}H) CdR of specific activity 1100 mc per mmole and enzyme solution taken from one of the following:- redissolved, lyophylised cell-free extracts of normal rat liver (3.7 mg. protein); redissolved, lyophylised extracts of Landschutz ascites tumour cells (3.7 mg.protein); a mixture of both types of extract containing equal proportions of liver protein and ascites protein (3.7 mg. : 3.7 mg. protein) or containing liver protein and ascites protein in the proportions 3 : 1 (11.1 mg. : 3.7 mg. protein). Incubation was for 60 minutes in a shaking water-bath at 37°. For reaction mixtures containing (^{3}H) TdR, further treatment was as given in the text (Section 2.2.4 (a)). Reaction mixtures containing (^{3}H) CdR were deproteinised and neutralised in the usual manner to give solutions of total volume 7.0 ml., 5.0 ml. of which were submitted to deamination with nitrous acid as described in the text (Section 3.2.3 (d)). The resulting deoxyuridine derivatives were separated on columns of EXTEOLA cellulose and the results were expressed

Table 5. (contd.): as the percentage of the total radioactivity recovered in UdR, dUMP, dUDP and dUTP and taken as equivalent to the respective deoxycytidine derivatives produced by the enzymic phosphorylation.

of thymidine nucleotidase activity in extracts of normal rat liver has previously been noted (Bollum, 1958; Manstavinos and Canellakis, 1959) and implicated as a reason for the failure of such extracts to form TTP from TdR and TMP.

The present experiments, however, did not carry quantitative authority and the interpretations given above must, as a result, remain somewhat speculative. In particular, no firm evidence has been recorded with a direct bearing on the mechanism of formation of TTP from TdR, neither has it been established whether the inhibition caused by the liver extract is specifically directed towards any single reaction in this process. Present evidence tends to indicate that all the relevant reactions are so inhibited, the phosphorylation of TMP being possibly more seriously effected than the thymidine kinase activity.

3.3.8. Experiments with the thymidine kinase from cell-free extracts of Landschutz ascites tumour cells

The evidence so far collected (see above and Section 2.3) demonstrated clearly that the thymidine and thymidylate kinases of Landschutz ascites tumour cells were capable of phosphorylating thymidine to TTP and while experiments with crude enzyme extracts sufficed to reveal the overall pattern of this process, it became apparent that precise elucidation was hampered by the lack of information on the individual enzyme reactions.

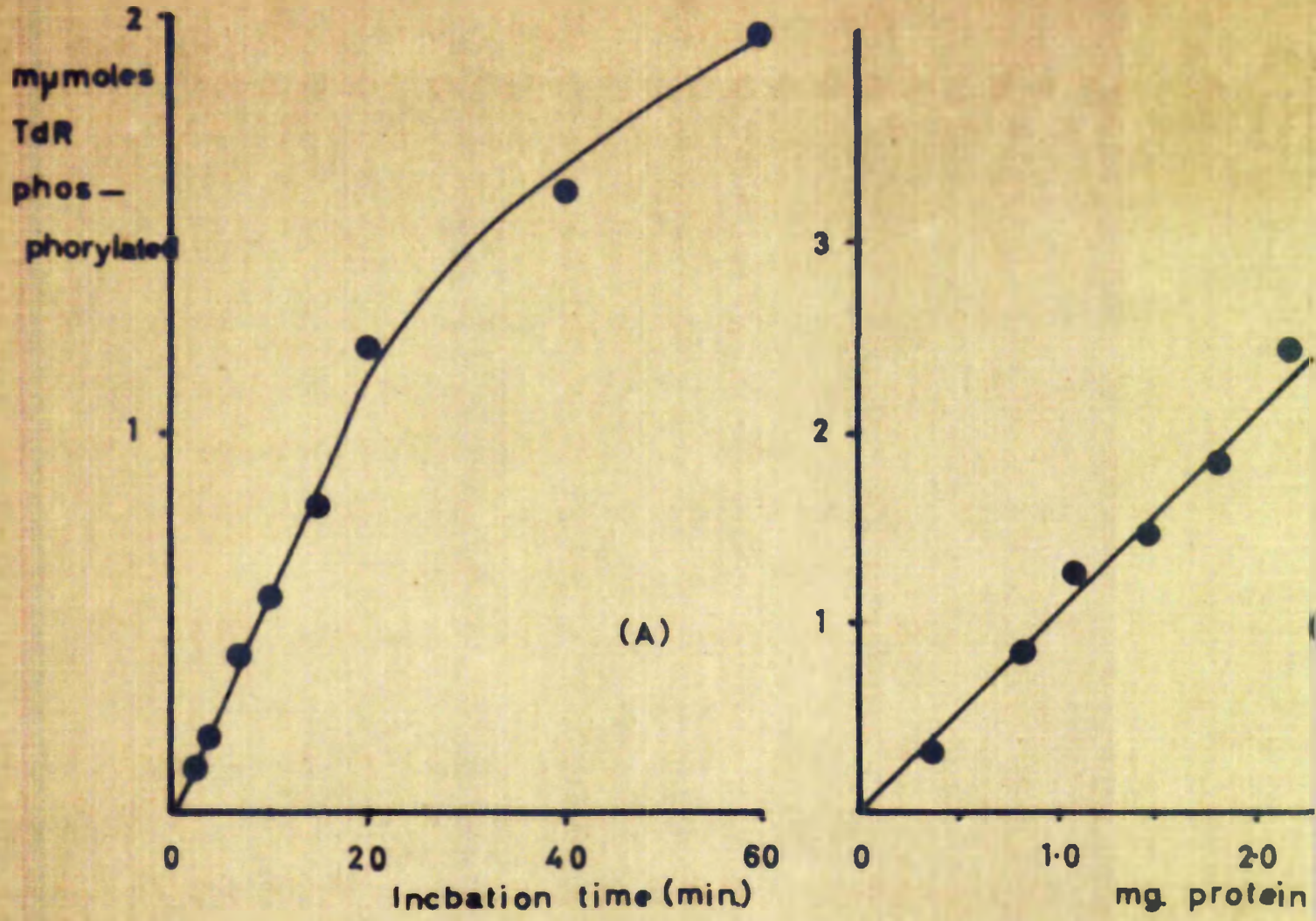
Thus several types of experiment were performed in order to characterise and, if possible, resolve the separate kinase activities co-operating in the

phosphorylation of thymidine to TTP; such studies being initially concentrated on the reaction catalysed by thymidine kinase (TdR kinase). Earlier studies (see Section 2.3.2.) had indicated that the formation of TMP from TdR was catalysed relatively efficiently by the crude cell-free extracts of Landschutz ascites tumour cells; thus when (^{3}H) TdR was used as substrate, over 80 per cent of the total radioactivity was recovered as phosphorylated thymidine derivatives (TMP + TDP + TTP) after incubation for 60 minutes with the crude ascites extracts as enzyme source (see Fig. 22). By using the total radioactivity recovered in (TMP + TDP + TTP) as the criterion for measurement of TdR kinase activity, relatively reliable values could be obtained as the system was thus internally compensated for losses of (^{3}H) -label (Goutier, 1964) by thymidine catabolism. This criterion was also the basis of a means of expressing the specific activity of TdR kinase (for definition of a unit of TdR kinase activity, see legend to Fig. 31).

Preliminary experiments were performed in order to formulate the optimal requirements of the TdR kinase; the parameters being determined by the use of material from the lyophylised pool of cell-free extracts of Landschutz ascites carcinoma (see Section 3.2.2. (a)).

Experiments exploring the effect of pH on the action of TdR kinase showed (Fig. 31 (C)) that the reaction exhibited a relatively narrow pH optimum giving maximal activity between pH 7.8 and pH 8.1 when in a reaction medium containing 5 x 10^{-3} M $MgCl_2$ and 5 x 10^{-3} M ATP. The ATP and Mg^{++} requirements of the reaction were also determined and the results indicated that the reaction catalysed by TdR kinase operated optimally in the presence

Fig 31


2
mμmoles
TdR
phos—
phorylated
1
0
20
40
60
Incbation time (min)
(A)
3
2
1
0
1·0
2·0
mg. protein

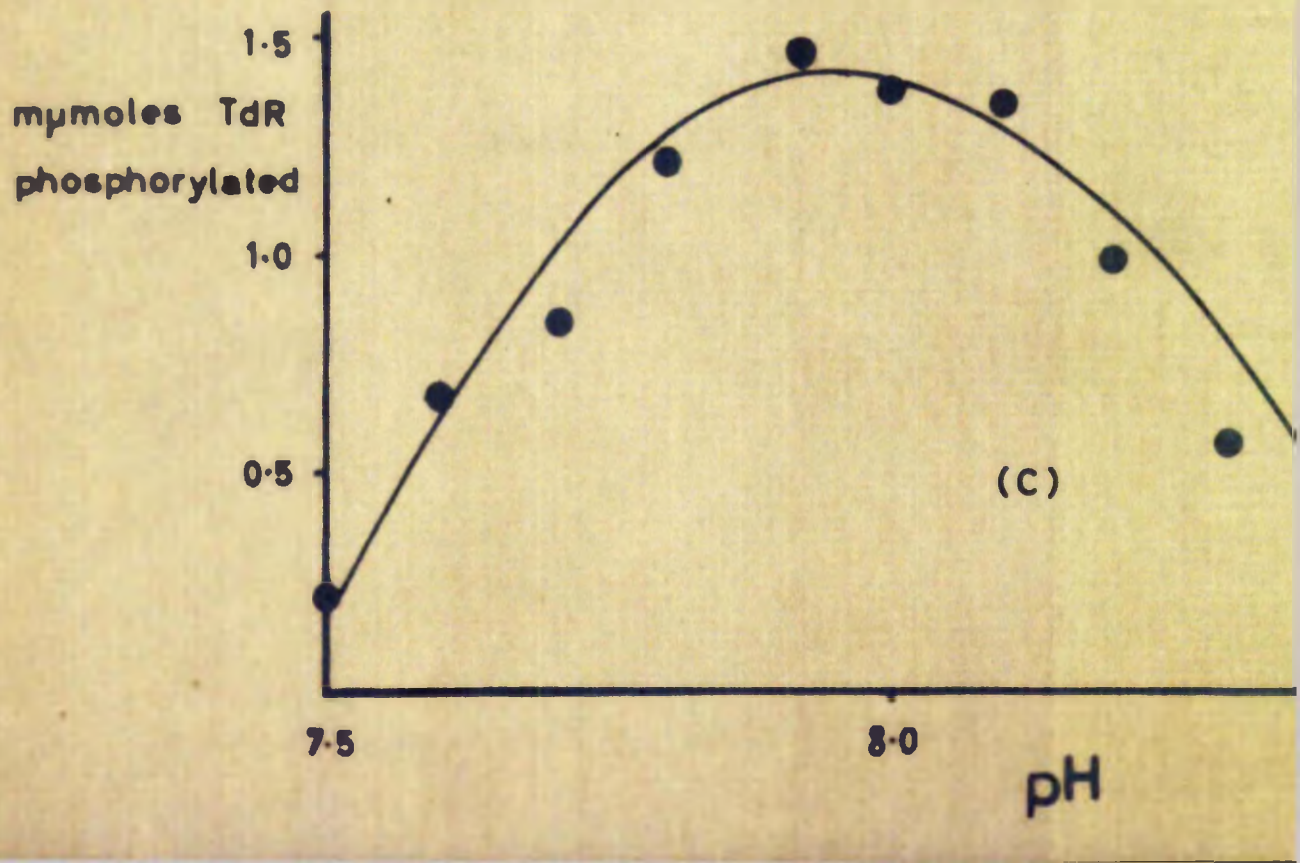

1·5
mμmoles TdR
phosphorylated
1·0
0·5
7·5
8·0
pH
(C)

Fig. 31. The action of TdR kinase from extracts of Landschutz ascites tumour cells as measured by the total amount of (^{3}H) TdR phosphorylated.

(A) Time course of total phosphorylation of (^{3}H) TdR.

The reaction mixtures contained the following components in total volume of 1.0 ml.:- 100 µmoles tris buffer, pH 8.0 0.1 µmoles 2-mercaptoethanol, 5 µmoles $MgCl_2$, 5 µmoles AT 1 µc (^{3}H) TdR of specific activity 360 mc per mmole and enzyme solution (derived from lyophylised cell-free extra of ascites tumour cells) containing 1.0 mg. protein. Th mixtures were incubated for the times indicated in a shaking water-bath at 37°. Further treatment was as described in the text (Section 3.8.3 (a)). Fractionatio on columns of ECTEOLA cellulose yielded two fractions, on containing (^{3}H) TdR and the other containing (^{3}H) -labell (TMP + TDP + TTP). Results were obtained as the percentage of the total radioactivity recovered in (TMP + TDP + TTP) from which the number of mmoles of TdR phosphorylated were calculated as follows:

$$\frac{2.78 \times X}{Y \times K} = \text{mmoles TdR phosphorylated}$$

where the numerical factor represents the amount of TdR present initially (mmoles), X is counts per minute recovered as (TMP + TDP + TDP), Y is the fraction of total initial radioactivity recovered and K is the total radio-

Fig. 31. (contd.): activity of the TdR present initially. One unit of TdR kinase activity was defined as that which phosphorylates 1 mumole TdR in 15 minutes.

(B) The effect of protein concentrations on the total phosphorylation of (^{3}H) TdR.

The conditions were as described above except that the reaction mixtures contained protein in the quantities given on the diagram. The incubation time was 15 minute

(C) The effect of pH on the total phosphorylation of (^{3}H) TdR.

The conditions were as described above except that the reaction mixtures contained 100 µmoles tris buffer of the pH indicated on the diagram and 1.0 mg. protein. The incubation time was 15 minutes.

of ATP : Mg^{++} concentrations in the ratio 1 : 1. The reaction thus exhibited a set of requirements closely resembling those previously shown to ensure optimal efficiency of production of TTP from TdR (Section 3.3.1), the resemblance of the two systems being further emphasised by the marginal stimulation of TdR kinase activity resulting from the presence of 0.1 x 10^{-3} M 2-mercaptoethanol. No stimulation was observed in the presence of bovine serum albumin in contrast to the findings of Okazaki and Kornberg (1964a).

The time course of TdR kinase action (Fig. 31 (A)) was shown to be a linear function of the incubation time from time zero up to about 30 minutes after which time reaction product inhibition became manifest. The specific activity of the TdR kinase under these conditions was about 0.06 mumoles TdR phosphorylated per minute per mg. protein over the initial 15 minutes of the reaction, more than 60 per cent of the total amount of TdR being recovered in the form of phosphorylated derivatives at the end of a 60 minutes' incubation period. The initial reaction rate was routinely measured prior to any new series of experiments in order to allow for fluctuations in the TdR kinase activity due to storage of the lyophylised cell-free ascites extract pool. The specific activity of TdR kinase did not vary significantly in material drawn from this pool over a period of 12 months, values of 0.05 - 0.07 mumoles TdR phosphorylated per minute per mg. protein being representative.

Given the set of optimal requirements for TdR kinase action described above, it was important to ascertain that the assay used for the determination of TdR kinase activity (Section 3.2.3 (a)) was valid under the conditions

chosen. The results shown in Fig. 31 (B) reveal that the phosphorylation of TdR was a linear function of the protein concentration over the selected range and thus indicate that the enzyme remained fully saturated with substrat

A report in the work of Weissman, Smellie and Paul (1960) that treatment of crude Ehrlich ascites extracts with dilute acid led to an enrichment of the TdR kinase of that tissue was further investigated. Experiments in which the crude ascites extracts were fractionated with dilute acetic acid have shown that a major proportion of the TdR kinase activity remained in the supernatant fluid when the pH was lowered to 4.5. The proportion of the tota initial TdR kinase activity so recovered was found to be dependent on a number of factors, chief among which were a strict control of pH and the presence of stabilising factors. Thus, while 80 per cent of the total TdR kinase activity was frequently lost when the lyophylised crude extract preparation was dissolved in distilled water, experiments performed in the presence of 0.01 M phosphate buffers, pH 7.5 - 7.8, resulted in recoveries of 60 - 80 per cent of the total TdR kinase activity. One such experiment (see Section 3.2.4 (a)), performed in 0.01 M phosphate buffer, pH 7.8, is recorded in Table 6 from which it is seen that over-lapping fractionation of the enzyme solution results in an enrichment of the TdR kinase activity, 95 per cent of the total activity being recovered in the fraction remaining soluble at pH 4.5 (AS 4.5 fraction). The material so obtained contained TdR kinase purifie 3-fold over the original extract. Moreover, examination of the recovery values makes it clear that the total measurable enzyme activity in the fractio recovered at pH 4.5 exceeds that present initially, thus indicating perhaps a

Table 6.

Fraction	Units per ml.	total no. of units	Protein concentration mg. per ml.	Per cent recovery of protein	Per cent recovery of activity	Specific activity units per mg.	Purification
Control	8.20	123.1	7.3	100	100	1.13	1
AS 5.5	3.63	54.5	4.48	61	45	0.81	0.7
AR 5.5	1.43	21.41	3.2	44	17	0.45	
AS 5.2	3.40	51.00	3.12	43	42	1.09	1.0
AR 5.2	1.47	22.00	3.52	48	18	0.47	
AS 5.0	4.95	74.2	2.93	40	61	1.69	1.5
AR 5.0	1.90	28.5	4.04	55	23	0.47	
AS 4.5	7.73	111.6	2.52	35	95	3.05	3
AR 4.5	2.87	43.13	4.16	63	35	0.69	

Table 6. The effect of fractionation with dilute acetic acid on TdR kinase of cell-free extracts of Landschutz ascites tumour cells.

The fractionation experiments were performed as described in Section 3.2.4 (a). Enzyme solution was prepared by dissolving lyophylised cell-free extracts of Landschutz ascites tumour cells in 0.01 M phosphate buffer, pH 7.8, 15 ml. portions being transferred to centrifuge tubes. Each portion was then adjusted to either pH 5.5, 5.2, 5.0 or 4.5 with 1.0 N acetic acid, centrifuged to give the appropriate acid-soluble (AS) fraction or acid-insoluble (AR fraction and the pH of each individual fraction readjusted to 7.8 with 1.0 N sodium hydroxide. Samples were taken for measurement of TdR kinase activity using the radioactivity assay (Section 3.2.3 (a)) with (^{3}H) TdR as substrate. One unit of TdR kinase activity was defined as that which is capable of phosphorylating 1 mumole TdR in 15 minutes (calculated from the formula given in the legend to Fig. 31). Specific TdR kinase activity was given as units per mg. protein.

differential precipitation of a TdR kinase inhibitor whenever the pH is lowered beyond 5.0. The phenomenon may be due to the removal of dephosphorylating enzymes or to the unblocking of repressed enzyme molecules, both of which events would be expected to result in the gradual unmasking of TdR kinase activity. However, it seemed clear also that some destruction of enzyme activity occurred in the acid medium, only 60 per cent of the TdR kinase activity being recovered when the precipitation was performed at pH 5.5 - 5.0. A slight improvement in the recovery values was observed when the fractionation was performed in the presence of 0.2×10^{-3} M 2-mercaptoethanol or 0.1 M KCl while the presence of NaCl appeared to cause some destruction of TdR kinase activity.

While these results largely confirmed the observations of Weissman, Smellie and Paul (1960), other experiments indicated that the TdR kinase of Landschutz ascites tumour cells behaved differently from the Ehrlich ascites enzyme. Thus it was shown that the TdR kinase did not respond favourably to fractionation with ammonium sulphate (Table 7) under the conditions employed by Weissman, Smellie and Paul (1960). Best recoveries were obtained under conditions where the pH of the medium and of the saturated ammonium sulphate solution was strictly controlled, the most favourable pH being 7.8. In such fractionations with the crude ascites extracts, a 2-fold purification of the TdR kinase activity was achieved in the fraction precipitated between zero and 30 per cent ammonium sulphate saturation. Unfortunately, this represented no more that 20 per cent of the total kinase activity, the remainder being spread over the succeeding fractions. Ammonium sulphate

Table 7 (B).

Fraction. Per cent acetone (V/V)	Units per ml.	Total no. of units	Protein concentration mg. per ml.	Per cent recovery of protein	Per cent recovery of activity	Specific activity units per mg.	Purification
Control	7.35	735	8.1	100	100	0.907	1
0 - 45	0.75	3.0	0.72	4	4	1.042	1.2
45 - 52.5	0.512	5.12	1.39	17	7	0.369	
52.5 - 60	2.47	24.7	5.45	66	34	0. 42	
60 - 75	0.523	2.62	1.26	8	4	0.415	
Supernatant fluid	0	0	0.05	1	0	0	

Table 7 (A).

Fraction. per cent ammonium sulphate saturation	Units per ml.	Total no. of units	Protein concentration mg. per ml.	Per cent recovery of protein	Per cent recovery of activity	Specific activity units per mg.	Purification
Control	5.44	108.8	6.00	100	100	0.901	1
0 - 30	2.01	20.1	1.34	11	19	1.496	1.6
30 - 40	0.888	8.88	1.55	12	8	0.574	
40 - 50	1.405	14.05	3.3	27.5	13	0.426	
50 - 60	0.535	5.35	0.96	8	5	0.777	
Supernatant fluid	0.523	26.15	0.84	35	24	0.623	

Table 7. Effects of ammonium sulphate and acetone fractionations on TdR kinase of cell-free extracts of Landschutz ascites tumour cells.

(A) Ammonium sulphate fractionation at pH 7.6.

Fractionation was performed as described in Section 3.2.4 (d) with an enzyme solution prepared by dissolving the lyophylised cell-free extracts of Landschutz ascites tumour cells in 0.05 M phosphate buffer, pH 7.6. Fractions precipitated following the addition of saturated ammonium sulphate (preadjusted to pH 7.6) were redissolved in 0.01 M tris buffer, pH 7.7. Samples were taken for measurement of TdR kinase activity using the radioactivity assay (Section 3.2.3 (a)) with (^{3}H) TdR as substrate. One unit of TdR kinase activity was defined as that capable of phosphorylati 1 mumole of TdR in 15 minutes.

(B) Acetone fractionation at pH 7.8.

Fractionation was performed as described in Section 3.2.4 (e). Enzyme solution was prepared by dissolving lyophylised cell-free extracts of Landschutz ascites tumour cells in 0.01 M phosphate buffer, pH 7.8, and fractions precipitated by acetone treatment were dissolved in the same buffer. Measurement of TdR kinase activity was performed as above.

fractionation of the AS 4.5 fraction, similarly, did not result in significant enrichment in the TdR kinase activity in contrast to the observations of Weissman, Smellie and Paul (1960). Fractionation with acetone under conditi strictly controlled with respect to temperature and pH (see Section 3.2.4 (e), as shown in Table 7 resulted in marginal enrichment of TdR kinase activity only at the expense of the recovery of total enzyme activity.

While the present experiments did no more than demonstrate the presence of TdR kinase activity in the crude ascites extracts, some additional studies (described in the following Sections) have clearly shown the separate existence of this kinase which, by virtue of its relatively greater stability, may readily be separated from the thymidine nucleotide kinases.

3.3.3. Stability of the thymidine and thymidylate kinases of cell-free extracts of Landschutz ascites tumour cells

Several types of preliminary experiment had demonstrated that the thymidine and thymidylate kinases were highly unstable in aqueous solution and precautions invariably had to be taken in order to reduce treatments in aqueous media to a minimum. Thus, the crude ascites extracts retained their kinase activities for a few hours only and extracts aged for 18 - 24 hours at 0° frequently lost all detectable TMP kinase and TDP kinase activities while retaining 40 per cent only of their TdR kinase activity. Similarly, frozen extracts stored for 24 hours at -10° lost 40 per cent of their TdR kinase activity. However, it was found that the thymidine and thymidylate kinase activities from such extracts could be preserved by lyophylisation and

subsequent storage of the dried material at -50°. In this form, the TMP and TDP kinases retained their activities for a few months while the TdR kinase activity remained unimpaired after storage for 18 months. In general, therefore, the crude cell-free ascites extracts were lyophylised immediately after preparation unless specifically required for enzyme experiments in which case incubation was carried out within 1 - 8 hours of the preparation. In the course of enzyme purification also, kinase activities were preserved by lyophylisation whenever appropriate. The instability of the thymidine and thymidylate kinases from such extracts was further illustrated by their sensitivity to dialysis. The fractionation experiments involving the use of saturated ammonium sulphate and acetone, it was particularly desirable to have access to procedures for the removal of excess salt or organic solvent (see Tables 7 and 10). However, several experiments performed in both the presence of and the absence of high ammonium sulphate concentrations revealed that the kinase activities were quickly destroyed when dialysis was performed against distilled water at 0°. In the most favourable circumstances, when dialysis was performed with the use of thoroughly washed and boiled dialysis tubing against three changes of 0.01 M tris buffer, pH 7.8, in 0.1×10^{-3} M 2-mercaptoethanol at 0°, 30 per cent only of the total TdR kinase activity was recovered after 18 hours in contrast to the experience of Bojarski and Hiatt (1960) and Weissman, Smellie and Paul (1960) while TMP and TDP kinase activities were all but completely destroyed by this treatment. The instability of the thymidylate kinases to dialysis has also been observed by Ives, Morse and Potter (1963). However, both TdR and TMP kinases appeared

to be unaffected by the inclusion of low concentrations of ammonium sulphate in enzyme experiments (Tables 7 and 8).

It would appear, therefore, that the thymidine and thymidylate kinase of the crude ascites extracts exhibited variable relative stability under different conditions, the TdR kinase being relatively more stable to almost all types of treatment than were the TMP and TDP kinases. This conclusion was further substantiated in experiments designed to study the response of the different kinases to treatment with dilute acid. In the fractionation experiments described earlier (Section 3.3.2.), it was observed that a proportion of the TdR kinase activity was lost in the course of treatment with dilute acetic acid, the recovery of kinase activity being dependent on the duration of the period in which the enzyme was in direct contact with dilute acid. Satisfactory recovery of kinase activities by this type of treatment was therefore only achieved when the period in acid solution was reduced to a minimum and the acid soluble fractions so obtained were immediatel neutralised with dilute alkali (see Section 3.2.4 (a)). Another interesting observation made in the course of such preliminary experiments was that the TMP and TDP kinases appeared to lose their activities more rapidly in the presence of dilute acetic acid than did TdR kinase. Thus it seemed conceivabl that this apparent differential stability could be utilised as a means of separating the relevant kinase activities and experiments were initiated to study the fate of kinase activities as a function of the duration of the acid treatment. In such experiments (Fig. 32), the percentage of the total TdR, TMP and TDP kinase activities recovered in the fraction remaining soluble at

Table 8.

Source of enzymes	Reaction mixture	Concentration of additions	TMP kinase specific activity units per mg. protein
		x 10^3 M	
Crude soluble extracts of ascites tumour cells.	Complete	-	1.93
	+ EtSH	0.1	1.87
	+ EtSH	0.5	1.72
	+ EDTA	0.05	1.75
	+ EDTA	0.40	1.85
	+ $(NH_4)_2SO_4$	2.0	1.86
	+ $(NH_4)_2SO_4$	10.0	1.93
AS 4.7 Fraction	Complete	-	6.22
	+ EtSH	0.05	5.92
	+ EtSH	0.15	4.53
	+ EtSH	0.5	3.21
	+ EDTA	0.05	6.52
	+ EDTA	0.4	6.29
	+ $(NH_4)_2SO_4$	2.0	6.36

Table 8. Effect of various ions on the phosphorylation of (^{32}P) TMP by cell-free extracts of Landschutz ascites tumour cells and by the A3 4.7 fraction.

TMP kinase activity was measured by the radioactivity assay (Section 3.2.3 (b)) with (^{32}P) TMP as substrate. The reaction mixtures contained the following components in a total volume of 1.0 ml.:- 100 µmoles tris buffer, pH 7.6, 5 µmoles ATP, 5 µmoles $MgCl_2$, 20 mumoles (^{32}P) TMP of specific activity 5 x 10^6 counts per minute per µmole, 1.0 mg. protein from enzyme solutions prepared from the sources given in the Table in 0.01 M phosphate buffer, pH 7.6, and 2-mercaptoethanol (EtSH), EDTA or $(NH_4)_2SO_4$ in the concentrations indicated. Incubation was for 25 minute at 37°. One unit of TMP kinase activity was defined as that which catalysed the phosphorylation of 1 mumole TMP in 25 minutes (calculated from the formula given in the legend to Fig. 33) and the specific enzyme activity was defined as units per mg. protein.

Fig. 32.

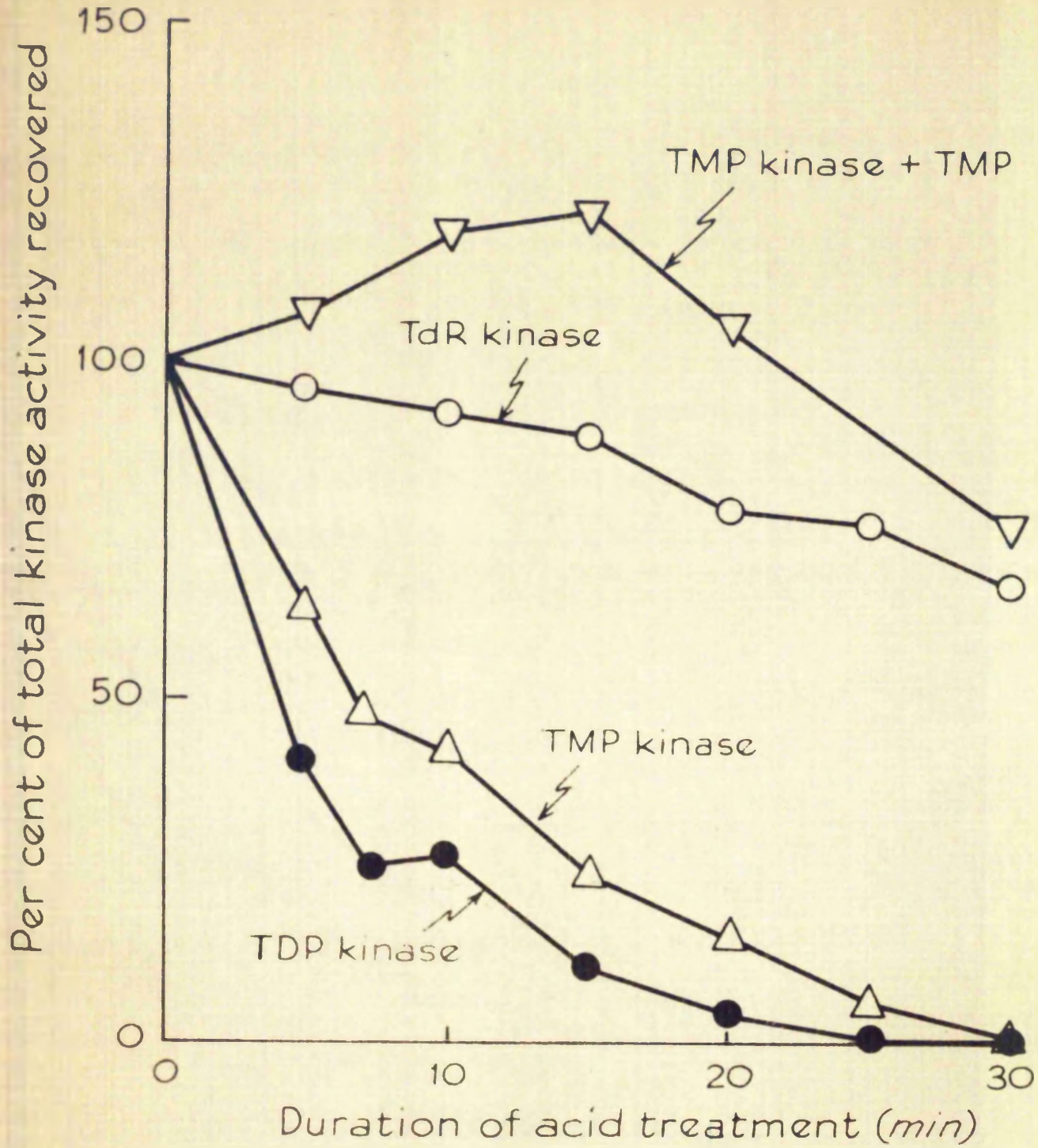

150
100
50
0
Per cent of total kinase activity recovered
TMP kinase + TMP
TdR kinase
TMP kinase
TDP kinase
0
10
20
30
Duration of acid treatment (min)

Fig. 38. The effect of treatment with dilute acetic acid on the activities of TdR kinase, TMP kinase and TDP kinase of cell-free extracts of Landschutz ascites tumour cells.

Two enzyme solutions were prepared by dissolving lyophylised cell-free extracts of Landschutz ascites tumour cells in 0.01 M phosphate buffer, pH 7.6. Both solutions contained 8.5 mg. protein per ml. To one solution was added 80 mumoles of TMP per 1.5 mg. protein whereupon both solutions were added 1.0 M acetic acid until pH 4.5 was attained. Samples of the solutions were either removed immediately, centrifuged and the pH of the supernatant fluids readjusted to 7.6, or allowed to stand at 0° for the times indicated on the diagram before being similarly neutralised. The details of the acid treatment were those given in Section 3.2.4 (a). Enzyme activities were determined as indicated; TdR kinase (Section 3.2.3 (a)), TMP kinase (Section 3.2.3 (b)) and TDP kinase (Section 3.2.3 (b)) and the results expressed as the percentage of total initial enzyme activity recovered in the neutralised supernatant fractions. For the solution containing added TMP, only the curve for TMP kinase is recorded, no change being observed in the recovery of the TdR or TDP kinases.

pH 4.5 was determined at different time intervals after precipitation with 1.0 N acetic acid, the time intervals being measured as the time elapsing between the initial attainment of pH 4.5 and the readjustment of the fractions to pH 7.8. The results showed that the ability of such solutions to catalyse the formation of TTP from TdR was progressively lost over a 30 minute period of acid treatment, the TMP and TDP kinase activities being lost at a more rapid rate than was the TdR kinase activity. Indeed, enzyme solutions exposed to acid treatments extending beyond 30 minutes still retained their ability to phosphorylate TdR while all measurable activity on TMP and TDP had been destroyed, the TdR kinase preparation so obtained retaining some 60 per cent of the total initial TdR kinase activity. A preparation with TdR kinase alone could, therefore, be conveniently obtained by judicious timing of the duration of acid treatment and provided strong evidence for the separate existence of the TdR kinase in the cell-free extracts of Landschutz ascites carcinoma. Although experimental details are not available, it seems probable that a similar procedure was applied to extracts of Novikoff hepatoma (Ives, Morse and Potter, 1963) in the preparation of a TdR kinase uncontaminated with thymidylate kinase activities.

Subsequent experiments in the present investigation have focussed attention on the TMP kinase, the instability of which proved to be a major obstacle in its characterisation. It was shown, however, that addition of excess TMP to the enzyme solutions appeared to lend increased stability to the TMP kinase component of such solutions (see Section 3.3.4.). Thus, the addition of excess TMP to the crude ascites extracts prior to fractionation

with dilute acid resulted in the recovery of 80 - 120 per cent of the TMP kinase activity in the supernatant fraction following acidification to pH 4.7 while much of the TDP kinase activity was destroyed. The degree of stabilisation afforded to TMP kinase by its substrate was nicely illustrated by experiments such as that shown in Fig. 32 from which it may be deduced that significant loss of TMP kinase activity did not occur within the initial 20 minutes of dilute acetic acid treatment at pH 4.5. The relative stability of the TdR and TDP kinases remained unaffected by the addition of excess TMP.

3.3.4. Experiments with the TMP kinase from cell-free extracts of Landschutz ascites tumour cells

The results of *in vitro* experiments on the mechanism of the formation of TTP (see Section 2.3.2.) provided strong circumstantial evidence in favour of the hypothesis propounded by Reissman, Smellie and Paul (1960) that the process proceeds by stepwise phosphorylation from TdR through TMP and TDP. In the view of Bianchi, Butler, Crathorn and Shooter (1961), formation of TTP from TMP occurs by pyrophosphorylation and the argument thus hinges on whether or not the formation of TTP from TdR requires the participation of a kinase specifically responsible for the phosphorylation of TMP to TDP. The remainder of the present investigation was devoted to the search for unequivocal proof of the existence of a TMP kinase in the cell-free extracts of Landschutz ascites carcinoma.

Earlier experiments (Fig. 26) had indicated that the cell-free extracts were capable of phosphorylating TMP to TDP and TTP in a reaction where the

production of TDP appeared to be rate limiting. When (^{32}P) TMP was used as substrate, over 50 per cent of the total radioactivity was recovered as phosphorylated derivatives (TDP + TTP), only 10 per cent of which was associated with the TDP, after incubation for 60 minutes with the enzymes of crude ascites tumour extracts. As in the case of TdR kinase activity (see Sections 3.2.3 (a) and 3.3.2.), it was found convenient to use the total radioactivity recovered in (TDP + TTP) with (^{32}P) TMP as substrate as a criter for the measurement of TMP kinase activity (Section 3.2.3 (b)), this criterion being also employed to express specific TMP kinase activity (for definition of a unit of TMP kinase activity, see legend to Fig. 33). While this type of assay could not distinguish between phosphorylating activity due to TMP kinase alone and that due to the combination of TMP kinase with TDP kinase, corroborative evidence on the relative activities of the two kinases was obtained by suitable correlation with paper chromatography as described elsewhere (Section 3.2.3 (b)).

The results recorded in Section 3.3.2. indicated that the requirements for optimal activity of the TdR kinase coincided with those promoting maximal formation of TTP from TdR when the reactions were catalysed by the crude extracts of Landschutz ascites carcinoma. It was thus of interest to discove whether the optimal requirements for TMP kinase activity were similarly governed.

A number of preliminary experiments had borne out the fact that the rat of phosphorylation of TMP by the crude extracts was relatively low compared wi the ability of such extracts to phosphorylate TdR (Fig. 31) and TDP (Fig. 25).

Enrichment of the TMP kinase activity was, therefore, a necessary prelude to the determination of optimal requirements and the parameters quoted hereafter refer to the partially purified AS 4.7 Fraction (see Fig. 38 and Table 9) rather than to the crude cell extract.

Experiments on the time course of the phosphorylation of TMP with partially purified enzyme preparations have shown (Fig. 33 (A)) that the reaction proceeds as a linear function of the incubation period over time intervals from zero to 30 minutes when studied in a reaction medium containing 5×10^{-3} M $MgCl_2$ and 5×10^{-3} M ATP. With (^{32}P) TMP-5' as substrate in a concentration of 2×10^{-5} M, the initial rate of phosphorylation as measured by the proportion of the total radioactivity appearing in (TDP + TTP), was of the order of 0.05 mumoles TMP phosphorylated per minute, the specific enzyme activity being most conveniently given as mumoles TMP phosphorylated per 25 minutes per mg. of protein. A falling off in the reaction rate was observed with incubation periods extending beyond 30 minutes, presumably due to inhibition of the thymidylate kinases by accumulating reaction products combined with the effect of endogenous phosphatases. It was observed with interest that no reaction occurred under these conditions when (^{32}P) TMP-3' replaced (^{32}P) TMP-5' as substrate, the enzyme therefore exhibits specificity for the deoxyribonucleoside 5'-monophosphate. In subsequent experiments it was shown (Fig. 33 (B)) that the rate of phosphorylation of TMP by the partially purified TMP kinase of the AS 4.7 Fraction was directly proportional to the protein concentration over the range 0.1 - 2.0 mg. per ml., thus showing that the assay used for determination of TMP kinase activity was valid within this range.

Table 9.

Fraction	Units per ml.	Total no. of units	Protein concentration mg. per ml.	Per cent recovery of protein	Per cent recovery of activity	Specific activity units per mg.
Control	28.96	1445	11.5	100	100	2.52
AS 5.5	21.61	1079	7.47	65	75	2.89
AS 5.2	17.81	891	4.75	41	62	3.75
AS 5.0	15.92	797	4.36	38	55	3.66
AS 4.7	36.11	1818	4.25	37	125	8.51
AS 4.5	23.45	1117	4.03	35	81	5.75

Table 9. The effect of fractionation with dilute acetic acid on TMP kinase of the cell-free extracts of Landschutz ascites tumour cells in the presence of added TMP.

The details of the fractionation procedure were those described in Section 3.2.4 (a). Enzyme solution was prepared by dissolving lyophylised cell-free crude extracts of Landschutz ascites cells in 0.01 M phosphate buffer, pH 7.6, to which was added 80 mumoles of TMP per 1.5 mg. protein and the solution was divided into six equal portions. Each portion was then adjusted to either pH 5.5, 5.2, 5.0, 4.7 or 4.5 with 1.0 M acetic acid, centrifuged immediately and the pH of the supernatant fractions readjusted to 7.6 with 1.0 M sodium hydroxide. Samples were taken from each neutralised fraction and the TMP kinase activity measured using the radioactivity assay (Section 3.2.3 (b)) with (^{32}P) TMP as substrate (after pretreatment on a column of Sephadex G-25 to remove added TMP; see Fig. 36). One unit of enzyme activity was defined as that capable of phosphorylating 1 mumole TMP in 25 minutes.

Fig. 33.

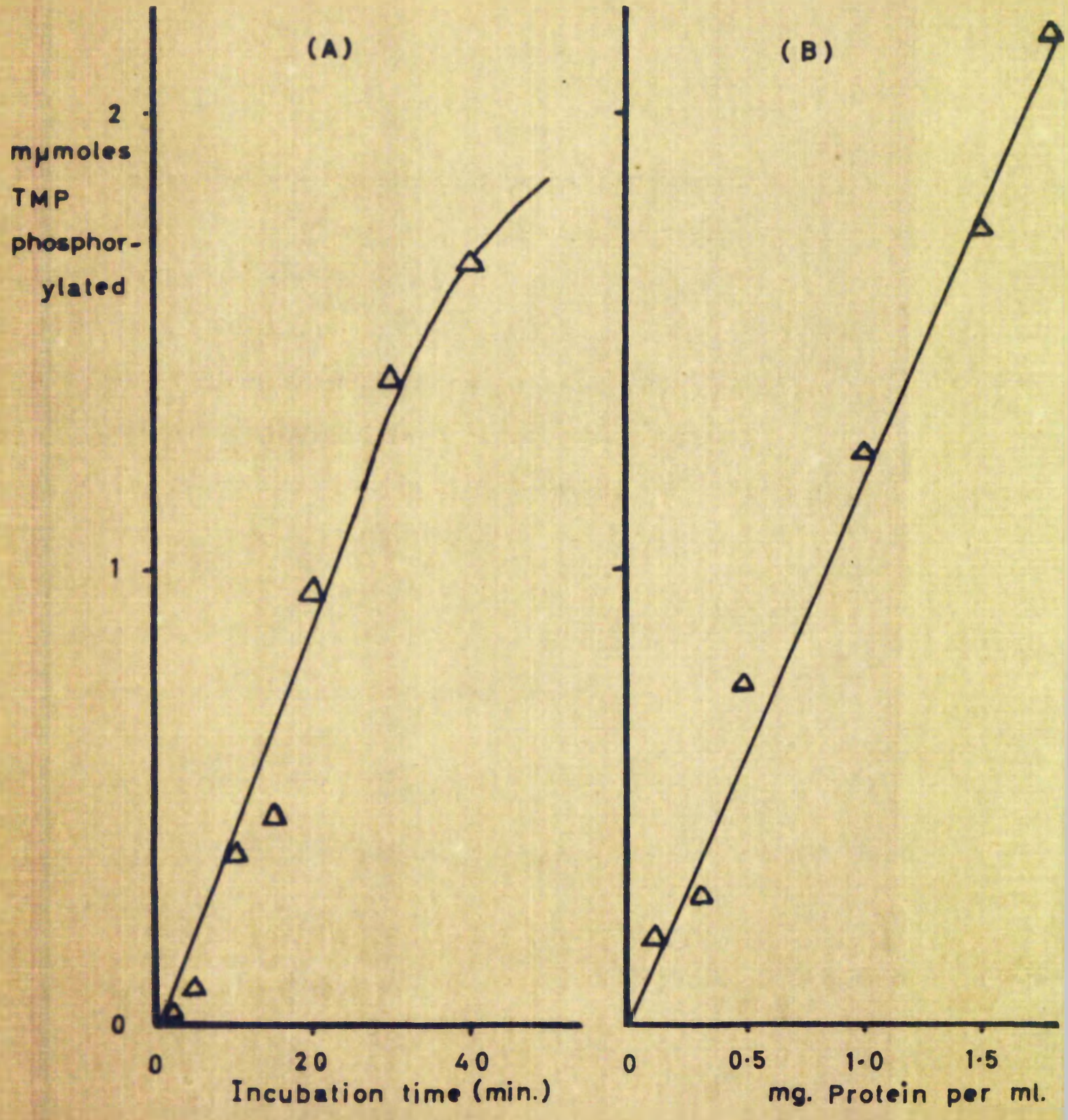

(A)
(B)
2
1
0
mμmoles TMP phosphor-ylated
0
20
40
Incubation time (min.)
0
0·5
1·0
1·5
mg. Protein per ml.

Fig. 33. The action of TMP kinase from extracts of Landschutz ascites tumour cells as measured by the total amount of (^{32}P) TMP phosphorylated.

(A) The time course of the total phosphorylation of (^{32}P) TMP.

The reaction mixtures contained the components given in the text (Section 3.2.3 (b)), the (^{32}P) TMP having the specific activity 5×10^6 counts per minute per μmole. Each reconstituted ascites tumour cell-free extracts and incubation was carried out for the times indicated on the diagram in a shaking water-bath at 37°. After the separation of reaction products, the results were obtained as the percentage of the total radioactivity recovered in (TDP + TTP) from which the number of μmoles of TMP phosphorylated could be calculated as follows:

$$\frac{X \text{ (counts per minute)}}{Y \times Z \text{ (counts per minute)}} = \text{μmoles TMP phosphorylated}$$

where X is the total number of counts per minute recovered in (TDP + TTP), Y is the fraction of the total initial radioactivity recovered and Z is the number of counts per minute corresponding initially to 1 μmole TMP. One unit of TMP kinase activity was defined as that which phosphorylates 1 μmole of TMP in 25 minutes.

Fig. 33 (Contd.): (B) The effect of enzyme concentration on the total phosphorylation of (^{32}P) TMP.

The reaction mixtures contained the components described in the text (Section 3.2.3 (b)) with enzyme concentration as given on the diagram. The incubation time was 25 minutes.

In experiments designed to study the effect of pH on the phosphorylatio of TMP, it was shown (Fig. 34) that the reaction exhibited a particularly sharp pH optimum at pH 7.6 when catalysed by the AS 4.7 Fraction. The rate of the reaction declined rapidly above pH 7.7 and below pH 7.5 so that when the phosphorylation was conducted in the presence of 0.1 M tris buffer, pH 7.3 or pH 8.0, the amount of (TDP + TTP) produced within 25 minutes was only one-half of that formed at pH 7.6. Thus it would appear that the pH optimum for the TMP kinase differs from those of the TdR kinase and of the over-all phosphorylation of TdR to TTP which are in the range pH 7.8 - 8.1. The significance of this observation remains uncertain, but it seems conceivable that the differentiation of pH optima so indicated may provide a partial explanation for the rate limiting effect of the phosphorylation of TMP in the formation of TTP as catalysed by the crude ascites extracts. It seems clear, however, that these optima represent the most favourable balance between phosphorylation and dephosphorylation in the complex ascites extract enzyme mixture and the pH optima of highly purified kinases may prove to be different from those presented here.

If, as has been supposed, the true cosubstrate for the TMP kinase were the ATP - Mg^{++} chelate, it would be of some interest to examine the effects of variation in the magnesium concentration on the phosphorylation of TMP by the partially purified AS 4.7 Fraction. In the experiment shown in Fig. 35, the ATP concentration was kept constant at 5×10^{-3} M, a concentration which appeared to be optimal for the TMP kinase, and the Mg^{++} concentration was varied. While the results indicated that the net rate of TMP phosphorylation

Fig 34.

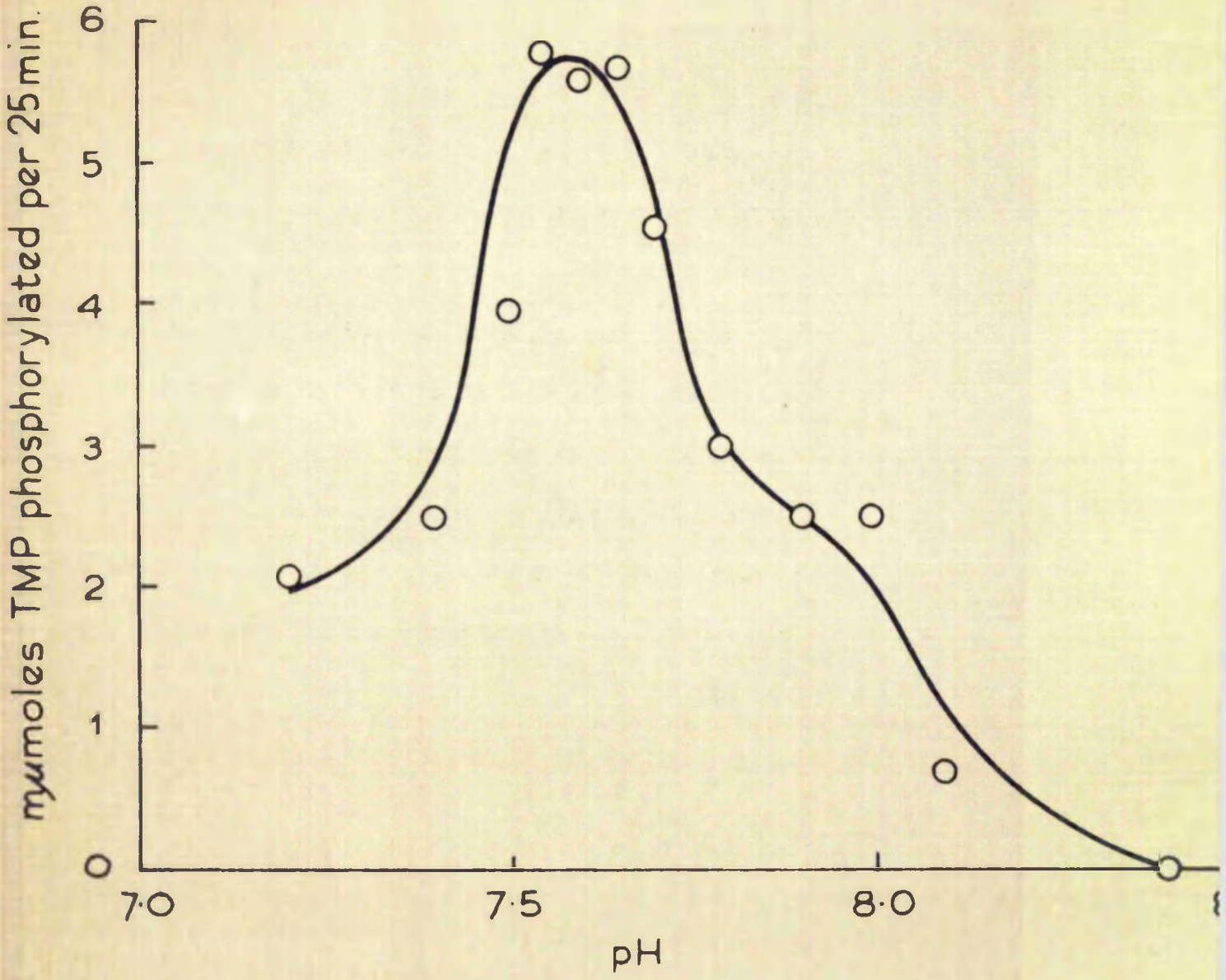


Fig. 34. The effect of pH on the action of TMP kinase.

The reaction mixtures contained the following components in a total volume of 1.0 ml.:- 5 µmoles ATP, 5 µmoles $MgCl_2$, 20 mµmoles (^{32}P) TMP of specific activity 3.5 x 10^6 counts per minute per µmole, 1.0 mg. protein and 100 µmoles tris buffer of the pH indicated on the diagram. Incubation was for 25 minutes in a shaking water-bath at 37° and further treatment was as described in the text (Section 3.2.3 (b)). The source of enzyme was the AS 4.7 Fraction (see Section 3.3.5; Methods I and II).

Fig 35.

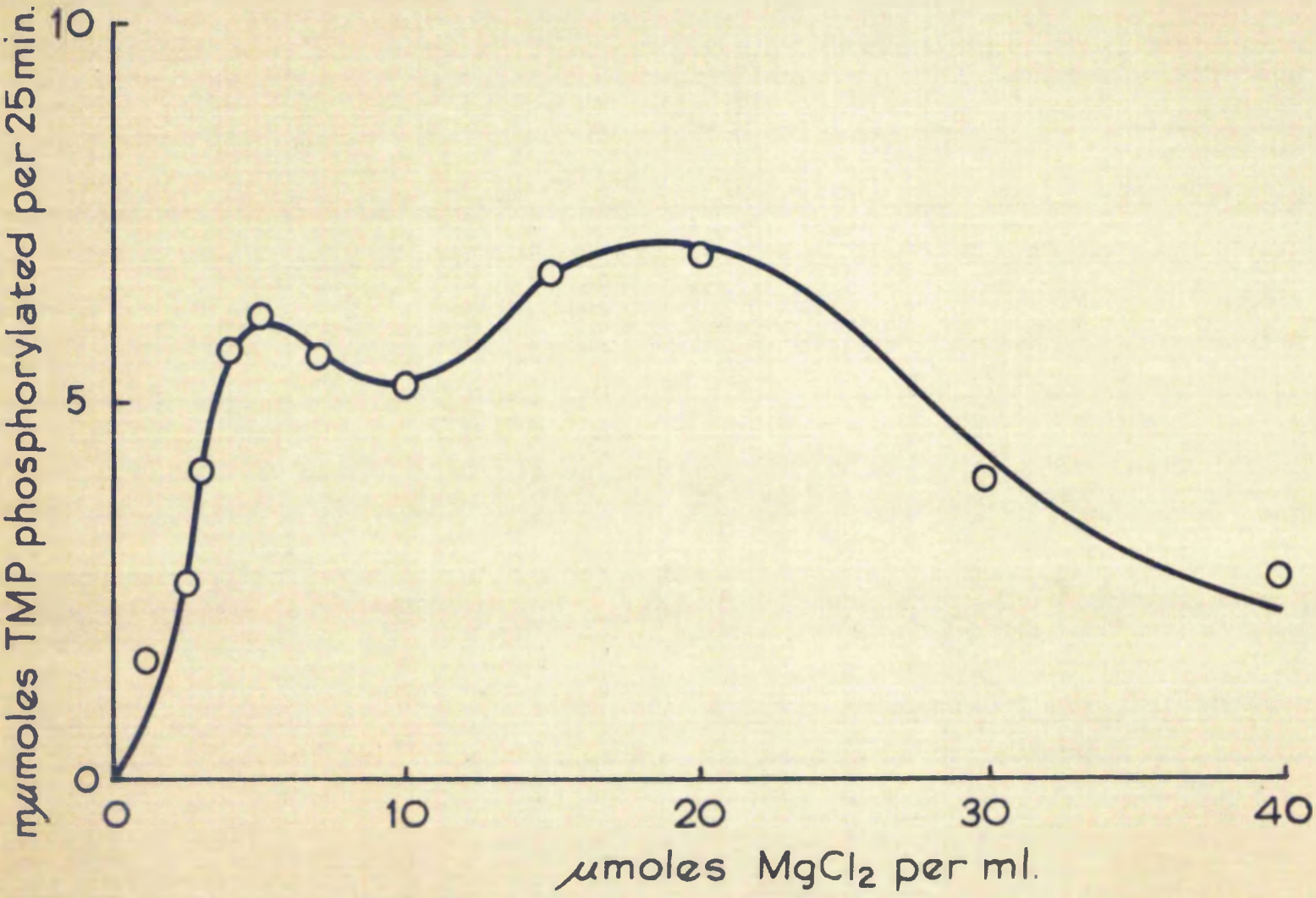

μmoles TMP phosphorylated per 25 min.
10
5
0
0
10
20
30
40
μmoles MgCl2 per ml.

Fig. 35. The effect of Mg^{++} concentration on the action of TMP kinase.

The reaction mixtures contained the following components in a total volume of 1.0 ml.:- 5 µmoles ATP, 100 µmoles tris buffer, pH 7.6, 20 mµmoles (^{32}P) TMP of specific activity 3.5 x 10^6 counts per minute per µmole, 1.0 mg. protein and $MgCl_2$ in the quantities indicated on the diagram. Incubation was for 25 minutes in a shaking water-bath at 37° and further treatment was as described in the text (Section 3.2.3 (b)). The source of enzyme was the AS 4.7 Fraction (See Section 3.3.5; Methods I and II).

was optimal at 5×10^{-3} M $MgCl_2$, there were also indications that there occurred a second optimum between 15×10^{-3} and 20×10^{-3} M $MgCl_2$ concentrati No TMP was phosphorylated in the absence of added $MgCl_2$, thus showing that any endogenous Mg^{++} was insufficient to sustain the reaction, while the relatively slow decline in the TMP kinase activity with higher levels of Mg^{++} could be indicative of the availability of unchelated Mg^{++} to nucleotide phosphatases. The double Mg^{++} optimum which seems to be exhibited may be due to different Mg^{++} requirements of the TMP and TDP kinases. In subsequent routine kinase assays, the ATP and Mg^{++} concentrations were maintained in a ratio of 1 : 1.

The instability of the TMP kinase in aqueous solution (see Section 3.3.3.) necessitated a search for possible stabilising factors. Factors were sought which could fulfil the two separate functions of minimising denaturation of the enzyme during incubations at 37° and of lending added stability to the TMP kinase component during enzyme fractionations. 2-Mercaptoethanol was previously (Section 2.3.1) shown to stimulate the overall phosphorylation of TdR to TTP by the crude ascites extracts and seemed a possible candidate to act as stabilising factor for the TMP kinase also. Experiments which investigated the effect of this compound, as well as some other ions, on the phosphorylation of TMP have shown (Table 8) that the TMP kinase derived from the crude lyophylised ascites tumour extract was unaffected by the presence of 2-mercaptoethanol, EDTA and 1×10^{-2} M $(NH_4)_2SO_4$. It was observed, however, that the rate of phosphorylation of TMP by the partially purified AS 4.7 Fraction declined markedly in the presence of

2-mercaptoethanol, the specific activity of the TMP kinase being reduced to one-half that of untreated preparations in the presence of 0.5×10^{-3} M 2-mercaptoethanol. EDTA and $(NH_4)_2SO_4$ had no significant effect on the phosphorylation of TMP by the AS 4.7 Fraction. The addition of bovine serum albumin to the reaction mixtures, similarly, had no effect on the rate of phosphorylation of TMP even when the activity of highly purified kinase preparations was measured.

An original observation made by Hiatt and Bojarski (1960) that the administration of thymidine resulted in a stimulation of the thymidylate kinase activity of rat tissues suggested thymidine as a possible stabilising factor for TMP kinase. However, in the experiments of Hiatt and Bojarski (1960) thymidine was presumably converted to TMP within the cell thus suggesting that TMP was the true stimulatory agent. The present series of experiments have confirmed the validity of this assumption and have shown that addition of excess TMP to the TMP kinase derived from extracts of Landschutz ascites tumour cells results in increased kinase stability. This device has proved particularly valuable in the characterisation and purification of TMP kinase as described in the following pages. Thus, the redissolved lyophylised, crude extract, which previously had been observed to lose its ability to phosphorylate TMP over a period of 2 - 3 hours, showed retention of TMP kinase activity up to 24 hours when dissolved in 0.01 M phosphate buffer, pH 7.6, containing 80 mumoles TMP per 1.5 mg. protein. The protection of TMP kinase by its substrate raised several important questions as to the precise nature of this effect. Some knowledge of this

was necessary because it was clear that the assay employed to measure TMP kinase activity became quantitatively invalid once applied to enzyme solutions containing high concentrations of TMP. It seemed probable that the association of TMP with TMP kinase under such circumstances would represent a situation akin to an enzyme - substrate complex in which case association should be a reversible process. The reversibility of the TMP - TMP kinase association was demonstrated by experiments in which enzyme solution containing TMP was fractionated on columns of Sephadex G-25 (see Section 3.2.4 (c In such experiments, (^{32}P) TMP replaced TMP and elution of the mixture with 0.01 M phosphate buffer, pH 7.6, revealed that unlabelled TMP kinase appeared early in the elution, followed, in subsequent fractions, by protein-free (^{32}P) TMP. A perfect separation of the two components was thus achieved as shown in Fig. 36. As this provided a convenient and rapid means of removing the added TMP, the procedure was routinely resorted to prior to the assay for TMP kinase.

The investigations of Weissman, Smellie and Paul (1960) had given some indication that the TMP kinase responded favourably to fractionation with dilute acetic acid. Early experiments with the enzyme from Landschutz ascites tumour cells, however, demonstrated variable but low retention of TMP kinase activity when exposed to treatment with dilute acid. However, when fractionation (described in Section 3.3.3) with dilute acid was conducted in the presence of excess TMP (80 mumoles TMP per 1.5 mg. protein), 80 - 120 per cent of the total initial TMP kinase activity was recovered in the fraction remaining soluble at pH 4.5 (AS 4.5 Fraction). The recovery of enzyme

Fig. 36.

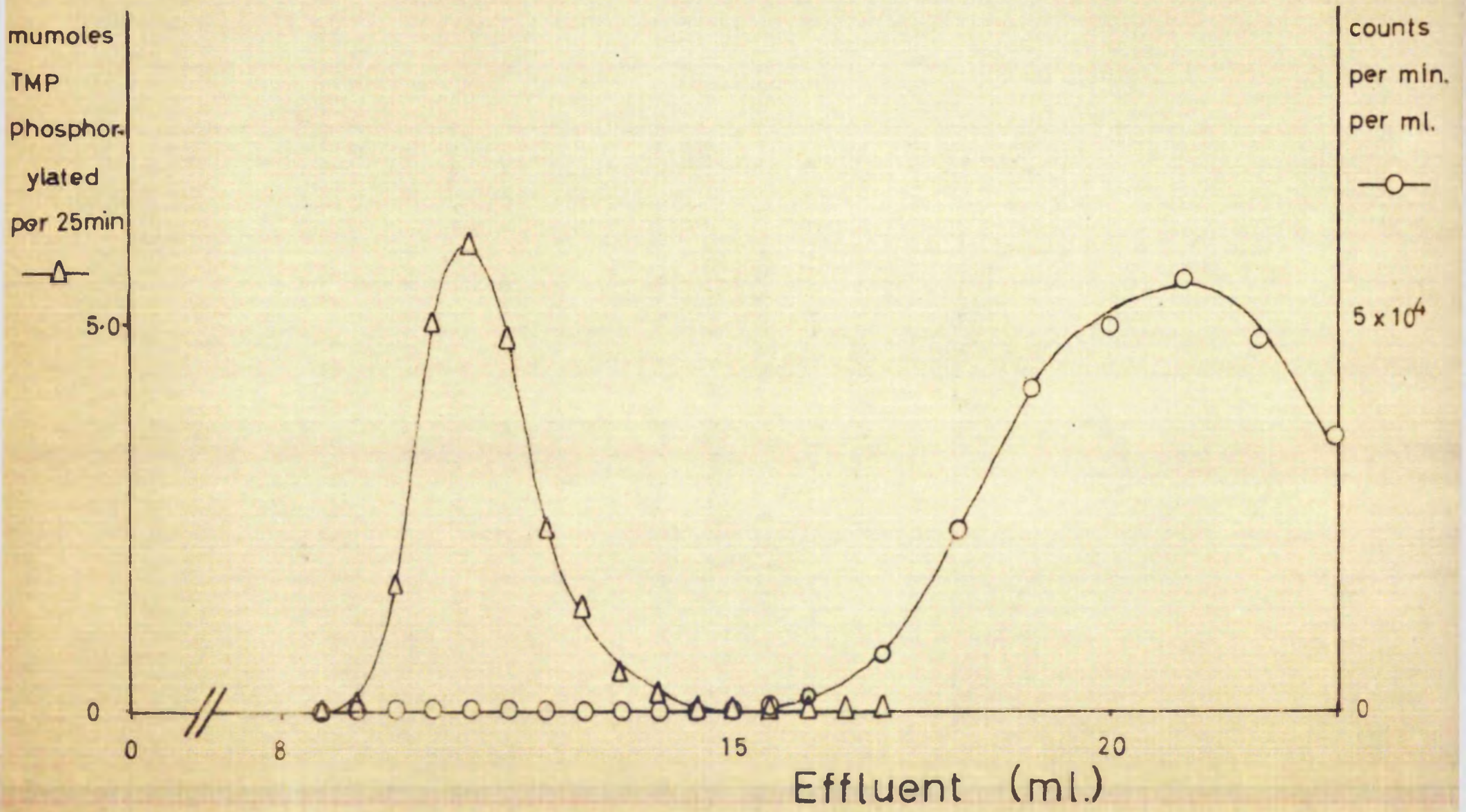

mumoles
TMP
phosphor-
ylated
per 25min
5·0
0
counts
per min.
per ml.
5 x 10⁴
0
0
8
15
20
Effluent (ml.)

Fig. 36. Resolution of a mixture of TMP kinase and TMP on a column of Sephadex G-25.

Enzyme solution was prepared by dissolving the lyophylised cell-free crude extracts of Landschutz ascites tumour cells in 0.01 M phosphate buffer, pH 7.6, to give a protein concentration of 17.25 mg. per ml. To this solution was added 40 mumoles (^{32}P) TMP being 0.6 x 10^6 counts per minute per μmole. A portion (1.0 ml.) of the enzyme solution, now containing 17 mg. protein per ml., was fractionated on a column of Sephadex G-25 (20 x 1 cm., dia.) previously equilibrated with 0.01 M phosphate buffer pH 7.6, and fractions of 0.5 ml. were collected. Enzyme activity was assayed as described elsewhere (Section 3.2.3 (b)) and other samples were taken for the direct measurement of radioactivity. The column V_o was 8.0 ml. The recovery of TMP kinase activity was 96 per cent while that of (^{32}P) TMP, after collection of 35 ml. effluent, was 95 per cent.

activity, nevertheless, declined as a reciprocal function of the time in contact with dilute acid and this period was never allowed to exceed 15 minut (see Fig. 32). Overlapping fractionation of redissolved, lyophylised crude ascites extract with 1.0 N acetic acid showed that the proportion of the tota initial TMP kinase activity recovered in the acid soluble fraction declined steadily when the pH was lowered from 7.6 to 5.0, below which value retention of TMP kinase increased sharply so that 125 per cent of the total activity was recovered at pH 4.7 (see Table 9). A possible explanation for this observation is that lowering of the pH beyond 5.0 causes a preferential precipitation of contaminating dephosphorylating enzymes or a progressive removal of endogenous inhibitors. While it seems clear that the addition of TMP protects the TMP kinase activity over a wide range of pH values, this effect does not appear to extend beyond pH 4.7, presumably due to the increased rate of enzyme - substrate complex dissociation at lower pH values. The AS 4.7 Fraction retained TMP kinase activity purified 2 - 3-fold over the lyophylised crude extracts and contained TdR kinase activity together with low, but measurable TDP kinase activity.

Subsequent experiments were focussed on methods leading to the elimination of contaminating kinase activities from the TMP kinase preparation One such possible fractionation procedure involved the use of ammonium sulphate and preliminary experiments were performed in order to discover the type of fractionation most favourable. Strict control of pH and temperature was shown to be a prerequisite of even moderate success, enzyme solutions being prepared in 0.01 M phosphate buffer, pH 7.6, with saturated

ammonium sulphate solutions at the same pH. Such fractionations, however, met with little quantitative success and the pattern of fractionation obtainable is well illustrated by the results recorded in Table 10 using the AS 4.7 Fraction. While a fraction precipitated between 50 and 55 per cent ammonium sulphate saturation showed a 2-fold enrichment of TMP kinase activity, this fraction represented no more than 10 per cent of that present initially, the remainder being distributed over the other fractions. Moreover, some 40 per cent of the total TMP kinase activity was unaccounted for and was not found in the supernatant liquor remaining after 70 per cent ammonium sulphate saturation, thus indicating destruction of enzyme activity. This pattern did not appear to be effected by the addition of excess TMP prior to fractionation and further attempts to purify TMP kinase by this method were abandoned. Fractionation with acetone at -15°, similarly, resulted in serious inhibition of TMP kinase activity.

Efforts were directed, next, to the possible use of adsorbent gels as a means of fractionation of the complex ascites extract enzyme mixture. C γ-alumina is well established as a potentially powerful adsorbent of phosphatases (Courtois, 1947) and the ease with which this gel can be manipulated makes it a convenient tool in enzyme fractionations. Several exploratory experiments carried out on material from the lyophylised cell-free extract pool (Section 3.2.2 (a)) revealed that the optimal adsorption of protein on C γ-alumina occurred at pH 6.0 - 6.3 and that other requirements included low temperature (0 - 2°), high protein concentration in the enzyme solutions and low ionic strength in the buffers used for the adsorption. However,

Table 10.

Enzyme fraction per cent ammonium sulphate saturation	Units per ml.	Total no. of units	Protein concentration mg. per ml.	Per cent recovery of protein	Per cent recovery of activity	Specific activity units per mg.
Control	26.39	210.9	6.85	100	100	3.84
0 - 30	8.87	17.74	2.42	9	8	3.66
30 - 35	4.42	8.85	1.92	7	4	2.31
35 - 40	5.33	10.66	1.50	5	5	3.55
40 - 45	9.77	1954	2.56	9	9	3.82
45 - 50	7.99	15.99	2.86	10	7	2.8
50 - 55	10.17	2o.34	1.62	6	10	6.2
55 - 70	7.33	36.64	1.70	17	16	4.31

Table 10. The effect of ammonium sulphate fractionation at pH 7.6 on TMP kinase derived from the AS 4.7 Fraction in presence of added TMP.

Ammonium sulphate fractionation was carried out according to the procedure described in Section 3.2.4 (d). Lyophylised AS 4.7 Fraction was dissolved in 0.01 M phosphate buffer, pH 7.6, (to which was added 80 mumoles TMP per 1.5 mg. protei and fractions precipitated by the addition of saturated ammonium sulphate (preadjusted to pH 7.6) wereredissolved in the same buffer. TMP kinase activity was measured using the radioactivity assay (Section 3.2.3 (b)) and one unit of TMP kinase activity was defined as that capable of phosphorylating 1 mumole of TMP in 25 minutes.

as the TMP kinase could not be expected to tolerate lengthy operations conducted at pH 6, a compromise set of conditions was employed with both the enzyme solutions and the C γ-alumina suspension equilibrated at pH 7.6. Under these conditions, treatment of the AS 4.5 Fraction with increasing concentrations of C γ-alumina gel led to adsorption of TMP kinase along with much of the protein as shown in Fig. 37, the progress of the adsorption being followed by measuring the proportion of the TMP kinase activity remaining unadsorbed (see Section 3.2.4. (b)). Such measurements, suitably correlated with paper chromatography (Section 3.2.3 (b)), indicated that TDP kinase activity was similarly adsorbed. Thus, addition of 5 ml. of C γ-alumina suspension to the enzyme solution (1.0 ml.) resulted in the adsorption of all but a few per cent of the TMP kinase activity and further additions of gel did not markedly alter the proportion of adsorbed and unadsorbed enzyme. Significant differences in the adsorption pattern of TMP kinase were observed if excess TMP were added to the AS 4.7 Fraction prior to adsorption on C γ-alumina gel. Under these circumstances, the rate of adsorption of TMP kinase was markedly reduced so that after the addition of 3 - 5 ml. of gel suspension to the enzyme solution (1.0 ml.), there remained in solution 40 - 80 per cent of the total TMP kinase activity. The relative rates of adsorption of protein and TDP kinase were not so effected. This operation thus led to a supernatant enzyme fraction containing TMP kinase practically uncontaminated with TDP kinase activity. The fraction remaining in the supernatant fluid after the addition of 3 ml. of gel suspension per ml. of enzyme solution was purified 60 - 80-fold over

Fig. 37.

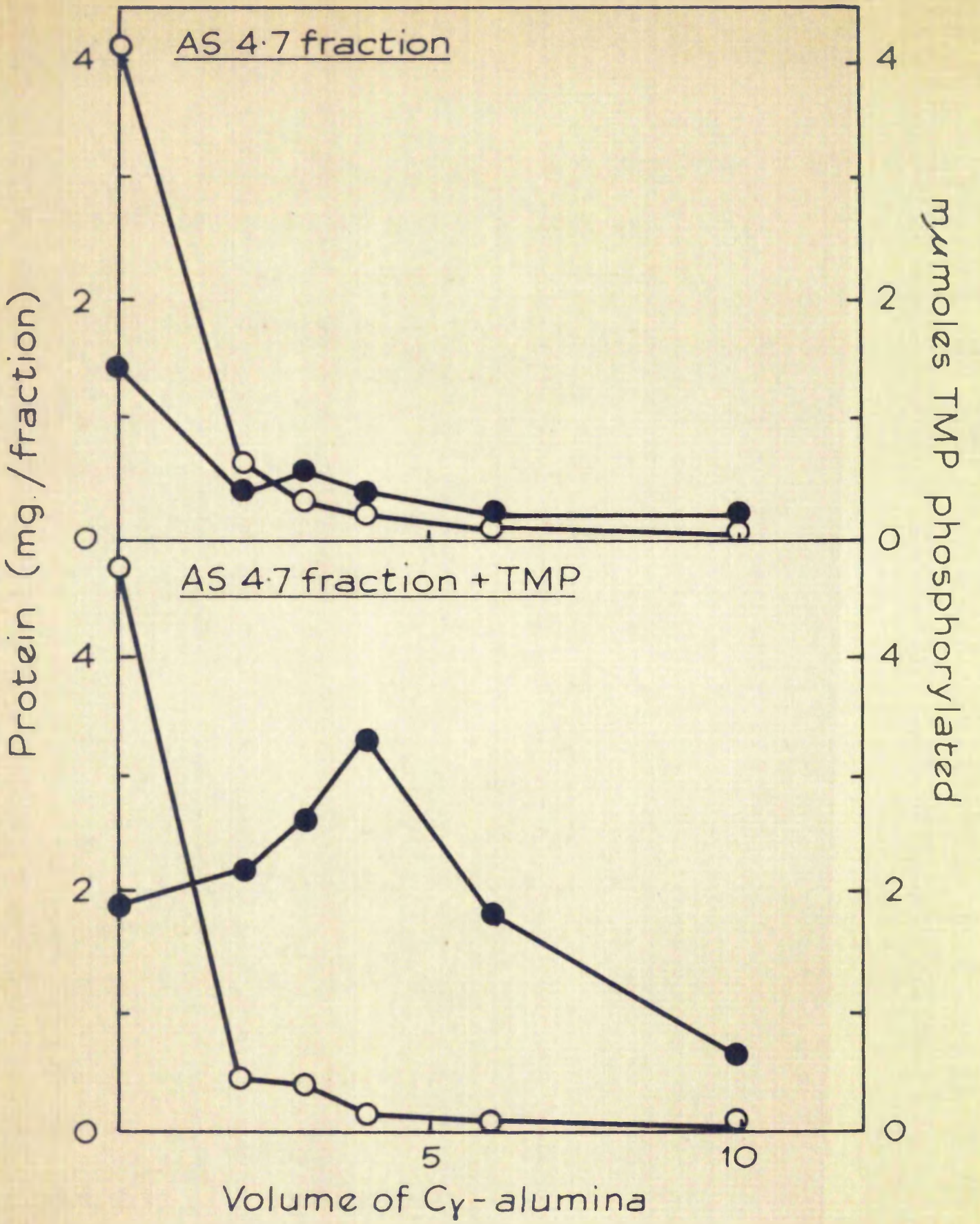

AS 4·7 fraction
AS 4·7 fraction + TMP
Protein (mg./fraction)
μmoles TMP phosphorylated
Volume of Cγ-alumina
0
2
4
5
10

Fig. 37. The adsorption of TMP kinase on C γ-alumina gel in the presence and absence of added TMP.

The lyophylised AS 4.7 Fraction was dissolved in 0.01 M phosphate buffer, pH 7.6, and the resulting solution divided into two parts, to one of which was added 80 mumoles TMP per 1.5 mg. protein. Portions (1.0 ml.) of these solutions were transferred to 15 ml. centrifuge tubes containing the volumes of C γ-alumina gel indicated on the diagram. Adsorption was carried out for 30 minutes at 0° after which the mixtures were centrifuged and the protein and TMP kinase activity remaining in the supernatant fluid were measured (Sections 3.2.3 (a) and 3.2.3 (b)). The details of the fractionation using C γ-alumina gel are described in Sections 3.2.4 (b) and 3.3.5; Method I. The results were expressed as mumoles TMP phosphorylated per 25 minutes per supernatant fraction. Protein concentrations were likewise expressed as mg. per supernatant fraction. Open circles denote protein concentration and the closed circles refer to TMP kinase activity.

the crude ascites extracts and was designated the C γ AS 4.7 Fraction. It seems possible that the pattern of adsorption of TMP kinase observed in the presence of excess TMP is due to rearrangements on the enzyme surface resulting in increased repulsion of fixed charges. That some adsorption does take place is shown by the decline in supernatant TMP kinase activity at the higher levels of C γ-alumina gel concentration.

3.3.5. Partial purification of thymidine 5'-monophosphate kinase from cell-free extracts of Landschutz ascites tumour cells

The results described in the previous Section demonstrated that substantial gains in the specific activity of TMP kinase could be achieved by suitable fractionation with dilute acetic acid and by treatment of the enzyme solutions with C γ-alumina gel provided excess TMP was present as a stabilising factor. It seemed possible, therefore, that such operations might provide the basis of a method for the purification of the TMP kinase present in the crude ascites tumour extracts. Additional preliminary experiments had indicated that some enrichment of the TMP kinase could be achieved by lyophylisation of the crude extracts followed by redissolution of the dry powder in 0.01 M phosphate buffer, pH 7.6, the insoluble portion of the protein being discarded. This observation may be related to the increased thymidylate kinase activities observed in "aged" extracts of calf thymus (Bojarski, 1968) and of Novikoff hepatoma (Ives, Morse and Potter, 1968) and it may also have some connection with the release of TMP kinase activity from inactive complexes with small cellular particles as observed by freezing

and thawing of mouse liver homogenates (Kielley, 1963a). Additional enzyme fractionation techniques were required for the removal of various contaminating activities and advantage was taken of the recently developed procedures depending on molecular sieving (Flodin, 1962) with uncharged dextran gels.

(a) Method I

All operations were carried out in the cold (0 - 2°).

Fractionation with dilute acetic acid: The lyophylised cell-free extracts of Landschutz ascites tumour cells (prepared as described in Section 2.2.1) was dissolved in 0.01 M phosphate buffer, pH 7.6, to give a solution containing 12 - 14 mg. protein per ml. and the residual protein and other insoluble material was removed by centrifugation for 20 minutes at 2000 x g. To the supernatant fluid was added TMP solution to a concentration of 80 mumoles per 1.5 mg. protein and the bulk of the solution diluted to some specified volume from which samples were removed for the estimation of protein and the measurement of TMP kinase activity. The enzyme solution, now containing 10 - 12 mg. protein per ml. in a volume not exceeding 200 ml., was submitted to fractionation with dilute acetic acid. To the solution, cooled in an ice-bath and with continuous stirring, was added 1.0 M acetic acid drop by drop at the pH meter until the pH reached 4.7. The suspension was then quickly transferred to centrifuge bottles and centrifuged at 1000 x g for 6 minutes after which the supernatant was returned to the pH meter and the pH readjusted to 7.6 with the addition of 1.0 M sodium hydroxide. The duration of the whole operation involving contact with acetic acid should not be allowed to exceed 15 minutes. After the removal of samples for

protein estimation and measurement of kinase activity, the main bulk of the solution (designated the AS 4.7 Fraction) was preserved by lyophylisation and stored at -30°. Since TMP had been added to the solution, a preliminary treatment (Section 3.2.4 (c)) on a column of Sephadex G-25 (20 x 1 cm., dia.) was required prior to the measurement of TMP kinase activity. The fractionation with acetic acid yielded a TMP kinase preparation purified 3 - 5-fol over the crude extracts. These preparations also contained partially purified TdR kinase and relatively low TDP kinase activity. Lyophylisation did not result in loss of activity.

Fractionation with C γ-alumina: The lyophylised AS 4.7 Fraction containing TMP was dissolved in 0.01 M phosphate buffer, pH 7.6, to give a solution containing 6 - 10 mg. protein per ml. and a portion was reserved for the measurement of TMP kinase activity. The main bulk of the solution was added to C γ-alumina gel pretreated as follows: - a volume of C γ-alumina suspension (11 mg. per ml.) corresponding to three times the volume of enzyme solution to be fractionated was transferred to a 1500 ml. centrifuge bottle and centrifuged at 1000 x g for 10 minutes after which the supernatant fluid was discarded. The residual gel was washed three times with 300 ml. portions of ice-cold 0.01 M phosphate buffer, pH 7.6, the gel being resuspended and centrifuged between each washing. The gel was finally dispersed in a further 400 ml. of buffer and allowed to equilibrate overnight at 0°. Prior to use, the suspension was sedimented by centrifugation at 1000 x g for 15 minutes and the supernatant liquor was discarded.

To the packed gel contained in the centrifuge bottle was added the

AS 4.7 Fraction and the gel was dispersed manually. Manual stirring was continued intermittently over a 5 minute period after which the mixture was allowed to stand in the cold for 20 minutes to complete the adsorption. The gel was then packed tightly in the centrifuge bottle by centrifugation at 1500 x g for 15 minutes and the supernatant fluid was decanted into a precooled measuring cylinder. The volume was noted and, after the removal of samples for estimation of protein and measurement of TMP kinase activity, the main bulk of the solution was lyophylised and stored at -50°. Measurement of TMP kinase activity required pretreatment of the preparation on a column of Sephadex G-25 in order to separate the enzyme from TMP. The fraction obtained by the C γ -alumina treatment was designated the C γ AS 4.7 Fraction and contained 60 - 80 per cent of the total kinase activity of the AS 4.7 Fraction while practically no TdR kinase or TDP kinase activity could be detected. The TMP kinase so obtained was purified 80 - 100-fold over the crude ascites extract.

Fractionation on Sephadex G-100:- The lyophylised C γ AS 4.7 Fraction was carefully extracted with 3 - 4 ml. of 0.01 M phosphate buffer, pH 7.6, and any insoluble material was removed by centrifugation at 2000 x g for 20 minutes. The enzyme solution so prepared contained 30 - 40 mg. protein per ml., 2 ml. of which was transferred to a column of Sephadex G-100 (95 x 1 cm., dia.) previously prepared and equilibrated with ice-cold 0.01 M phosphate buffer, pH 7.6, as described elsewhere (Section 3.2.4 (c)), the remainder of the enzyme solution being used for the estimation of protein and measurement of the TMP kinase activity by the radioactivity assay (Section

3.2.3 (b),. The void volume of the column had previously been determined by the volume required to move a sample of γ-globulin from the top to the bottom of the column and was generally found to be 25 - 30 ml.

Gradient elution with 0.01 M phosphate buffer, pH 7.6, was started after all the enzyme solution had passed into the column bed and proceeded at a flow rate of 15 - 20 ml. per hour, fractions of 2.0 ml. being collected from the point where a volume corresponding to V_0 had appeared in the effluent Samples were withdrawn from each fraction and assayed for TMP kinase activity. A representative chromatographic profile is given in Fig. 38 from which it is seen that TMP kinase activity appears in the effluent as an irregularly shaped peak some time after the appearance of the main protein peak. 30 Fractions were collected in this way and the six tubes of the TMP kinase peak with the highest specific activity were combined and designated the SG γ AS 4.7 Fraction The kinase activity of such fractions was sufficiently high to merit the use of the spectrophotometric assay for TMP kinase (Section 3.2.3 (e)). However, the use of this assay technique revealed also that superimposed upon the TMP kinase peak there was a highly active ATPase which, when measured with the spectrophotometric procedure given in Section 3.2.3 (e), was shown to hydrolyse ATP at the rate of 2 µmoles per 25 minutes per mg. protein. Nevertheless, 40 - 60 per cent of the total TMP kinase activity in the SG γ AS 4.7 Fraction was recovered in the SG γ AS 4.7 Fraction purified 1000-fold over the crude extracts and having a specific activity of 0.6 - 0.9 µmoles TMP phosphorylated per 25 minutes per mg. protein. No TDP kinase could be detected in this preparation, neither was there any evidence of phosphorylation of TdR.

Fig 38

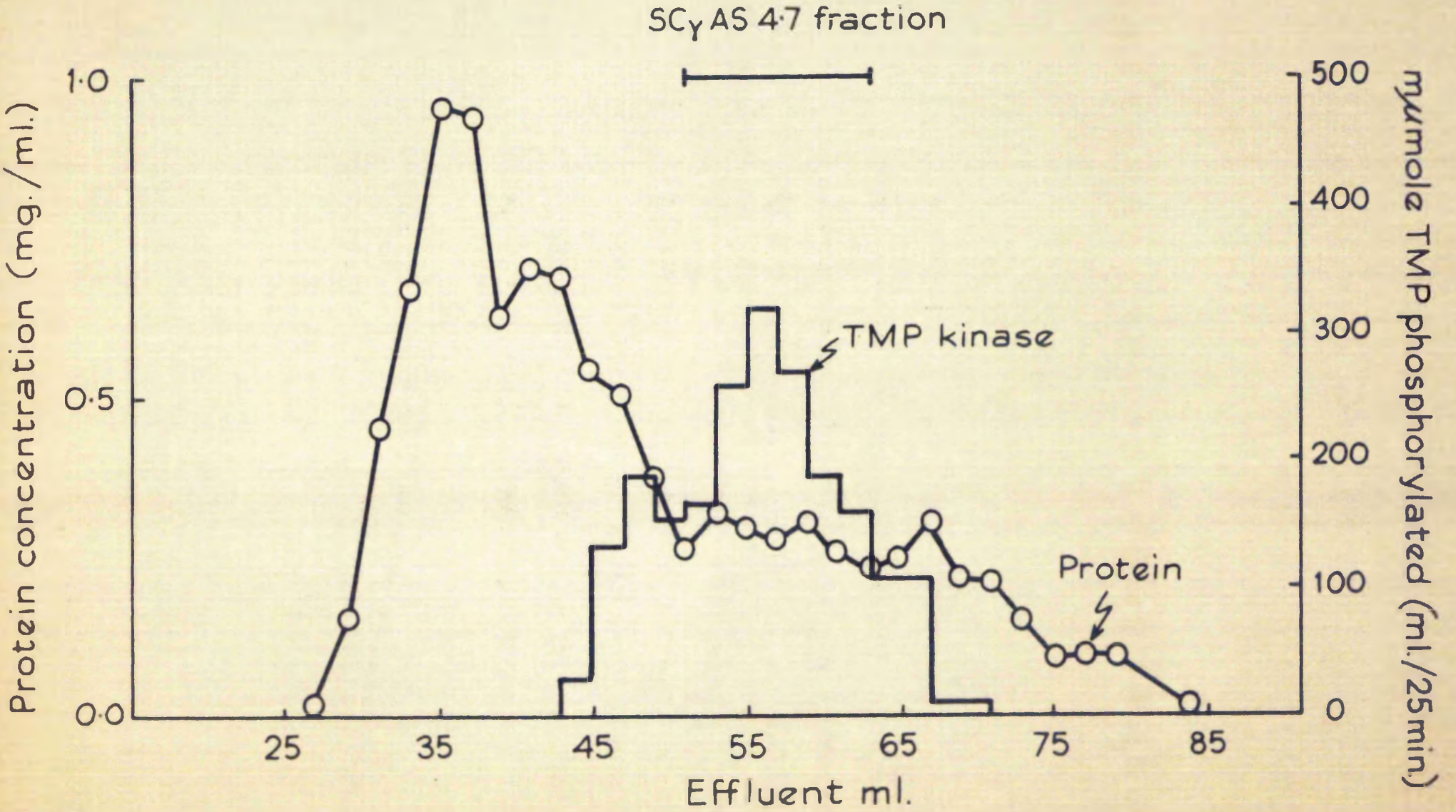

SCγ AS 4·7 fraction
1·0
0·5
0·0
Protein concentration (mg./ml.)
500
400
300
200
100
0
mμmole TMP phosphorylated (ml./25min.)
TMP kinase
Protein
25
35
45
55
65
75
85
Effluent ml.

Fig. 38. Chromatography of the G$_\beta$ AS 4.7 Fraction on a column of Sephadex G-100 dextran gel (90 x 1 cm., dia.).

The eluant was 0.01 M phosphate buffer, pH 7.6, and the details of the fractionation were those described in the text (Section 3.3.5; Method I) and the preparation and equilibration of the dextran column were given in Section 3.2.4 (c). The column V_o was 27 ml. TMP kinase activity was measured with the spectrophotometric assay (Section 3.2.3 (c)) and the fractions within the brackets were combined and constituted the SG$_\beta$ AS 4.7 Fraction.

However, because of the presence of ATPase as a contaminant in the SCγAS 4.7 Fraction, it seemed possible that the ATPase or some other phosphatase present in the preparation was capable of hydrolysing TTP. If this were so, no TTP would be expected to appear among the reaction products in assays wher (^{32}P) TMP was used as substrate since the activity of a possible TDP kinase would be masked by dephosphorylating activity, the TTP being reconverted to TDP as quickly as it was being formed. Several experiments in which this possibility was investigated have shown that no such TTP dephosphorylating activity (Section 3.8.3 (e)) was present in the SCγAS 4.7 Fraction (see Table 13).

The purification of TMP kinase by this method thus demonstrated that the kinase responsible for the phosphorylation of TMP to TDP was a discrete enzyme and was separable from the TdR kinase and TDP kinase responsible, respectively, for catalysing the initial and the ultimate steps in the formation of TTP from TdR. The serious contamination of the TMP kinase with ATPase, unfortunately, minimised the usefulness of the SCγAS 4.7 Fraction as a source of TMP kinase for future kinetic characterisation.

The over-all results of the purification scheme are summarised in Table 11.

(b) Method II

Another type of purification scheme was developed with the express purpose of eliminating phosphatase contaminants from the TMP kinase. In the scheme described below, the initial step was identical to that employed in Method I, more care being taken, however, to elucidate the fate of the TdR

Table 11.

Enzyme fraction	Units per ml.	Total volume ml.	Total no. of units	Protein concentration mg. per ml.	Total amount of protein mg.	Specific activity units per mg.	Purification
Crude, soluble extracts	2.34	3335	7800	3.52	9510	0.82	1
Redissolved lyophylised crude extract	17.5	410	7000	14.12	5650	1.25	1.5
AS 4.7	19.4	400	7950	6.04	2475	3.21	4
CYAS 4.7	17.7	423	7500	0.22	91	82.4	100
SCYAS 4.7	415	10	4150	0.51	5	950	1150

Table 11. Purification scheme for TMP kinase derived from cell-free extracts of Landschutz ascites tumour cells (Method I).

TMP kinase activity was measured using the radioactivity assay (Section 3.2.3 (b)) throughout, and the values obtained by this means for the SC/AS 4.7 Fraction were checked against values obtained using the spectrophotometric method for the measurement of TMP kinase activity (Section 3.2.3 (c)). Good agreement between the two methods was obtained with purified TMP kinase preparations while there was considerabl divergency with more impure preparations.

and TDP kinases in the course of the enzyme fractionations.

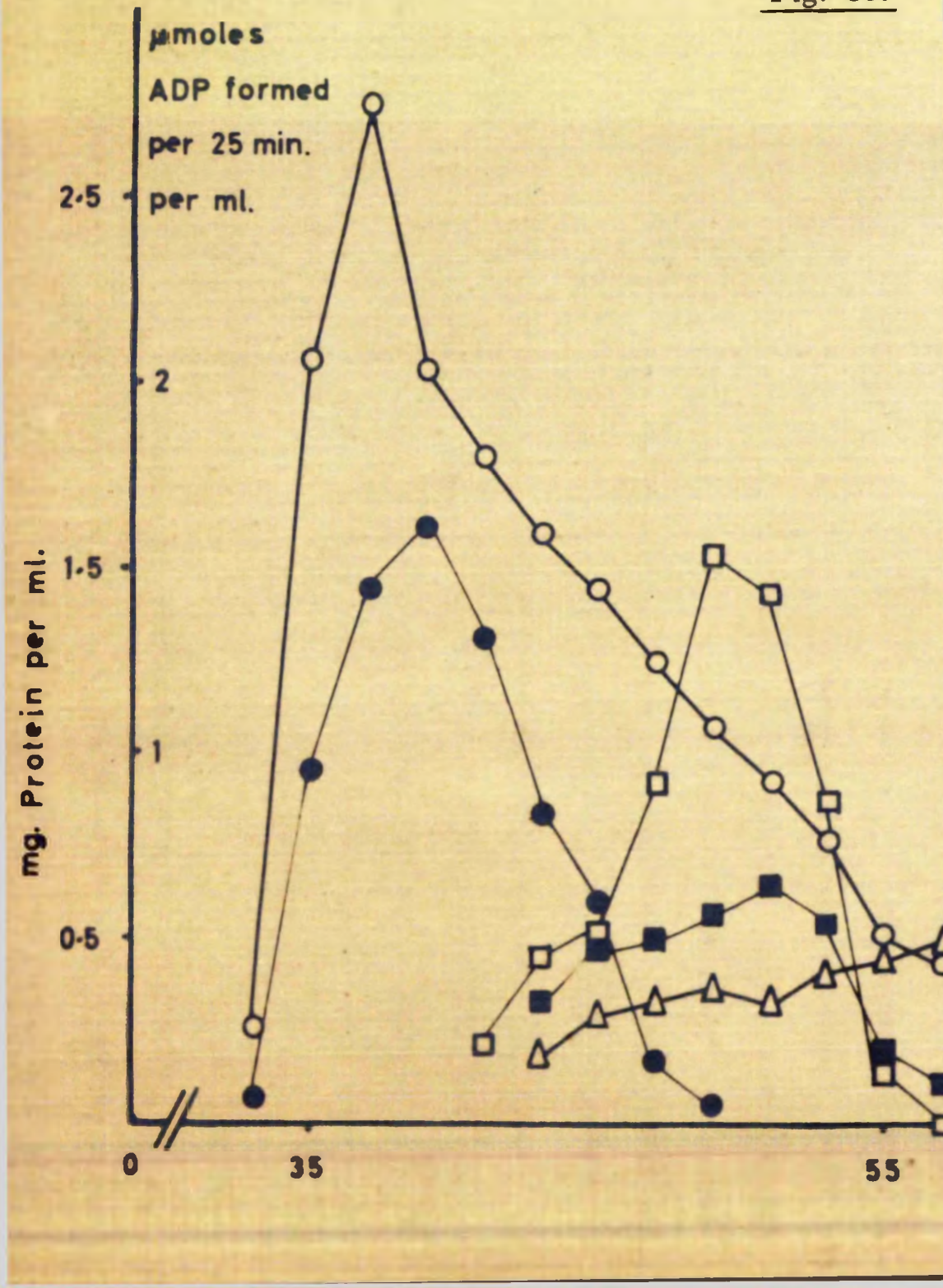
Fractionation with dilute acetic acid:- Lyophylised crude extract was dissolved in 0.01 M phosphate buffer, pH 7.6, and centrifuged to remove residual protein. To the supernatant fluid, containing 12 - 14 mg. protein per ml., was added TMP to a concentration of 80 mumoles per 1.5 mg. protein and fractionation with 1.0 N acetic acid was carried out as described above. The two fractions obtained by lowering the pH to 4.7 were preserved and immediately neutralised with the addition of 1.0 N sodium hydroxide. Samples were withdrawn from each of the fractions for the estimation of protein and measurement of kinase activities, the TMP being removed by treatment with Sephadex G-25 (Section 3.2.4 (c)) prior to measurement of TMP kinase activities. The main bulks of the two Fractions (AS 4.7 and AR 4.7) were lyophylised and stored at -50°. The AS 4.7 Fraction contained 120 per cent of the total initial TMP kinase activity purified 6 - 8-fold over the crude extracts together with 80 per cent of the total TdR kinase activity purified 2 - 3-fold and 5 per cent of the total initial TDP kinase activity. The AR 4.7 Fraction when similarly assayed for kinase activities, was shown to contain 25 per cent of the total initial TMP kinase activity, 40 per cent of the TdR kinase activity and 10 per cent of the TDP kinase activity of the lyophylised crude extracts. Thus some 80 per cent of the TDP kinase activity was destroyed by the acid treatment while the recoveries of TdR and TMP kinase activities exceeded the measured values of the lyophylised crude extracts by 20 and 80 per cent, respectively.

Fractionation on Sephadex G-100:- Lyophylised AS 4.7 Fraction (containing

TMP) was dissolved in 3 - 4 ml. of 0.01 M phosphate buffer, pH 7.6, and centrifuged, if necessary, to remove any residual protein. After portions had been reserved for the estimation of protein and measurement of kinase activities, 2.0 ml. of enzyme solution (containing 30 - 40 mg. protein per ml.) were run on to a column of Sephadex G-100 (90 x 1 cm., dia.) previously equilibrated with ice-cold 0.01 M phosphate buffer, pH 7.6 (see Section 3.2.4 (a)). The column void volume as measured with γ -globulin, was 30 ml.

Gradient elution was performed with 0.01 M phosphate buffer, pH 7.6, at a flow rate of 15 ml. per hour and fractions of 2.0 ml. were collected from the point where a volume corresponding to V_o had passed through the column. 30 Fractions were collected in this way, samples of each being withdrawn for estimation of protein and measurement of kinase activities. The remainder of each fraction was lyophylised and stored at -50°. No treatment with Sephadex G-25 was necessary prior to the TMP kinase assay since TMP was strongly retained on the Sephadex G-100 dextran gel and only appeared in the column effluent after some 200 ml. of eluant had been applied. A representative chromatographic profile incorporating effluent protein concentration and the results of assays of TdR kinase (Section 3.2.3 (a)), TMP kinase (Section 3.2.3 (b)), TDP kinase (Section 3.2.3 (b)) and ATPase (Section 3.2.3 (c)) is shown in Fig. 39. Three peaks of kinase activity were obtained. The first, representing TdR kinase, appeared in the effluent superimposed on the main protein peak and the second contained TDP kinase contaminated with ATPase and a small amount of TMP kinase accounted for by slight overlapping of the third peak of TMP kinase activity. The third

Fig. 39.


µmoles
ADP formed
per 25 min.
per ml.
2·5
2
1·5
1
0·5
mg. Protein per ml.
0
35
55

TdR kinase
activity
mμmoles TDP or TTP formed per 25 min. per ml.
10
5
40
20
Effluent (ml.)
75

Fig. 39. Chromatography of the AS 4.7 Fraction on a column of Sephadex G-100 dextran gel (98 x 1 cm., dia.).

The eluant was 0.01 M phosphate buffer, pH 7.6 and the details of the fractionation were those described in the text (Section 3.3.5; Method II). Fractions of 2.0 ml. were collected at 2° and at a flow rate of 15 ml. per hour. TdR kinase was measured as described earlier (Section 3.2.3 (a)) with (^{3}H) TdR as substrate and the results recorded as the percentage of total radioactivity recovered in (TMP + TDP + TTP) per ml. of effluent after an incubation period of 15 minutes. TMP kinase activity was measured by the radioactivity assay (Section 3.2.3 (b with (^{32}P) TMP as substrate. TDP kinase was measured by the use of paper chromatographic techniques (Section 3.2.3 (b)). ATPase activity was determined by the spectrophotometric assay given in Section 3.2.3 (c). The fractions containing TMP kinase uncontaminated with TDP kinase activity (after 64 - 75 ml. effluent had been collected) were combined and designated the SAS 4.7 Fract

Protein : ○

ATPase : □

TdR kinase : ●

TMP kinase : △

TDP kinase : ■

peak contained TMP kinase and the six fractions of thehighest specific activity were combined and designated the SAS 4.7 Fraction. The recovery of TMP kinase activity as represented by the SAS 4.7 Fraction was 40 per cent of that applied to the column which contained TMP kinase purified 60-fold over the crude ascites extracts. Moreover, the TMP kinase so obtained was capable of phosphorylating TMP to TDP only, thus indicating the absence of contamination with TDP kinase; neither was there any evidence of TdR kinase activity in this preparation. However, as (^{32}P) TMP was used as substrate for the measurement of TMP kinase (and TDP kinase), the absence of TTP from the reaction products could be due to contamination of the SAS 4.7 Fraction with nucleoside triphosphate dephosphorylating enzymes. No such dephosphorylating activity was found when ATP was omitted from the reaction mixtures and (^{32}P) TTP replaced (^{32}P) TMP as substrate as recorded in Table 1
A peak of ATPase activity, as measured by the spectrophotometric assay (Section 3.2.3 (e)), appeared in the effluent preceding the TMP kinase peak. There was evidence also of apparent tailing off of ATPase into the SAS 4.7 Fraction as samples taken from the latter Fraction catalysed a slight drop in the extinction at 340 mμ when incubated with the ATPase assay mixture. Subsequent experiments, however, showed that the oxidation of $NADH_2$ by the SAS 4.7 Fraction was independent of ATP and due, possibly, to flavoprotein contamination. It must be emphasised also that the TDP kinase as measured in the effluent need not represent more than a small proportion of the total TDP kinase present as no account was taken of possible TDP kinase activity appearing in the chromatogram independently of TMP kinase activity. The

progressive removal of TDP kinase from TMP kinase in the course of the purification is demonstrated in Fig. 40 where paper chromatograms of reaction products run at different stages of the purification of TMP kinase show the gradual disappearance of biosynthetically formed TTP.

The over-all results of the purification scheme are summarised in Table 12.

Fig. 40

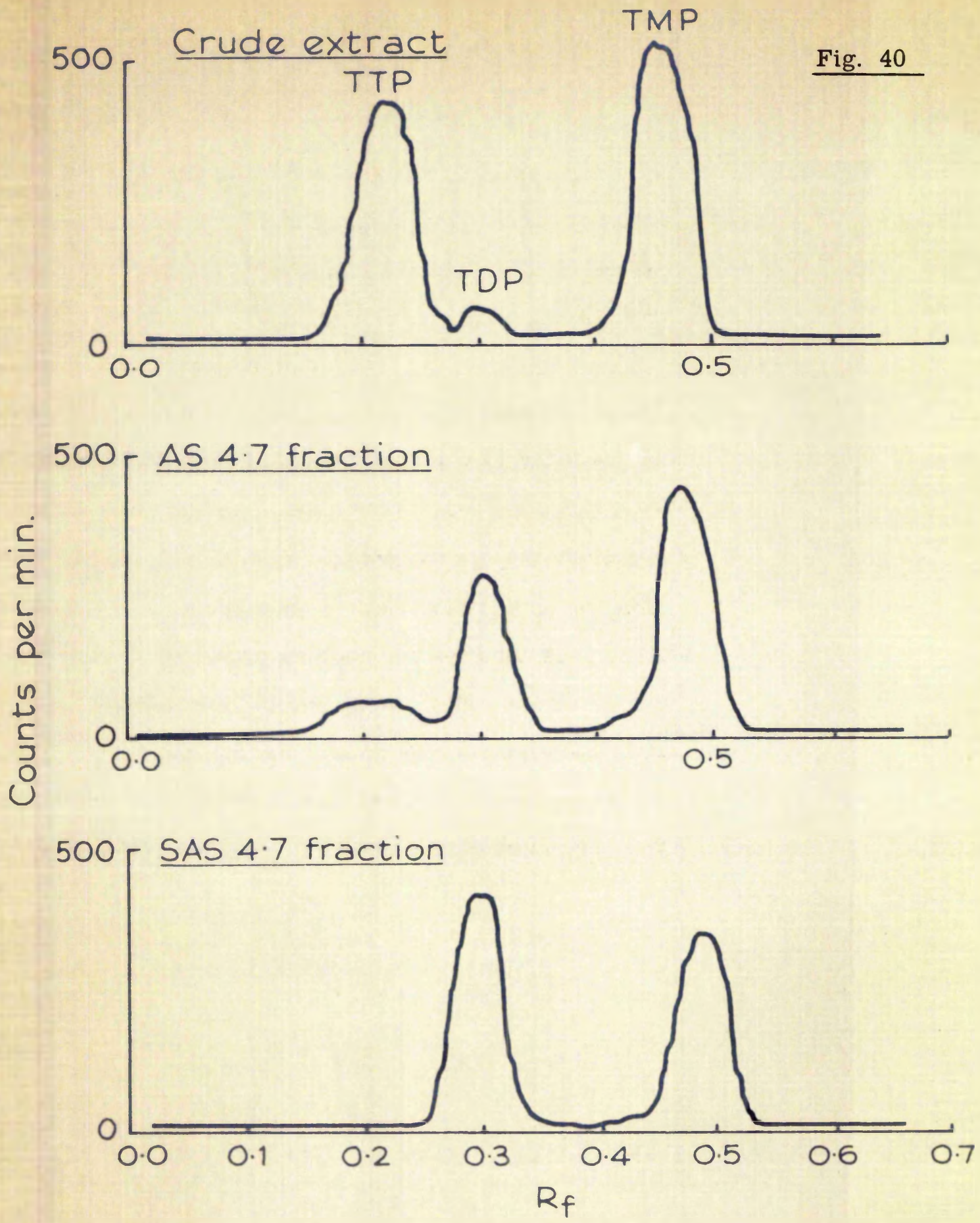

Crude extract
500
TTP
TDP
TMP
0
0·0
0·5
AS 4·7 fraction
500
0
0·0
0·5
SAS 4·7 fraction
500
0
0·0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
Rf
Counts per min.

Fig. 40. Paper chromatographic separation of reaction products obtained in the phosphorylation of (^{32}P) TMP by enzyme preparations at various stages of the purification of TMP kinase.

Enzyme experiments with (^{32}P) as substrate were carried out as described in the text (Section 3.2.3 (b)) followed by submitting the reaction products to descending paper chromatography in isobutyric acid/distilled water/ammonia EDTA (Section 2.2.3 (b)). Appropriate (^{32}P) -containing lanes were cut from the chromatograms and scanned for radioactivity using the Nuclear-Chicago Actigraph windowless gas-flow detector (Section 2.2.3 (d)). Marker compounds included in the chromatograms are shown. The sources of enzyme were as given on the diagram.

Table 12.

Enzyme fraction	Units per ml.	Total volume ml.	Total no. of units	Protein concentration mg. per ml.	Total amount of protein	Specific activity units per mg.	Purification
Crude, soluble extracts	1.91	75	143	3.01	227	0.63	1
Redissolved, lyophylised crude extracts	11.68	10	117	12.3	123	0.95	1.5
AS 4.7	13.41	10	134	3.87	387	3.46	5.5
SAS 4.7	8.47	12	101	0.23	2.7	37.2	60

Table 12. Purification scheme of TMP kinase derived from cell-free extracts of Landschutz ascites tumour cells (Method II).

TMP kinase activity was measured throughout using the radioactivity assay (Section 3.2.3 (b)).

Table 13.

Enzyme fraction	Per cent total radioactivity recovered	Per cent total radioactivity in thymidine nucleotides			TDP form mμmoles per 25 min.
		TTP	TDP	TMP	
Control (- enzyme; not heated)	95	95.5	4.5	-	0.90
Control (-enzyme; heated)	90	95.5	4.5	-	0.90
Control (+ enzyme)*	92	96	4	-	0.82
$SC_{\gamma}AS$ 4.7 Fraction					
fraction 9	95	96	4	-	0.82
10	90	96	4	-	0.82
11	89	96.5	3.5	-	0.73
SAS 4.7 Fraction					
fraction 18	91	97	3	-	0.6
19	93	95.5	4.5	-	0.9
20	90	96	4	-	0.82

* protein (0.5 mg.; fraction 10) of the $SC_{\gamma}AS$ 4.7 Fraction boile for 2 minutes before addition.

** calculated on the basis of the total radioactivity present ini tially.

Table 13. Determination of phosphatase activities in fractions eluted from columns of Sephadex G-100 in the course of TMP kinase purification.

Reaction mixtures were prepared containing the following components in a total volume of 1.0 ml.:- 5µmoles $MgCl_2$, 100 µmoles tris buffer, pH 7.6, 20 mµmoles (^{32}P) TTP of specific activity 10^7 counts per minute per µmole and 0.5 mg. protein from fractions eluted from columns of Sephadex G-100 comprising the 30% AS 4.7 Fraction (Method I) and the SAS 4.7 Fraction (Method II). Incubation was for 25 minutes at 37° and further treatment was as described for the radioactivity assay of phosphatases (Section 3.2.3 (
The results were calculated as the number of mµmoles TTP being hydrolysed in 25 minutes.

CHAPTER IV.

GENERAL DISCUSSION

4.1. The mechanism of formation of thymidine 5'-triphosphate from thymidine and thymidine 5'-monophosphate by enzymes from Landschutz ascites tumour cells

It is now widely accepted that the replicative synthesis of DNA shows an absolute requirement for the 5'-triphosphates of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine and that the synthetic reactions leading to the formation of these precursors are so controlled that they are produced in the correct amount, at the correct time and at the appropriate site. It seems equally certain that in the ultimate stages these mechanisms are identifiable with a set of deoxyribonucleotide phosphokinases, each component of which responsible for the phosphorylation of one of the deoxyribonucleoside 5'-monophosphates dAMP, dGMP, dCMP and TMP to their respective 5'-triphosphates. Of the deoxyribonucleotide phosphokinase systems, that concerned with the phosphorylation of TMP (and TdR) to TTP has been singled out for special attention, the uniqueness of this system being derived partly from the chemical dissimilarity of its substrates to ribonucleotide counterparts and partly from its powerful potential as a regulator of DNA synthesis and hence of cell division.

The present investigation has shown that cell-free extracts of Landschutz ascites tumour cells enclose the full complement of phosphokinases required to phosphorylate thymidine and thymidine 5'-monophosphate to thymidine 5'-triphosphate. Pertinent questions were asked as to the precise sequence of events in this phosphorylation process and several types of experiment were carried out with the express purpose of gauging the maximum potentialities

of the system. As a result, the conclusion can now be confidently drawn that the phosphorylation of TdR to TTP proceeds through the intermediates TMP and TDP. This conclusion was based on two independent lines of evidence; firstly the kinetic experiments performed with cell-free extracts of Landschut ascites tumour cells provided strong evidence for the role of TDP as an intermediate in the formation of TTP from TdR and TMP (Section 2.3.2) and secondly, the identification and resolution of three phosphokinases derived from such extracts, each responsible for catalysing one of the phosphorylations TdR to TMP, TMP to TDP and TDP to TTP (Sections 3.3.2., 3.3.4 and 3.3.5).

It is important, at this point, to consider to what extent these general conclusions are justified by the specific evidence presented, immediate implications of which have been noted elsewhere (Sections 2.3 and 3.3). It seems necessary also to reiterate some of the limitations inherent in the experimental techniques employed, particularly those concerned with the kinetics of thymidine and thymidylate kinase action. Thus, the reaction rate measurements invariably reflect net phosphorylation and, when operated under optimal conditions, represent the optimal net rate obtainable with the most favourable balance of phosphorylating and dephosphorylating activities. It follows that the formation of TTP from TdR, in general, and the individual intermediate reactions, in particular, may not have realised their maximum potential and the purified component enzymes may well exhibit different optimal requirements from those given here (Sections 2.3.1., 3.3.2 and 3.3.4).

From the time course experiments with (^{3}H) TdR and (^{32}P) TMP (Figures 22, 23 and 26), it was clear that labelled TDP was invariably found in the

reaction products prior to labelled TTP and the low relative radioactivity in TDP maintained throughout the incubations was consistent with the behaviour expected of a transient intermediate. These results also suggested other points. Examination of, for example, Fig. 22 indicates that the rate of formation of TDP from TMP was maximal when the concentration of TMP in the reaction mixtures was maximal also and the subsequent fall in the TMP concentration may suggest that the TMP kinase requires higher substrate concentration for full saturation than do the TdR and TDP kinases. Furthermore, from the experiments with (^{32}P - α) TDP (Fig. 24; Table 3) and (^{32}P - β) TDP (Fig. 25), it is clear that TTP was formed very rapidly without the intermediate formation of TMP or higher phosphates of TdR (or TMP) than TTP.

These conclusions are clearly consistent with the hypothesis of Weissman, Smellie and Paul (1960) that formation of TTP from TdR proceeds through TMP and TDP when catalysed by enzymes derived from cell-free extracts of Ehrlich ascites tumour cells. The enzyme fractionation experiments of those authors had indicated the existence of three different phosphokinases, each responsible for one step in the formation of TTP from TdR. Consistent with those findings is also the partial separation of TMP phosphokinase and TDP phosphokinase by the centrifugation of extracts of calf thymus in sucrose density gradients (Bojarski, 1962).

That the initial step in the formation of TTP from TdR is the phosphorylation of TdR to TMP has for some time been considered beyond dispute. Thus Bollum and Potter (1959) demonstrated a clear precursor-product relationship between TdR and TMP in time course experiments with extracts of

regenerating rat liver. The strongest evidence for the identity of the initial reaction, however, came from enzymological studies on TdR kinase which, by virtue of its relatively higher stability, was readily separable from the thymidylate kinases (see Sections 3.3.2 and 3.3.3; Figures 32 and 39). These conclusions were further supported by the findings of Ives, Morse and Potter (1963) using extracts of Novikoff hepatoma and, recently, by the purification of a TdR kinase from E.coli (Okazaki and Kornberg, 1964a). All these preparations have been shown to catalyse the phosphorylation of TdR to TMP without also forming thymidine polyphosphates.

While the initial reaction, catalysed by TdR kinase, is thus well established and characterised, the sequence of events in the phosphorylation of TMP resulting in the formation of TDP and TTP remains a matter of considerable argument. This existing uncertainty has not been alleviated by the fact that several of the reported investigations have neglected to make a precise distinction between the types of product formed by the action of the thymidylate kinases on TMP, one reason for this omission being the failure of the methods used to separate adequately the reaction products. Thus it has become customary to express the products of thymidylate kinase action as (TDP + TTP) (Bessman, 1959; Bollum and Potter, 1959; Maestavinos and Canellakis, 1959; Kielley, 1961, 1963a, b; Ives, Morse and Potter, 1963). Little valid information on the sequences of the individual reactions could be deduced from experiments using this approach. Some of the other studies with a direct bearing on the mechanism of the phosphorylation of TdR and TMP to TTP suffer from similar analytical deficiencies. It was therefore

necessary to exercise some caution in comparing the results of such studies with those presented here (cfr. resolution of thymidine nucleotides Section 2.2.4 (a); Fig. 17).

The general conclusion that TDP is an intermediate in the formation of TTP from TdR and TMP has been supported by experiments on HeLa cells and HeLa cells infected with the virus of cowpox (McAuslan and Joklik, 1962). In experiments on the time course of phosphorylation of (^{14}C) TMP with enzyme preparations from HeLa cells, radioactive TDP was detected prior to the formation of any TTP and the results thus resembled the pattern obtained with extracts of Landschutz ascites carcinoma (Figures 22, 23 and 26). Moreover, the percentage of total radioactivity in TDP always exceeded that in TTP as expected from steady state tracer kinetics and it was also shown that the accumulation of radioactivity in TDP from (^{14}C) TMP was independent of the addition of a 5-fold excess of unlabelled TDP. While these results all point to the reaction sequence TMP $\longrightarrow$ TDP $\longrightarrow$ TTP, an unequivocal conclusion does not appear justifiable from the evidence presented by McAuslan and Joklik (1962). Thus, for the reasons stated in Section 2.3.2, it does not seem conceivable that a closed <u>in vitro</u> system such as that employed by McAuslan and Joklik (1962) could approach the steady state claimed by these workers and, furthermore, measurements of specific radioactivity were not performed in any of the experiments quoted. The most acceptable evidence comes from the experiment in which the time course of phosphorylation of (^{14}C) TMP was studied in presence and absence of added, unlabelled TDP. The significance of this experiment lies in that if TDP were an intermediate in

the formation of TTP from TMP, then the addition of unlabelled TDP would be expected to result in significant reduction of TTP specific activity (as was in fact observed). However, the alternative mechanism of Bianchi, Butler, Grathorn and Shooter (1961) postulates the sequence TMP $\longrightarrow$ TTP $\longrightarrow$ TDP and it seems possible that the results observed by McAuslan and Joklik (1962) could be explained in terms of this sequence depending on the relative rates of the backward reactions. In the present investigation, crude cell-free extracts of ascites tumour cells were found to contain dephosphorylating enzymes acting on TDP and TTP and a experiment of the type quoted above could therefore not, by itself, be expected to provide valid evidence on the mechanism of formation of TTP. Somewhat surprisingly, McAuslan and Joklik (1962) state that nucleotidases acting on TDP and TTP were not present in the cell-free extracts of HeLa cells. This statement is unsupported by their evidence since phosphatase activity was being sought in the presence of ATP and thereby providing a regenerating system for thymidine nucleotide substrates.

Circumstantial evidence for the intermediate formation of TDP in the phosphorylation of TMP to TTP from a somewhat different precept has been provided by Delamore and Prusoff (1962) who studied the competitive antagonism of 5-iodo-2'-deoxyuridine (IUdR) of TdR phosphorylation under the influence of several human neoplastic tissues. This antagonism was presumed to operate by phosphorylation of IUdR to the corresponding 5'-mono-, 5'-di- and 5'-triphosphates and subsequent incorporation of IdUMP into DNA specifically in place of TMP. When the uptake of (^{3}H) TdR for DNA synthesis by

whole cells was used as a guide to the effect of IUdR, it was found that inhibition was directed towards specific steps in the phosphorylation of TdR depending on the type of cell employed. However, it was found with all the cell populations tested that the proportion of total radioactivity in TDP always exceeded that in TTP whether or not the phosphorylation was inhibited by IUdR and that IUdR had no effect on the ratio of radioactivity in TDP to that in TTP. This provided an indication that formation of TDP preceded formation of TTP and Delamore and Prusoff (1962) argued further that if TDP were not on the direct pathway for the biosynthesis of TTP, then the latter product must be particularly unstable in the system studied.

All the findings described so far are in direct contrast with those of Bianchi, Butler, Crathorn and Shooter (1961) who found, in experiments with cell-free extracts of regenerating rat liver, leukaemic mouse spleen and Ehrlich ascites tumour cells, that the formation of TDP was always preceded by accumulation of TTP. These observations led these workers to conclude that TTP was formed from TMP by a one-step reaction involving the addition of pyrophosphate and that TDP only arose by degradation of TTP by, presumably, phosphatase action. However, upon closer examination, it is apparent that this conclusion is kinetically untenable if based on the evidence presented.

Thus it is clear that the chromatographic procedure used by Bianchi, Butler, Crathorn and Shooter (1961) for the separation of thymidine nucleotides was inferior to the separation of such compounds on columns of ECTEOLA cellulose as employed in the present investigation (Fig. 17), particularly with regard to the resolution of TDP and TTP. Since at the best only small

amounts of TDP were detected at the early stages of time course experiments (Figures 22, 23 and 26), it seems quite possible that TDP might not have been detected in all the experiments of Bianchi, Butler, Crathorn and Shooter (1961). Moreover, close inspection of the time course experiments on the phosphorylation of (^{14}C) TdR as given by these authors revealed that inadequate data was presented for the radioactivity in TDP at time intervals of less than one hour in spite of the observation that little or no TdR remained in the reaction mixtures at the end of this time. Bianchi, Butler, Crathorn and Shooter (1961) admit that their results would be compatible with the reaction sequence: TdR $\longrightarrow$ TMP $\longrightarrow$ TDP $\longrightarrow$ TTP if the rate of phosphorylation of TDP to TTP were rapid compared with the rate of formation of TDP from TMP in which case the appearance of TDP would only be transient. However, the experiments which they carried out to investigate this possibilit seemed of doubtful value. Thus, in these experiments, the phosphorylation of TdR by diluted enzyme preparations was examined in order to slow down the initial stages of the reaction, no TDP being detected under these circumstance The conclusion that TDP was not an intermediate in the biosynthesis of TTP fro this data seems very questionable since it is difficult to believe that more dilution of the enzyme preparation would slow down the reaction leading from TDP to TTP to a greater extent than that leading from TMP to TDP. Another explanation which may be offered for the failure to detect TDP, at early time intervals, is that the crude extracts used by Bianchi, Butler, Crathorn and Shooter (1961) contained, in addition to TMP kinase, a diphosphokinase capable of phosphorylation TDP to TTP. The inclusion of an ATP regenerating

system such as that used would tend to drive the reaction to the right and thereby favour the production of TTP at the expense of the accumulation of TDP. The presence of a diphosphokinase acting on TDP has been demonstrated in extracts of Landschutz ascites tumour cells (Figures 24 and 25) and this possibility has also been favoured by Bessman (1963).

From the results given in Section 2.3.2, it is clear that TMP and TTP were formed very rapidly from TdR and that TTP was formed very rapidly from TDP. Under these circumstances, it seemed possible that the formation of TDP from TMP was the rate limiting step in the over-all reaction sequence, in which event TDP would only be expected to appear as a transient intermediat

It seems clear that the activity of the thymidine and thymidylate kinase system from Landschutz ascites carcinoma is markedly sensitive to accumulation of reaction products and hence the maintenance of a high rate of TTP formation is dependent on its efficient utilisation. This is in full agreement with the findings of Delamore and Prusoff (1962) that specific inhibition of DNA polymerase in Ehrlich ascites tumour cells and in calf thymus by IUdR led to a drastic reduction in the radioactivity recovered in TTP from (^{14}C) TdR. The negative feedback inhibition of TdR kinase by TTP demonstrated by Ives, Morse and Potter (1962, 1963) is of considerable importance in this connection and will be discussed later (see Section 4.3). In the present experiments, the presence of excess unlabelled TDP and TTP was found to inhibit markedly the formation of TTP from (^{32}P) TMP (Fig. 27) while it was not certain whether this effect was due to a general mass action inhibition by both TDP and TTP on TMP kinase or a more specific inhibition

by either TDP or TTP. It is of interest to note also that in phosphorylation experiments with (^{14}C) TMP by extracts of HeLa cells, the proportion of total radioactivity recovered in TDP was unaffected by the addition of a 5-fold excess of unlabelled TDP (McAuslan and Joklik, 1962) thus suggesting that the inhibition of TMP kinase observed in the present experiments might be due to TTP alone or to a combination of TTP with TDP.

Little is known as to whether the mechanisms here proposed are compatible with the patterns obtained in the intact cell. Only one investigation is on record in which the phosphorylation of TdR has been studied in vivo with simultaneous determination of the pool sizes of the thymidine nucleotides. The relationship between precursors and products in the spleen and thymus of the rat was studied by Potter and Nygaard (1963) following the intraperitoneal injection of (^{14}C) TdR to rats. The results of such experiments showed that in the spleen, the specific activity - time curves for the thymidine derivatives were compatible with the sequence:

$$\text{TdR} \longrightarrow \text{TMP} \longrightarrow (\text{TDP} \rightleftharpoons \text{TTP}) \longrightarrow \text{DNA}$$

Thus it was not possible to distinguish which of the thymidine polyphosphates, TDP or TTP, was the direct precursor of DNA - thymine and no simple precursor-product relationship was established between TMP, TDP and TTP as a result of very rapid equilibration of radioactivity between TDP and TTP.

In view of the complicating factors mentioned above and the possible interference from nucleotidases suggested by Bianchi, Butler, Crathorn and Shooter (1961), it seemed clear that significant evidence related to the

mechanism of formation of TTP would most readily be obtained if it were possible to separate and characterise the individual reaction steps. As stated elsewhere (Section 3.3.4), it seemed evident that the hypothesis claiming TDP as an intermediate in the formation of TTP from TMP would receive powerful support were it possible to demonstrate unequivocally the presence in the crude ascites tumour extracts of a phosphokinase capable of phosphorylating TMP to TDP.

The enzyme fractionation experiments of Weissman, Smellie and Paul (1960) had indicated the presence of a TMP kinase in cell-free extracts of Ehrlich ascites tumour cells and this prediction has been amply substantiated in the present investigation using cell-free extracts of the related Landschut ascites tumour cells (see Sections 3.3.4 and 3.3.5). Thus it was demonstrated that a phosphokinase responsible for the phosphorylation of TMP to TDP was present in the crude cell-free ascites tumour extracts, and several experiments indicated that the TMP kinase activity so measured was the property of a discrete protein separable from the TdR and TDP kinases (Figures 32 and 39). Two different types of purification procedure (Section 3.3.6) led to TMP kinase preparations purified 60-fold or 1000-fold over the crude ascites tumour extracts. Such preparations were shown to catalyse the transformation of TMP into TDP while being incapable of forming TMP from TdR or of forming TTP from TDP (Fig. 40). In one procedure, TMP kinase was purified by removing inactive protein by dilute acetic acid precipitation followed by adsorption of further inactive protein by treatment with C γ -alumina gel and, finally, by fractionation on a column of Sephadex G-100 dextran gel (Table 11).

While this preparation contained TMP kinase purified 1000-fold over the crude extracts, it was shown to contain also a highly active enzyme hydrolysing ATP. This, unfortunately, made the preparation unsuitable as a source of TMP kinase for stoichiometry experiments and for kinetic elucidation of the reaction leading from TMP to TDP. However, the ATPase was shown to be incapable of dephosphorylating TTP (Table 13) so that the appearance of TDP as the end product of the reaction could not have been caused by a nucleotidase mediated degradation of TTP.

The second procedure led to a TMP kinase preparation purified 60-fold over the crude extracts by acid precipitation (as above) followed directly by fractionation on a column of Sephadex G-100 (Table 12). The treatment with Sephadex G-100 dextran gel proved of particular significance in that it provided a means of separating the relevant kinase activities on the basis of the molecular weight of their constituent proteins. The TMP kinase preparation so obtained was free of TdR kinase, TDP kinase and ATPase, all of which activities appeared in the effluent prior to the TMP kinase activity (Fig. 39). According to Flodin (1962), polymers of molecular weight exceeding 100 000 will pass through a column of Sephadex G-100 unhindered while polymers having a molecular weight of less than 100 000 will exhibit partition coefficients for the gel of between zero and one. An approximate measure of the molecular weight of a particular protein may thus be obtained with the aid of appropriate marker polymers. In the present experiments, the TMP kinase was observed to appear in the effluent imperfectly separated from the haem containing band of crude sheep blood haemoglobin and a molecular

weight of 50 000 - 60 000 may therefore be tentatively ascribed to the partially purified TMP kinase. By the same token, the TdR kinase would appear to have a molecular weight exceeding 100 000 while the extremely low levels of TDP kinase activity present at this stage of the purification made its precise position on the chromatograms somewhat more uncertain.

TdR kinase has likewise been purified (4 - 5-fold) by virtue of the greater stability of this enzyme in dilute acid media under which circumstances the TMP and TDP kinase activities appear to be destroyed (Fig. 38; Table 6). The TdR kinase obtained after removal of inactive protein by dilute acetic acid precipitation was shown to catalyse the formation of TMP from TdR without being capable of phosphorylating TMP to TDP and TTP.

The instability of the thymidine and thymidylate kinases in aqueous solution proved a major obstacle in the characterisation of those enzymes (see Section 3.3.3). However, the evidence was consistent with the view expressed by Weissman, Smellie and Paul (1960) that the kinases were not equally labile and it was generally found that in order of decreasing stability were TdR kinase, TMP kinase and TDP kinase. The addition of excess TMP to enzyme solutions was found to protect TMP kinase activity while being without effect on the TdR or TDP kinase activities. This protection of TMP kinase by its substrate originally predicted by Hiatt and Bojarski (1960) was thus confirmed. It is difficult to see what form the association of TMP kinase with TMP takes but presumably the specific protection of active groups on the enzyme surface is involved. The formation of a definite enzyme - substrate complex under these circumstances cannot be

excluded, but this seems unlikely since the stabilising effect of TMP was observed in the absence of ATP and Mg^{++} and whatever molecular event does occur, the TMP - TMP kinase association was shown to be freely reversible (see Fig. 36).

The properties of the TdR kinase of Landschutz ascites carcinoma generally concur with those ascribed to the Ehrlich ascites enzyme by Weissman Smellie and Paul (1960) although the TdR kinase in the present investigation did not respond to ammonium sulphate fractionation. However, the partially purified TdR kinase appeared to be different from the TdR kinase of Novikoff hepatoma (Ives, Morse and Potter, 1963) since it was more labile and showed different requirements for ATP and Mg^{++} (see Fig. 31). Comparison with the highly purified TdR kinase of E.coli (Okazaki and Kornberg, 1964a) was somewhat inappropriate owing to the exceptionally high reaction velocity of that enzyme; however, the pH optima of the two enzymes appeared to be similar as were the Mg^{++} requirements and the stabilisation with 2-mercaptoethanol. As found in the present investigation (see Fig. 32), Okazaki and Kornberg (1964a) showed that it was possible to separate the TdR kinase from TMP and TDP kinases by treatment with dilute acid and the purified E.coli enzyme was shown to catalyse stoichiometrically the reaction:

$$TdR + ATP \rightleftharpoons TMP + ADP$$

The TMP kinase had several properties which distinguished it from the non-specific nucleoside monophosphate kinase of Strominger, Heppel and Maxwell (1959). Thus the TMP kinase was very much more labile, had a lower reaction velocity and was destroyed by acetone and ammonium sulphate fractionations.

Similarly, the ascites tumour TMP kinase differed considerably from the E. coli TMP kinase described by Hurwitz (1959) in that acetone and ammonium sulphate fractionations resulted in serious loss of activity. The TMP kinase also appears to be distinct from the thymidylate kinase found in extracts of Novikoff hepatoma (Ives, Morse and Potter, 1963) which had different ATP and Mg^{++} requirements. Only one report is available in the literature of a TMP kinase which stoichiometrically catalyses the reaction:

$$TMP + ATP \rightleftharpoons TDP + ADP$$

Bello and Bessman (1963a) have described the purification of a new enzyme induced in E.coli following infection with the T2 bacteriophage. This enzyme was readily seperable from the thymidylate kinase of uninfected bacteria but, unlike the latter enzyme which appeared to be specific for TMP (Hurwitz, 1959), the newly formed TMP kinase was shown to be tri-functional and it was capable of phosphorylating TMP, dGMP and 5-MeOH-dCMP to the corresponding diphosphates, all of which activities appeared to be the property of single protein. Bello and Bessman (1963a) described the kinetic properties of this enzyme in some detail. Thus both ATP and dATP could serve as phosphoryl group donor, Mg^{++} or Mn^{++} were required for optimal activity and the enzyme had a broad pH optimum in the range 7.0 - 9.3. This latter property clearly distinguished it from the TMP kinase of Landschutz ascites tumour cells which exhibited a particularly sharp pH optimum at pH 7.6 (Fig. 34), and while the partially purified T2 bacteriophage-infected E.coli TMP kinase remained stable for several months at 4°, the Landschutz ascites tumour enzyme was destroyed in a matter of hours at this temperature unless

the enzyme solutions were supplemented with TMP. The substrate specificity of the TMP kinase was not studied in the present investigation, partly because of possible phosphatase contamination, but mainly because of the low absolute activity of the enzyme. It is somewhat sobering to note that the TMP kinase studied by Bello and Bessman (1963a) had a specific activity in the crude extracts some ten times higher than that of the TMP kinase purified 1000-fold from cell-free extracts of Landschutz ascites tumour cells. For similar reasons, the determination of K_m values for phosphoryl group donor and phosphoryl group acceptor must await further purification of the enzyme, but there is reason to believe that the TMP kinase shows absolute specificity for TMP (Weissman, Smellie and Paul, 1960; Hurwitz, 1959). In spite of the lack of kinetic information, the present instance appears to be the only TMP kinase from a mammalian source shown specifically to catalyse the formation of TDP from TMP, with the possible exception of the TMP kinase from calf thymus extracts (Bojarski, 1962) about which little is yet known.

The TDP kinase was not studied in detail but in so far as it was found to be extremely unstable (Sections 3.3.3 and 3.3.5), it resembles the enzyme from regenerating rat liver (Breitman, 1963) and that from extracts of Ehrlich ascites tumour cells (Weissman, Smellie and Paul, 1960). The TDP kinase was further characterised by its relatively high reaction velocity in the crude cell-free extracts (Fig. 25). Nucleoside diphosphokinases are generally regarded as exhibiting wide substrate specificity as is illustrated by the recently purified and crystallised enzyme from Brewers' yeast. This enzyme (Hetliff, Heaver, Lardy and Kuby, 1964) was shown to utilise as phosphoryl

group donors ATP, GTP, ITP, CTP, dATP and dCTP with ADP as acceptor and GDP, IDP, CDP and UDP as acceptors with dATP and ATP as donors; TDP was not tested with the enzyme. While the existence of a substrate specific TDP kinase in the cell-free extracts of Landschutz ascites carcinoma cannot be excluded, it seems rather unlikely in view of the large number of non-specific nucleoside diphosphokinases alluded to in extracts of mammalian, plant and bacterial cells (Bessman, 1963).

The demonstration that the extracts of Landschutz ascites tumour cells contain three separable phosphokinases, each responsible for one step in the reaction sequence: TdR → TMP → TDP → TTP does not of itself prove that this is the mechanism which obtains in the ascites tumour extracts or *in vivo*, it merely shows that all the enzymes required by such a mechanism are present *de facto* while alternative mechanisms are not precluded. Intensive purification and kinetic characterisation of the different kinases will be necessary in order to elucidate unambiguously the pathway for the formation of TTP from TdR. Nevertheless, the results given in Section 2.3 of kinetic experiments on the formation of TTP from TdR and TMP when augmented by the evidence presented on the separation of the TdR, TMP and TDP kinases and the purification of TMP kinase (Section 3.3).make an impressive case for a mechanism such as that proposed by Weissman, Smellie and Paul (1960) that the phosphorylation of TdR proceeds in a stepwise manner through TMP, TDP and TTP, in that order. The mechanism most consistent with the present evidence is given in Fig. 41.

There remains the question as to which, if any, of the reaction steps

Fig. 41

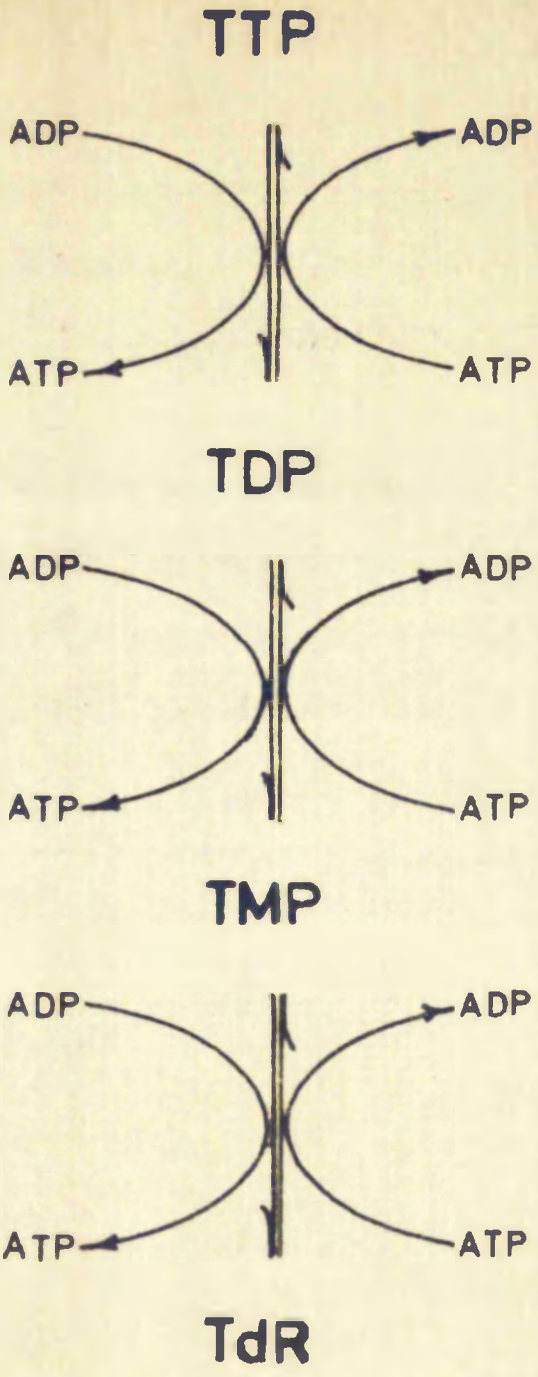


Fig. 41. The mechanism of formation of thymidine 5'-triphosphate as suggested by experiments with enzymes from Landschutz ascites tumour cells.

In the presence of the phosphoryl group donor, the phosphorylation of thymidine 5'-triphosphate precursors is essentially non-reversible and the over-all equilibrium favours production of thymidine 5'-triphosphate. With crude extracts of Landschutz ascites tumour cells, the reverse reactions are catalysed by nucleotidases.

in the formation of TTP from TdR (or TMP) is likely to be concerned in the regulation of such synthesis. The present evidence (Section 2.3.2) clearly favours the reaction catalysed by the TMP kinase as the rate limiting step in TTP biosynthesis. One of the ways in which this regulation may operate has been suggested by the different pH optima of the TdR kinase and the overall reaction, on the one hand, and of TMP kinase, on the other (compare Figures 18, 31 (C) and 34). Somewhat similar differences in the pH optima of the TdR and the thymidylate kinases were observed in extracts of Novikoff hepatoma (Ives, Morse and Potter, 1963). Conflicting evidence is available on this question elsewhere in the literature. Thus Bianchi (1962) has claimed that human tissues contain only small amounts of TdR kinase and that all human tissues convert TMP completely to TTP within 30 minutes; however, those experiments were carried out in the presence of an ATP regenerating system and with the tacit acceptance of the view that the phosphorylation of TMP to TTP occurred by pyrophosphate addition and the results could thus be due to the presence of extremely high TDP kinase activities in the cell-free extracts of human leukaemic tissue used. The conclusions of Bianchi (1962) were not supported by the experiments of Delamore and Prusoff (1962) in which several types of human leukaemic tissue were examined for their kinase activities. High TdR kinase activity was found by these workers in whole cells and the results were generally consistent with the possibility that the formation of TDP from TMP was rate limiting in the synthesis of TTP from TdR. If TMP kinase were the agent responsible for limiting the rate of TTP synthesis then useful confirmation might be expected from the infection of resting cells

with DNA-containing viruses or as a result of a sudden change in mammalian cell populations from a resting to a rapidly proliferating state (as in the regeneration of rat liver). As would be expected, an increased rate of production of TTP from TMP has been demonstrated as a result of these events. However, most of the reported investigations have neglected to take any account of intermediate reactions and in only one case has a detailed study of the relative activities of the TMP and TDP kinases been conducted. The infection of E.coli with T2 bacteriophage was shown by Bello and Bessman (1963a) to result in the induction of a new TMP kinase and the consequent rate of TTP production was greatly increased compared with that of uninfected bacteria while the TDP kinase activity remained undisturbed (Bello and Bessman, 1963b), yet the TMP kinase activity of the infected cells was only one-thirtieth of the TDP kinase activity of the uninfected cells. It seems possible to interpret these findings as an indication that high TDP kinase activities were already available in the uninfected cells while an increase in the TMP kinase activity was necessary to sustain the rate of TTP production required by the viral genome, thus also indicating that the TMP kinase activity was rate limiting in the formation of TTP from TMP in the uninfected cells. The rate limiting effect of TMP kinase in TTP biosynthesis may be a general phenomenon and if a regulating step were necessary in the formation of TTP from TdR or TMP, it is also the more likely in view of the generally accepted theory that de novo formation of thymidine nucleotides occurs by methylation of dUMP to TMP.

4.8. Intracellular location of the thymidine and thymidylate kinases

Current concepts on the replication *in vivo* of DNA seem to imply that such synthesis is restricted to sites located within the nuclei. During the period of interphase when DNA synthesis has been demonstrated by autoradiographic techniques such synthesis is localised and restricted to nuclear regions at a time when the nuclear membrane remains intact. However, recent studies on cellular morphology have shown that the nuclear membranes of several types of mammalian cells possess pores of measurable size, these findings being not inconsistent with the possibility that relatively large molecules may traverse the membrane under various circumstances. Indeed, the Jacob-Monod concept of the genetic control of protein synthesis stipulates the passage of metabolically unstable "messenger" RNA from nuclei-localised DNA to cytoplasmic microsomes. In bacteria, this type of transfer presents few structural problems as such organisms do not possess definable nuclear membrane structures while in mammalian cells such transfer would be governed by the permeability of the membrane barrier.

The current concepts on the synthesis of DNA appear to imply, further, that the formation of deoxyribonucleoside 5'-triphosphates is a sufficient as well as a necessary condition for such synthesis. The two types of process may therefore be intimately connected *in vivo* and it seems conceivable that the deoxyribonucleotide kinases and the DNA polymerase may be structurally linked so as to allow the passage of precursor material through an integrated chain of reactions in the course of DNA replication. This may not represent a true *in vivo* situation but, in the absence of firm alternatives, it is

intriguing to examine the available information on the intracellular location of the thymidine and thymidylate kinases in the light of the DNA polymerase distribution in mammalian cells.

In view of the relatively dependable evidence in favour of DNA synthesi being localised in conjunction with the chromosomes in the nucleus, it seems strikingly anomalous that the enzyme which is thought to be responsible for this process, DNA polymerase, should frequently be found in a highly active form in cytoplasmic extracts of mammalian cells (Smellie, Keir and Davidson, 1959; Bollum and Potter, 1959). One possible explanation for this observati was that this enzyme, as a nuclear constituent, had traversed the nuclear membrane in the course of the preparation of cytoplasmic extracts or that the isolation procedure employed sufficed to rupture a considerable proportion of nuclear membranes. The problem was investigated by Keir, Smellie and Siebert (1962) who compared the DNA nucleotidyltransferase activities in regenerating rat liver nuclei and cytoplasm isolated in non-aqueous media. The findings were interpreted as an indication of the existence of DNA polymerase in both nuclei and cytoplasm, the cytoplasmic enzyme activity being augmented by DNA polymerase leaching from the nucleus when such cellular fractionations were performed in aqueous media. The extreme solubility of the DNA polymerase thus indicated (Siebert, 1963) makes it unnecessary to stipulate a movement of DNA molecules back and forth from the nucleus for replicative DNA synthesis to occur. These conclusions were further amplified by the work of Smith and Keir (1963) who, working with calf thymus tissue, found that nuclei isolated in non-aqueous solvents retained a DNA polymerase of high specific activity

while the corresponding cytoplasmic extracts also contained measurable DNA polymerase activity. Thus it seems probable that the capacity and apparatus for the synthesis of DNA is not restricted to nuclear regions of the cell and that a certain proportion of the cellular DNA polymerase activity occurs in cytoplasmic localities also.

The question then arises as to what function is served by the cytoplasmic DNA polymerase. At least two possible answers have recently presented themselves. The first of these is the possible involvement of DNA polymerase with the synthesis of the cytoplasmic component of DNA which in mammalian cells apart from chick embryos, appears to be exclusively associated with mitochondria (Nass and Nass, 1963a, b). Chevremont (1962) has presented autoradiographic and cytochemical evidence for the incorporation of (^{3}H) TdR into mitochondrial structures of certain mammalian cells in tissue culture. However, as pointed out by Nass and Nass (1963b), the total amount of DNA-like material in the mitochondria of, for example, rat liver cells represents no more than 1 per cent of the total DNA content of those cells and, while a possible function for this component cannot be excluded, the present evidence appears to suggest that the mitochondrial DNA fibres are metabolically inert structures consistent with their being vestigial.

The second important possibility has suggested itself from the experiments of Littlefield, McGovern and Margeson (1963) who studied the changes in the intracellular distribution of DNA polymerase during the cell cycle of mouse fibroblasts. The results of such studies indicated that a proportion of the polymerase activity present in the cell-free cytoplasmic extracts in

the G_1 period, become associated with a particulate fraction when the cells entered the S period. The particulate fraction was presumably mainly nuclear and the observations thus suggest that cytoplasmic DNA polymerase enters the nucleus prior to, or in the course of, DNA synthesis, becomes particle associated and in this form catalyses DNA replication. However, as pointed out by Smith and Keir (1963), the interpretation of such results is made difficult by the presence in the homogenates and cell-free extracts of several types of nucleases which may be expected to degrade and fragment the added DNA primer in various ways and thus influence the measured polymerase activity.

While evidence regarding the intracellular location of the DNA polymerase is thus far from precise, even less is known about the location of the thymidine and thymidylate kinases. The present investigation provided little evidence with a bearing on this problem. Thus, while the kinases were obtained from cell-free extracts of Landschutz ascites tumour cells via a procedure which provided for the recovery of a mainly cytoplasmic fraction, no rigorous precautions were taken to exclude possible osmotic disruption of some nuclei. It seems clear also that all the reservations regarding the origin of the DNA polymerase in aqueous cytoplasmic extracts (Keir, Smellie and Siebert, 1962; Smith and Keir, 1963) apply with equal force to the present isolation of the thymidine and thymidylate kinase system. The possibility that the kinases studied were originally derived from the nuclei and appeared in the cytoplasmic extracts by passage across the nuclear membrane, therefore, not be excluded.

Although most of the evidence from other sources is of uncertain significance, several intriguing possibilities have emerged as to the location and function of the thymidine and thymidylate kinase system in mammalian cells. In most of the studies referred to above, the substrates for DNA polymerase action were provided in the form of (^{32}P) -labelled deoxyribonucleoside triphosphates, thus making the presence of kinases an unnecessary condition for incorporation. Several other types of study have been reported, however, in which incorporation of radioactive label into acid insoluble polynucleotides was investigated by the use of (^{3}H) TdR. Bekki and Schneider (1963) have examined the distribution of DNA polymerase activity in normal and regenerating rat liver and in Novikoff hepatoma using a polymerase assay which thus depended on the presence of thymidine and thymidylate kinases, and have shown that incorporation of (^{3}H) TdR was particularly effective with hepatoma nuclei isolated in non-aqueous solvents. In the case of the other cell types examined, <u>in vitro</u> incorporation was mainly confined to the cytoplasmic fractions. However, regenerating rat liver nuclei were capable of catalysing the rapid incorporation of (^{32}P) TMP from (^{32}P) TTP into acid insoluble polynucleotide material, this observation being interpreted as evidence of the absence of TdR, TMP and TDP kinases from the nuclear regions of such cells. The cytoplasmic origin of the kinases in several other types of mammalian cell has been circumstantially indicated. Of particular interest are the experiments of Bach (1962) who allowed HeLa cells to grow in a medium containing (^{3}H) TdR and found that the radioactivity became associated with a microsomal and a nuclear fraction, the label appearin

in the microsomes prior to its detection in the nuclei. Subsequent analysis showed that 10 per cent of the total DNA polymerase activity was associated with the microsomal fraction as measured by incorporation from (^{3}H) dGTP. While these experiments provided little direct evidence for the specific location of thymidine and thymidylate kinases, within the cytoplasm, the findings were consistent with the possibility that these enzymes also became associated with particles at some stage of the cell cycle. A different line of evidence with a bearing on this question has been provided by Billen (1963) who isolated a DNA polymerase - DNA complex from the nucleoprotein component of E.coli 15 T^{-}A^{-}U^{-}. This complex contained variable quantities of thymidine and thymidylate kinases as shown by incorporation from (^{32}P) TMP and (^{3}H) TdR. Centrifugation of the crude complex in a sucrose gradient brought about apparent dissociation of the polymerase activity and TdR kinase activity while part of the TMP kinase activity remained with the complex thus suggesting a difference in the "complex affinity" of the two kinases. It is significant also that no dAMP, dGMP or dCMP kinase activities were found in association with the DNA polymerase - DNA complex. The binding of thymidylate kinase in this way is not inconsistent with the conclusion of Firshein (1963) that DNA polymerase may have a much higher affinity for TTP than for dATP, dGTP or dCTP. Another type of evidence suggesting that thymidylate kinase may occur bound to cytoplasmic particles was provided by studies on mouse liver homogenates (Kielley, 1963a). While a considerable proportion of the TMP kinase activity of such homogenates appeared in a high-speed supernatant fraction, the resulting residue fraction upon freezing and thawing several

times released TMP kinase activity representing three times the total activity in the original supernatant fraction. It was observed, moreover, that the total TMP kinase activity thus recovered was nearly equal to that found in cell-free extracts of mouse ascites hepatoma. This evidence appears to suggest that part of the TMP kinase activity is structurally bound in an inactive form in non-proliferating tissues while in proliferating cells this component is released into the cytoplasm. A somewhat similar conclusion was reached by Bianchi, Orethorn and Shooter (1962) who studied the appearance of the thymidine and thymidylate kinases *in vivo* in rat liver following partial hepatectomy. If this were true, it should be possible to argue that the transformation of TMP kinase from a bound, inactive form to a soluble, active form occurs in response to specific events in the cell cycle, presumably at the time of DNA synthesis. How, thereafter, the TMP and TDP kinases co-operate (with TdR kinase ?) in the formation of TTP is largely a matter of speculation, but the sharp increase in the concentration of intranuclear deoxyribonucleotides which occurs in regenerating rat liver at the time of DNA synthesis (Behki and Schneider, 1962) together with the absence of thymidine and thymidylate kinase activities within such nuclei (Behki and Schneider, 1963) suggest that TTP may be produced in the cytoplasm and be subsequently transported to intranuclear sites of active DNA synthesis.

Such speculations, however, remain abstract in the face of the deficiencies in present day cell fractionation techniques and until vastly improved methods become available, there is little prospect of resolving problems of specific intracellular enzyme location in an unequivocal manner.

At present, relatively reliable information on the intracellular location of enzymes is confined to cases where electron microscopy or autoradiography can be successfully applied.

4.3. The possible involvement of the thymidine and thymidylate kinases in the control and regulation of DNA synthesis

The biosynthesis of DNA appears to be a process governed by two different types of control (see Section 1.4), one temporal in concept (Fig. 9) and the other quantitative. Several lines of evidence now available indicate (Lark, 1963) that each type of control operates in response to a different set of circumstances and that it may, therefore, be convenient to separate them conceptually. In the following discussion, it is proposed to refer to the temporal type as the control of DNA synthesis and to the quantitative type as the regulation of DNA synthesis.

That the formation of TTP may be rate limiting in DNA synthesis has been suggested by several studies in which the efficiency of endogenous deoxyribonucleotide kinase systems were compared in terms of the rates of formation of dATP, dGTP, dCTP and TTP. The findings have revealed striking differences in the ability of the deoxyribonucleotide kinases to phosphorylate dAMP, dGMP, dCMP and TMP, particularly when such studies were conducted on mammalian cells. Thus in normal rat liver, capability to phosphorylate TMP was found to be virtually absent (Canellakis, Jaffe, Manatavinos and Krakow, 1959; Gray et al., 1960) in agreement with the present findings (see Table 4), while the same tissue was found capable of efficient production of dATP, dGTP

and dGTP. Following-partial hepatectomy in the rat, the regenerating tissue was found to contain increased TMP phosphorylating activity whereas the deoxyadenylate, deoxyguanylate and deoxycytidylate kinase activities remained unchanged relative to the activities found in the resting organ (Canellakis, Jaffe, Manstavinos and Krakow, 1959; Gray et al., 1960). Increases in the activity of TdR kinase has also been found to be associated with the onset of DNA synthesis in regenerating rat liver (Weissman, Smellie and Paul, 1960; Belts, 1962), the elevation of TdR kinase activity being unaccompanied by any increases in the activities of AdR and GdR kinases (Belts, 1962). The other important point that emerged from these studies was that the activity of the thymidylate kinases remained low, even in rapidly dividing cells, when compared with the enzyme systems catalysing the formation of dATP, dGTP and dCTP. The evidence is thus clearly consistent with the view that TMP phosphorylating activity increases in synchrony with the mitotic index while still remaining effectively rate limiting in providing the ultimate precursors for DNA replication. A similar relationship in the phosphorylating activities acting on dAMP, dGMP and dCMP, on the one hand, and on TMP on the other, has been observed in mouse liver homogenates (Kielley, 1961) and in extracts of HeLa cells (Magee, 1962). The relationship does not appear to be universal since studies of this type carried out using bacterial extracts (Bessman, 1959) have indicated that formation of the four deoxyribonucleoside 5'-triphosphates proceeds at approximately the same rate.

In the experiments of Gray et al., (1960), the failure of extracts of normal rat liver to catalyse the phosphorylation of TMP and the inhibition

by normal liver extracts of the synthesis of TTP by extracts of Ehrlich ascites tumour cells were attributed to a non-dialysable, heat sensitive factor present in the liver extracts. These observations were confirmed in the present experiments (Section 3.3.1) and shown to be directed specifically towards the inhibition of the phosphorylation of TdR. No effect was observed on the phosphorylation of CdR (Table 5). The experiments of Gray et al. (1960) showed, moreover, that the rate of phosphorylation of dAMP, dGMP and dCMP by extracts of ascites tumour cells remained unaffected following the addition of normal rat liver extract and, while these findings were consistent with the view that the inhibition of the formation of TTP from TMP was caused by specific thymidine nucleotidases, the evidence did not permit precise interpretation. An interesting correlation between deoxyribonucleotide kinase activities and dephosphorylating activities in mouse liver and mouse ascites hepatoma has recently been provided by Kielley (1963b). This author confirmed the earlier conclusions (Manstavinos and Canellakis, 1959; Canellakis, Jaffe, Manstavinos and Krakow, 1959; Gray et al., 1960) that non-proliferating tissues contained active kinases transforming dAMP, dGMP and dCMP into the corresponding triphosphates while being deficient in demonstrable phosphorylation of TMP. With broken cell preparations of hepatoma, the thymidylate kinase activity was shown to be higher than in resting tissue while the deoxycytidylate kinase activity was markedly depressed in such a way that TMP kinase still appeared to be effectively rate limiting in the provision of substrates for the DNA polymerase. Measurements of nucleotidase activity indicated that the low TMP kinase activity of normal

mouse liver tissue could to some extent be accounted for by the high TMP nucleotidase of that tissue while, in contrast, little or no degradation of the other deoxyribonucleoside monophosphates was observed. This was taken to be compatible with the findings (Fiala, Fiala, Tobar and McQuillin, 1962) that TMP nucleotidase resides primarily in the microsomes while the nucleotidases of dAMP, dGMP and dCMP are restricted to mitochondria where they are latent (Kielley, 1961). The phosphorylation of TMP by broken cell preparations of mouse ascites hepatoma (Kielley, 1963b) was characterised by the absence of degradative reactions. However, the initial kinetics of TMP utilisation by mouse liver preparations did not indicate that TMP kinase was inherently deficient or lacking in that tissue. This conclusion was confirmed by the demonstration that potential thymidylate kinase activities in mouse liver were as high as those of liver ascites hepatoma and that a major part of the enzyme in liver was "particle-bound" (Kielley, 1963a). It seems possible that these observations may be related to the increased stability bestowed upon TMP kinase by its substrate (Section 3.3.4) which event, according to Hiatt and Bojarski (1961), may provide a means of regulating TMP kinase activity <u>in vivo</u>. In their view, TMP kinase is continuously formed in normal liver cells but is inactivated from lack of substrate which normally stabilises the enzyme. Neither Kielley (1963b) using mouse liver nor Weissman, Smellie and Paul (1960) using rat liver were able to demonstrate the induction of thymidylate kinase following intraperitoneal injection of TdR as suggested by Hiatt and Bojarski (1960) and it seems possible that the stabilisation of thymidylate kinase by TMP may operate <u>in vitro</u> to compensate

for the loss of some other stabilising agent present within the cell.

In addition to the evidence outlined above, there is a considerable weight of evidence from virus infected systems consistent with the view that the activity of the thymidine and thymidylate kinase system is effectively rate limiting in DNA synthesis. In most of the instances quoted below, increased production of TTP (with a consequent increase in the rate of DNA synthesis) was the result of true induction of one or more of the kinase components participating in the formation of TTP from TdR. However, as pointed out by Joklik (1962), the mere demonstration of an increased concentration of enzyme molecules as a result of viral infection need not imply a general deficiency of that enzyme, only that the required activity was not available in the relatively small region of the cell where replication of viral DNA is to be initiated. Elevation of thymidylate kinase activity has been demonstrated following infection of HeLa cells with vaccinia virus in which case this kinase activity was increased without comparable increases in dAMP, dGMP and dCMP kinase activities (Magee, 1962), neither was there evidence of an increase in deoxyribonucleoside kinases. Similarly, following infection of rabbit kidney cells with pseudorabies virus, a new thymidylate kinase appeared to be induced resulting in a 6-fold increase in activity over the thymidylate kinase of uninfected cells, while no increases were detected in other deoxyribonucleotide or deoxyribonucleoside kinases (Nohara and Kaplan, 1963). The induction of a new thymidylate kinase in E.coli following infection with the T2 bacteriophage (Bello, van Bibber and Bessman, 1961; Bello and Bessman, 1963a) has been described elsewhere (Section 4.1) In that

particular case, however, the TMP kinase is thought to perform a different role from that normally associated with thymidylate kinases in that the newly formed enzyme, following purification, exhibited equal affinity for TMP, dGMP and 5-MeOH-dUMP while the normal TMP kinase was specific for TMP.

The induction of new, active forms of thymidylate kinase would be expected to result in an increase in the efficiency of the de novo pathway leading to TTP, this pathway being dependent of the methylation of dUMP to TMP with subsequent phosphorylation of TMP to TTP (see Fig. 11). However, several instances are on record also of the induction of TdR kinase following viral infection. The over-all effect of such induction would be to elevate the levels of TMP available through the preformed pathway, thus presumably indicating that production of TMP by methylation of dUMP was insufficient in such cell populations to sustain the required rate of TTP (and DNA) synthesis.

The induction of TdR kinase in HeLa cells following infection with the virus of cowpox (McAuslan and Joklik, 1962; McAuslan, 1963) was found to be under the control of the viral genome as were the TdR kinases induced in mouse fibroblast cells by infection with vaccinia virus (Kit, Piekarski and Dubbs, 1963) or with herpes simplex virus (Kit and Dubbs, 1963). It was found with interest that UdR kinase was simultaneously induced, thus indicating that the newly formed TdR kinases were difunctional as has been demonstrated with the TdR kinase of E.coli (Okazaki and Kornberg, 1964a). In none of the systems quoted above was the induction of TdR kinase accompanied by increases in TMP kinase activity. The weight of evidence and the large number of instances in which induction of TdR kinase has been demonstrated makes the

traditional concept that the phosphorylation of TdR represents a preformed or "salvage" pathway appear decidedly tenuous. Instead, it may well be that some hitherto unknown mechanism allows TdR kinase to participate in a *de novo* pathway for the synthesis of TTP.

No instances are on record of the induction of a nucleoside diphosphokinase following viral infection of mammalian cells, and the failure to detect an increase in this enzyme may perhaps be taken as another indication of the highly active and non-specific nature of nucleoside diphosphokinases in uninfected cells (see Sections 3.1 and 4.1).

All these observations may be regarded as the consequence of control rather than the manifestation of a control mechanism *per se*. The possibility cannot be lightly dismissed, however, that true control or part of the regulation of DNA synthesis in a given cell population resides in the ability of the thymidine and thymidylate kinases of those cells to provide TTP. It is of considerable interest, therefore, to examine in more detail the potentialities of the formation of TTP from its precursors TdR and TMP as a regulator system.

In the view of Davidson (1962), DNA synthesis occurs at critical concentrations of thedeoxyribonucleoside 5'-triphosphates which are themselves components of a homeostatic mechanism. Pertinent features of such a mechanism have become apparent over recent years and the picture which is emerging is of startling complexity comprising several interconnected regulatory devices, the combined effect of which is to provide sophisticated and flexible control of internucleotide metabolism.

One type of model regulatory mechanism which has assumed particular importance in defining the balance between synthesis and degradation of nucleotides is that termed negative feedback inhibition (see Section 1.4). By this term is meant a situation in which accumulation of an end metabolite causes a specific inhibition of enzymes catalysing an early step in the metabolic pathway leading to its production. The first indications that such devices were involved in nucleotide metabolism were given by Yates and Pardee (1956b) who discovered that the first enzyme specific to pyrimidine synthesis de novo, aspartate transcarbamylase, was competitively inhibited by CTP, one of its distal products. In the subsequent years, there were described a large number of other negative feedback mechanisms which were shown to intervene at several points in internucleotide metabolism, in general, and at critical stages of de novo and preformed pathways leading to deoxyribonucleoside triphosphates, in particular. Some of the relevant negative feedback mechanisms are given in Fig. 48 from which can be deduced, among other things, the extent to which the de novo pathways for the synthesis of purine (Wyndgaarden and Ashton, 1959; Henderson, 1962) and pyrimidine (Yates and Pardee, 1956a, b; Gerhart and Pardee, 1962) ribonucleotides are specifically regulated by distal metabolites in the form of ribonucleoside monophosphates and triphosphates. Among the purine ribonucleotides, several additional feedback mechanisms appear to regulate the cycle of interconversions between IMP, GMP and XMP while histidine inhibits competitively the first enzyme specific to its synthesis, phosphoribosyl-ATP pyrophosphorylase (Magasanik and Karibian, 1960; Ames, Hartman and Jacob, 1963).

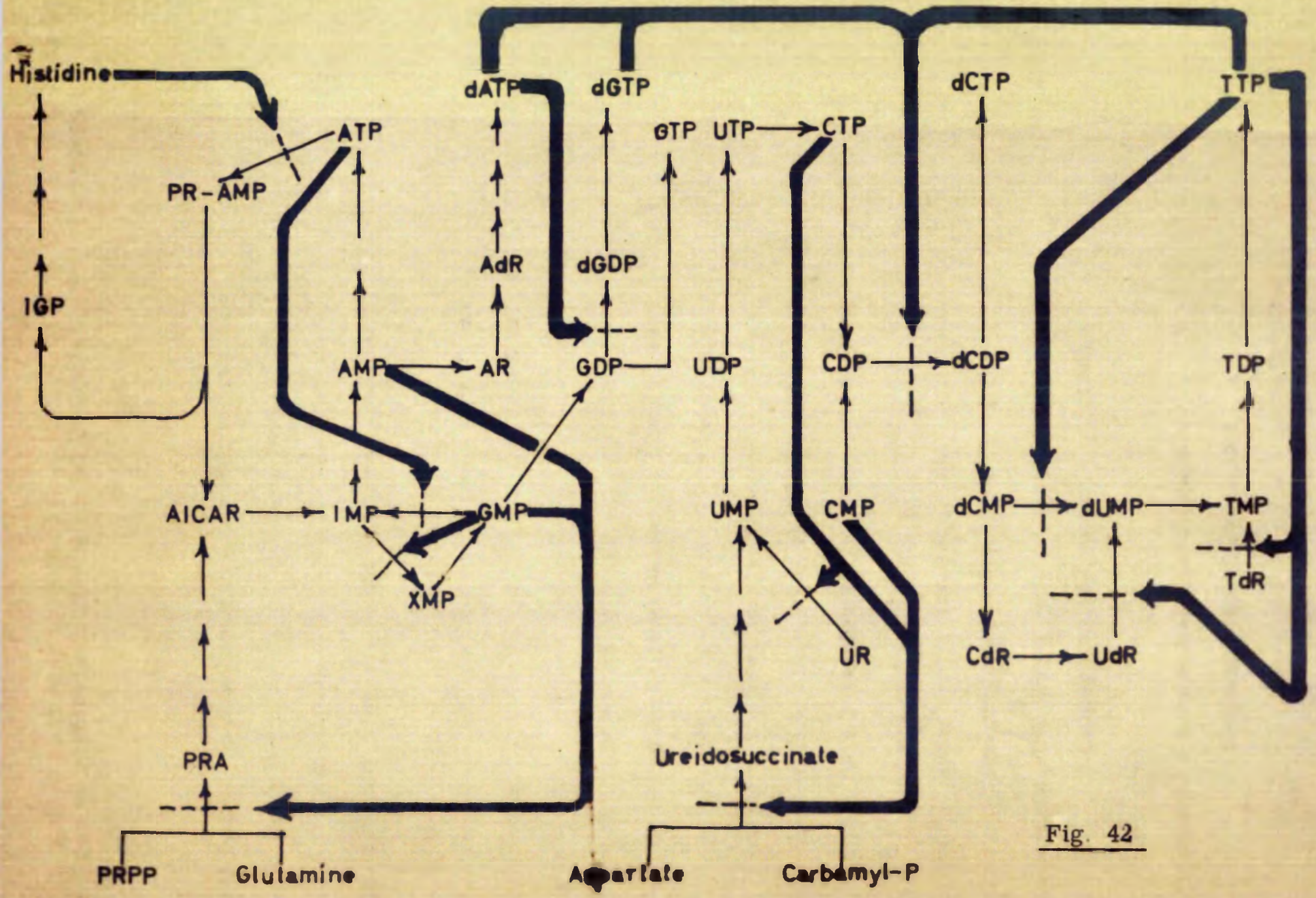

Histidine
IGP
PR-AMP
ATP
AMP
AR
AdR
dATP
dGDP
dGTP
GDP
GTP
UTP
CTP
UDP
CDP
dCDP
dCTP
TTP
TDP
AICAR
IMP
GMP
XMP
UMP
CMP
dCMP
dUMP
TMP
TdR
UR
CdR
UdR
PRA
Ureidosuccinate
PRPP
Glutamine
Aspartate
Carbamyl-P

Fig. 42

Fig. 42. Regulation of internucleotide metabolism by negative feedback control.

Thick arrows indicate negative feedback control and the thin broken lines denote specific reactions so controlled. Only relevant intermediates are included. (For more detailed schemes of internucleotide conversions, see Figures 10 and 11).

Three types of negative feedback inhibition mechanism may be recognised subject to the same molecular principles:- (a) Feedback inhibitions which regulate the activity of the first enzymes involved in purine and pyrimidine biosynthesis. (b) Feedback inhibitions by deoxyribonucleoside triphosphates of the reductive sequences leading to deoxyribonucleoside diphosphates. (c) Feedback inhibition of enzymes involved in the utilisation of preformed nucleosides for nucleic acid synthesis (in addition to the mechanisms illustrated, dGTP has been found to exert negative feedback control of dGR kinase (Maley and Maley, 1962b)). All negative feedback inhibition mechanisms, apart from those included in the last category, regulate essentially irreversible conversions.

PR-AMP = phosphoribosyl-1-adenosine 5'-monophosphate

IGP = imidazolylglycerol phosphate

Carbamyl-P = carbamyl phosphate.

While these mechanisms strongly influence the performance of the de novo pathways for the synthesis of ribonucleotides, a further set of feedback mechanisms is directly concerned in regulating the supply of deoxyribonucleoside 5'-triphosphates. This regulation is directed towards several critical stages in the passage of metabolites, derived from the ribonucleotid pool, through the series of reactions leading, ultimately, to the substrates for the DNA polymerase (see Figures 10 and 11). One crucial step which involves the departure of metabolites from the ribonucleotide pool is the reductive mechanism for the conversion of ribonucleotides to deoxyribonucleotides and which, according to present evidence, appears to take place exclusively at the diphosphate level (Reichard, 1962; Moore and Hurlbert, 1962; Bertani, Häggmark and Reichard, 1963; Larsson, 1963). In vitro studies conducted by Reichard, Canellakis and Canellakis (1961) with extracts of chick embryos have indicated that such reductive mechanisms may be governed by a singular type of negative feedback inhibition. Of the reducti systems studied, that concerned with the conversion of GDP to dGDP was observed to be inhibited by extremely low concentrations of dATP while that reducing CDP to dCDP was strongly inhibited by low concentrations of dATP, dGTP, and, to a lesser extent, of TTP but not by dGTP. In these experiments, the interplay between the reductive mechanism and DNA synthesis was examined as a function of incorporation of (^{32}P) from (^{32}P) CMP and it became apparent that a sensitive equilibrium existed between the two processes wherein the optimal requirements of one was contradictory to the optimal requirements of the other. Such feedback inhibition may have widespread significance in the

regulation of DNA synthesis and it has been connected with the inhibition of DNA synthesis in vivo following the addition of high concentrations of thymidine to tissue culture media and also appears to explain how CdR could relieve systems so inhibited (Morris and Fischer, 1960, 1963). A similar situation appears to obtain in certain bacteria (Lark, 1960).

Experiments on whole Ehrlich ascites cells have shown that whereas AdR inhibits DNA synthesis and leads to accumulation of dATP, this inhibition may be overcome by addition of CdR (Langer and Klenow, 1960). The type of inhibitory mechanism involved may be related to the negative feedback inhibiti by deoxyribonucleoside triphosphates of the reductive sequence leading to dCDP observed in cell-free systems (Reichard, Canellakis and Canellakis, 1960; Morris, Reichard and Fischer, 1963). Nevertheless, inhibition of DNA synthes by AdR has as yet only been observed in media containing very high concentrati of deoxyribonucleoside (Klenow, 1962). The fact that accumulation of purine deoxyribonucleotides does not appear to occur in normally growing cell populations (Lark, 1963) makes a direct relationship between the two mechanis appear tenuous.

A further indication that the thymidine nucleotides exert an influence over the reactions leading to their formation was obtained by Maley and Maley (1962b, 1963) who discovered that the deamination of dCMP with minces prepared from rat embryos and chick embryos was strongly inhibited by TTP. Several other important observations were made in conjunction with this discovery. Thus, in experiments on the utilisation of CdR by chick embryo mince, dCTP was observed to regulate the activity of CdR kinase (Maley and Maley, 1963) in a

manner reminiscent of negative feedback inhibition (Gerhart and Pardee, 1962) while in the presence of TTP, the inhibition of dCMP deaminase was accompanied by a stimulation of CdR kinase activity. In rat embryo mince (Maley and Maley, 1962b), TTP was observed also to inhibit the phosphorylation of UdR. The over-all effect of these interlaced regulatory mechanisms appeared to suggest that TTP might control its own synthesis by regulating the contribution of CdR (and deoxycytidine nucleotides) to the thymidine nucleotide pool.

Working on the assumption that none of the mechanisms given above could provide effective control of TTP synthesis in the presence of a substantial pool of thymidine, Ives, Morse and Potter (1962, 1963) examined the phosphorylation of TdR by cell-free extracts of Novikoff hepatoma and discovered that TTP exerted negative feedback control of TdR kinase activity. Such inhibition was demonstrated both by endogenously produced TTP (from (^{14}C) TdR) and by added TTP, neither TMP or TDP being effective. In contrast, thymidylate kinase was not inhibited by TTP under the conditions used, and the inhibition thus seemed to be specifically associated with the competitive inhibition by TTP of the first enzyme (see Section 4.1) in the metabolic pathway leading to its formation. A similar type of regulatory mechanism may operate in regenerating rat liver, from which a partially purified TdR kinase has been shown to be sensitive to added TTP or TDP (Breitman, 1963).

By far the most exhaustive study of the inhibitory effect of TTP on the phosphorylation of TdR has been that conducted by Okazaki and Kornberg (1964b). The use of a highly purified TdR kinase preparation from E. coli (Okazaki and Kornberg, 1964a) in this study permitted kinetic elucidation of many features

of the inhibitory mechanism involved.

Thus it was observed (Okazaki and Kornberg, 1964b) that the activity of TdR kinase was specifically inhibited by TTP, TMP or TDP being without effect. Several additional properties of the enzyme itself were examined and it was found to catalyse the phosphorylation of UdR at approximately the same rate as TdR, thus confirming an earlier report (Sköld, 1960b) that TdR kinase was bi-functional in action. The inhibition of UdR kinase by TTP as observed by Maley and Maley (1962b) was thus also confirmed. An extensive study (Okazaki and Kornberg, 1964b) was made of the acceptor and phosphoryl group donor specificities of the E.coli TdR kinase and such studies revealed that certain deoxyribonucleoside di- and triphosphates (dCDP, GDP, dATP, dGTP and dCDP) acted as activators, some triphosphates (dATP, dGTP) as both donors and activators and ATP as donor only. It appeared further that inhibition with TTP was not competitive with the phosphoryl group donor ATP, in contrast to the conclusion of Ives, Morse and Potter (1963), or with the activator dCDP, but rather with the substrate TdR. Okazaki and Kornberg (1964b) conclude, however, that TTP does not bind to the catalytic site for TdR, on the grounds that no inhibition was observed with TMP or TDP, but rather to an adjacent site close enough to compete sterically with the binding of TdR to its site. This conclusion is remarkably close to that of Gerhart and Pardee (1962) for the molecular mechanism of the feedback inhibition of aspartate carbamylase by CTP, and it seems quite possible that the provision of a so-called "allosteric site" (Monod, Changeux and Jacob, 1963) may have general significance in the regulation of intracellular enzyme systems. In

addition to this property (Okazaki and Kornberg, 1964b), it appeared that the TdR kinase bound and had, in the absence of activator, sites for 2 molecules of ATP while in the presence of the activator, dGDP, only one molecule of ATP was required, thereby suggesting that the second site might serve an activator function.

The biological significance of this kinetic data, in the view of Okazaki and Kornberg (1964b), lies in the fact that TdR kinase appears to be activated by those deoxyribonucleoside di- and triphosphates which accumulate when the supply to TTP is insufficient and that the activation may operate in effect by lowering the K_m of the enzyme for TdR thus favouring its efficien utilisation at low concentrations.

While TdR kinase might thus participate in an extremely powerful regulating mechanism governing the production of deoxyribonucleoside 5'-triphosphates, it is not yet possible to discover to what extent its potential is fulfilled in the intact cell. Nor is it possible to gauge its significanc relative to other negative feedback systems operating as regulators in the production of substrates for the DNA polymerase until those systems have been similarly scrutinised. However, even when solutions have been found to these questions, there remains the essential enigma of the function of the TdR kinase itself. The majority of the available evidence suggests that TdR is not on the direct pathway for the formation of TTP de novo, but that this pathway utilises instead the methylation of dUMP as an entrance to the thymidine nucleotide pool. The TMP thus formed is presumed to be phosphorylated directly to thymidine polyphosphates followed by the utilisation of

these compounds for DNA synthesis. The smallness of the intracellular thymidine pool (Lark, 1963), when considered together with the extremely poor utilisation of thymine in mammalian cells, suggests that if the TdR kinase functions in its traditional role, as part of a preformed pathway, its contribution to the thymidine nucleotide pool will be insignificant and insufficient to sustain the rate of TTP production required in DNA synthesis. Recently, a mechanism has been suggested which allows the participation of TdR in a normal pathway for producing TTP de novo. By incubating Ehrlich ascites tumour cells in a medium containing (^{32}P) -labelled inorganic phosphate, Langen and Liss (1962, 1963) observed that the specific activity of the DNA-thymidylic acid was considerably higher than that in the other deoxyribonucleoside monophosphates recovered from the DNA and they conclude from this that dilution of radioactivity was the less pronounced in the thymidine nucleotide pool and that this constituted the smaller of the deoxyribonucleotide pools. However, they (Langen and Liss, 1962) further observed that inhibition of DNA synthesis by fluorouracil (which is presumed to inhibit specifically thymidylate synthetase) could be reversed by adding TdR to the medium. Addition of (^{32}P) -labelled inorganic phosphate to a medium so composed gave DNA-deoxyribonucleoside monophosphates of relative specific activities similar to those obtained with cells to which no fluorouracil or TdR had been added. Langen and Liss (1963) argued from this that TdR itself may be a normal metabolite involved in de novo synthesis of TTP and they visualise a two-component system consisting of TdR kinase in association with TMP nucleotidase as the connecting link with the thymidine

nucleotides produced via dUMP. The wastage of energy resulting from the operation of a "salvage loop" of this type has been pointed out by Brumm, Potter and Siekevitz, (1956) and by Beltz, (1962), but some alternative mechan may well exist which allows the TdR kinase to realise to the full extent its remarkable potential as a regulator of deoxyribonucleotide metabolism.

While the thymidine nucleotides are thus intimately concerned in regulating the formation of the ultimate substrates for the DNA polymerase, few of the studies quoted so far have a direct bearing on whether they also contribute to the temporal control of DNA synthesis. In other words, are the thymidine nucleotides concerned with the event (or events) which constitute the initiation of DNA synthesis? Few attempts have been made to study the alterations in intracellular enzyme patterns in relation to the DNA synthesis cycle in mammalian cells. In bacteriophage-infected bacteria, however, de novo synthesis of the various enzymes necessary for the productio of specific types of DNA has been demonstrated (see Sections 2.1 (b) and 4.1)

Best known by far are the changes that occur in deoxyribonucleotide metabolism following partial hepatectomy in the rat, several gross results of which have been noted above (see p. 193). Thus, increases in several of the enzymes participating in the formation of deoxyribonucleoside 5'-triphosphates in rat liver have been demonstrated following such surgical intervention. It has been shown, moreover, that such increases in enzyme activity are discontinuous and linked specifically to events in the DNA synthesis cycle, more particularly, such elevation in synthetic activity has been observed to occur just prior to, or to coincide with, the onset of

DNA synthesis. The experiments of Maley and Maley (1960) showed that dCMP deaminase activity, which is very low in normal rat liver (Maley and Maley, 1961b), began to increase 12 hours post partial hepatectomy, followed at 18 hours by demonstrable thymidylate synthetase activity at which time there was also evidence of DNA synthesis. To account for these observations, Maley and Maley (1960) suggested the possibility of a sequential induction of dCMP deaminase and thymidylate synthetase, with the deaminase becoming elevated prior to and the synthetase concomitant with DNA synthesis. It was suggested further that the product of dCMP deaminase action, dUMP, might induce the formation of TMP synthetase, and that the product of this reaction (TMP) might, in its turn, induce the formation of TMP kinase. These suggestions correlate well with findings concerning the appearance of the thymidine and thymidylate kinases during regeneration of rat liver following partial hepatectomy. Thus, Bollum and Potter (1959) found that the highest uptake of (^{3}H) TdR into DNA occurred between 24 and 60 hours following the operation and the experiments of Weissman, Smellie and Paul (1960) revealed elevation in the levels of these enzymes at 20 - 24 hours post partial hepatectomy, just prior to the onset of DNA synthesis, the TdR, TMP and TDP kinases appearing sequentially, in that order. This timing of the appearance of thymidylate kinase was confirmed by Hiatt and Bojarski (1960) who observed an increase in the activity of this enzyme at 20 hours post partial hepatectomy. Weissman, Smellie and Paul (1960) suggest that since the increases in activity of these enzymes may only be observed during the growth phase, a sequential induction of the TdR, TMP and TDP kinase might be involved and

it seemed possible also that elevation of each kinase occurred in response to production of its substrate. This suggestion was clearly consistent with the experiments of Hiatt and Bojarski (1960) which demonstrated the induction of thymidylate kinase in rat liver following intraperitoneal injection of TdR. However, if the production of the thymidine and thymidylate kinases occurs in response to the endogenous formation of their respective substrates, and if this were a necessary as well as a sufficient condition for such increases, it seems logical to conclude with Hiatt and Bojarski (1960, 1961) that the metabolic event initiating the train of reactions leading to the synthesis of DNA must be manifest at a stage prior to the phosphorylation of TMP. Thus, it would appear that a metabolic pathway is involved proceeding from dCMP and TdR through TMP and TTP to DNA and that this sequence of reactions may be normally repressed. No evidence has yet appeared to show that this type of induction is due to <u>de novo</u> production of enzyme molecules, but rather that enzyme adaptation or positive feedback (see Section 1.4) may be the process involved. The molecular mechanisms which allow the cyclic appearance of thymidine kinase at the end of G_1 periods (Fig. 9) (Hotta and Stern, 1963) may be directly related to the particle-bound form in which such enzymes apparently occur in resting tissues (Kielley, 1963a; Bianchi, Grathorn and Shooter, 1962). It seems possible to conceive of a mechanism whereby relevant kinases might be released from an inactive, particle-bound complex specifically in response to the accumulation of the appropriate substrate and return to this inactive, latent state following the completion of the S period. Such activation-inactivatio

cycles, apparently, are not uncommon in mammalian cells (Perdee and Wilson, 1963) and suggest possibilities for control quite analogous to induction and repression.

Current concepts on the control of the initiation of DNA synthesis (Lark, 1963; Maalpe, 1963) do not visualise the thymidine and thymidylate kinase system as an essential component, but rather that both RNA and protein synthesis are required prior to DNA replication. Moreover, much of the evidence suggests that the controlling factor may be of a structural rather than a catalytic nature and that it may operate, not at the nucleotide level but at the level of the genome itself.

Considerations such as these are somewhat outside the scope of the present work, the object of which was manifestly not to elucidate how intracellular events are regulated along the time axis of the cell's life, but merely to indicate, through the *in vitro* system, which events in the formation of TTP could conceivably provide a regulating step in the synthesis of that compound and thus, in a wider context, influence DNA synthesis. The interpretations presented here can at best be no more than molecular concepts reflecting the potentiality of the enzyme system rather than the manner in which it operates within the intact cell. With these reservations in mind, the results presented are clearly consistent with the view that the thymidine and thymidylate kinase system forms an essential and integral part of the regulatory mechanisms which control the quantitative aspects of DNA replicati

SUMMARY

1. The phosphorylation of thymidine by enzymes derived from soluble extracts of Landschutz ascites tumour cells led to the formation of thymidine 5'-monophosphate (TMP), thymidine 5'-diphosphate (TDP) and thymidine 5'-triphosphate (TTP). The formation of TTP from thymidine with enzymes from this source was optimal at pH 7.8 to 8.1 and showed an absolute requirement for Mg^{++} and adenosine 5'-triphosphate (ATP) wi the maximal rate of TTP formation occuring in the presence of 5×10^{-3} M ATP and 5×10^{-3} M $MgCl_2$.

2. The mechanism of formation of TTP from thymidine by soluble extracts of Landschutz ascites tumour cells has been examined using an improved meth for the complete separation of thymidine nucleotides on columns of ECTEOLA cellulose.

3. Studies on the time course of formation of TTP from (^3H) -labelled thymidine have invariably shown that TMP and TDP may be detected in the reaction mixtures very soon after the start of the incubation. On no occasion was labelled triphosphate detected earlier than labelled diphosphate. In experiments with thymidine 5'-(^{32}P)-monophosphate, labell diphosphate was again detected before labelled triphosphate.

4. When experiments were performed with TDP labelled in either the α- or the β- phosphates with (^{32}P), TTP was formed very rapidly without the intermediate formation of TMP or any higher phosphates of thymidine than TTP.

5. It was observed that TMP and TTP were formed very rapidly from thymidine and that TTP was formed very rapidly from TDP in which circumstances it

seems possible that the formation of TDP is the rate-limiting step in the synthesis of TTP, thus also suggesting the involvement of thymidine kinase, TMP kinase and TDP kinase.

6. Extracts of normal rat liver were shown to phosphorylate thymidine only at 5 per cent the rate of Landschuts ascites tumour extracts. TMP was not phosphorylated by the liver extracts while deoxycytidine was transformed into phosphorylated derivatives six times the rate with thymidin Inhibition by normal liver extracts of the synthesis of TTP from thymidine and TMP by extracts of Landschuts ascites tumour cells was obser to occur in a manner which suggested specific competitive inhibition of thymidine and thymidylate phosphokinases since no inhibitory effect was observed on the phosphorylation of deoxycytidine.

7. An investigation was carried out in order to separate and characterise the three phosphokinases in extracts of Landschuts ascites tumour cells A preparation with thymidine kinase alone was obtained by treating crud extracts with dilute acid when TMP kinase and TDP kinase were lost progressively over a period of 30 minutes. The thymidine kinase so obtain was purified 3 to 4-fold from the crude extracts; it catalysed the phosphorylation of thymidine to TMP with ATP as phosphoryl group donor and optimal activity was obtained in presence of 5×10^{-3} M $MgCl_2$ and at pH 7.8 to 8.1.

8. If fractionation of the crude extracts with dilute acid was performed i the presence of excess TMP, 80 to 150 per cent of the initial TMP kinas (purified 2 to 3-fold) activity was recovered in the supernatant fracti (AS Fraction) following acidification to pH 4.7 while much of the TDP

kinase activity was destroyed. Phosphorylation of TMP by this fraction had a narrow pH optimum at 7.6 and an absolute requirement for ATP (5×10^{-3} M) and Mg^{++} (5×10^{-3} M or 20×10^{-3} M).

9. Treatment of the AS Fraction at pH 7.6 with increasing concentrations of C_γ-alumina gel led to adsorption of TMP kinase along with much of the protein. If excess TMP was added to the AS Fraction prior to adsorption of the alumina gel, however, most of the protein was adsorbed while 60 to 80 per cent of the TMP kinase remained in solution. Further purification on a column of Sephadex G-100 yielded TMP kinase purified 1000-fold. This preparation was devoid of TDP kinase activity but was contaminated with adenosine 5'-triphosphatase.

10. Fractionation of the AS Fraction directly on columns of Sephadex G-100 by gradient elution with phosphate buffer, pH 7.6, yielded three peaks of kinase activity. The first represented thymidine kinase, the second TDP kinase contaminated with adenosine triphosphatase and a small amount of TMP kinase which was accounted for by slight overlapping of the third peak of TMP kinase. This third fraction contained TMP kinase purified 60-fold and was shown to catalyse the formation of TDP from TMP. TTP was not detected in the reaction products and it was shown, further, that this failure to detect TTP was not due the presence of TTP dephosphorylating enzymes.

11. These results pointed to the existence of at least three phosphokinases in extracts of Landschutz ascites tumour cells and strongly support the view that TDP is an intermediate in the formation of TTP from thymidine and TMP in this system.

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