The inhibition of DNA nucleotidyltransferase by tissue extrac

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The synthesis of deoxyribonucleic acid (DNA) is catalyse by the enzyme DNA nucleotidyltransferase which requires prime DNA and incorporates monophosphate residues from the deoxyribonucleoside 5'-triphosphates into polydeoxyribonucleo tides. The transferase from ascites tumour cells is inhibited by extracts of liver and kidney and by calf serum and the present study was undertaken to determine the mode o action of these inhibitory factors.

DNA nucleotidyltransferase from calf thymus gland was employed as the test system and the characteristics of this enzyme were first determined. It requires magnesium ions and its activity is enhanced by 2-mercaptoethanol and ethylenediaminetetraacetic acid. The optimal concentration of primer DNA and deoxyribonucleoside triphosphates have been determined and the time course of incorporation of ^{32}P-TMP has been examined. The addition of salts such as sodium chloride inhibits the reaction.

It was confirmed that calf serum and extracts of rat liver and kidney inhibited the reaction. The inhibitory factor in calf serum was dialysable and heat-stable and the inhibition of the transferase could be accounted for by the monovalent cations present in the serum. However, the

inhibition of the transferase by extracts of rat liver and kidney was found to be due to a non-dialysable, heat-labile component.

Fractionation experiments have eliminated hydrolysis of the deoxyribonucleoside 5'-triphosphates by phosphatase as a mode of action of the inhibitor from rat liver, and the inhibitor has been purified to a point where it exhibits very low levels of hydrolytic activity towards deoxyribonucleoside triphosphates.

The influence on DNA nucleotidyltransferase of purified inhibitor preparations was found to be due, at least in part, to the action of a nuclease on the DNA primer. Thus, the deoxyribonuclease (DNase) and inhibitory activities parallelled one another very closely during the purification procedure, and showed a similar sensitivity to heat treatment. Part of the inhibition of the transferase was found to be due to the products of the action of the nuclease on the DNA primer, and not merely to the breakdown of the newly synthesised portions of DNA chains.

Relatively high concentrations of pancreatic DNase I were required to inhibit the transferase reaction, and concentrations of DNase I which produced a release of acid-soluble products from DNA similar to that produced by the purified inhibitor caused only slight inhibition. The addition of a small amount of splenic DNase II, which

produced only a small amount of acid-soluble products, inhibited the transferase reaction strongly.

While the inhibitor preparation caused 35% inhibition of DNA nucleotidyltransferase primed with denatured DNA, 105% stimulation was observed when native DNA was substituted for the denatured material.

The nuclease in the purified inhibitor preparation has an optimal pH between 6.5 and 8.5, and a requirement for either manganese or magnesium ions. Calcium ions cannot activate nor can they act synergistically in the presence of magnesium. Any increase in the ionic strength of the mediu inhibits the liver nuclease. The nuclease hydrolyses thermally denatured DNA approximately 2.5 times more rapidly than native DNA and exhibits some ribonuclease activity. These properties appear to distinguish this enzyme from any hitherto described nuclease.

The activities of various enzymes hydrolysing DNA were determined in subcellular fractions of rat liver. The relative activities of enzymes hydrolysing native and denatu DNA did not differ significantly in the fractions, but the recovery of enzymes hydrolysing native DNA was appreciably lower than those hydrolysing native DNA.

A comparison of the products of digestion of DNA by th liver nuclease after 1 and 22 hr. indicated an endonucleo-lytic rather than an exonucleolytic mode of action. Oligo

nucleotides isolated after digestion of DNA for 1 hr. inhibited the transferase reaction, but those obtained after 22 hr. did not. Removal of the terminal phosphate groups from the inhibitory oligonucleotides with alkaline phosphatase did not destroy their activity.

A "limit" digest of DNA by the liver nuclease contained deoxyribonucleosides and no other derivatives. A 24 hr. digest also contained a small amount of dinucleotides and the deoxyribonucleoside monophosphates of adenine, guanine, cytosine and thymine, in addition to deoxyinosine, thus indicating the presence of a phosphatase in the nuclease preparation. In the products of a 2 hr. reaction, all four bases seemed to be present at the phosphoryl terminal end of the oligonucleotides. Deoxyinosine also seemed to be present in this position, but no evidence was obtained for its occurrence at non-terminal sites. Deoxyadenosine, deoxyguanosine, deoxycytidine, thymidine and deoxyinosine 5'-monophosphate do not inhibit the transferase reaction.

Biochem. J. (1962) 84, 112P.

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Thesis presented for the
degree of Doctor of Philosophy,
The University of Glasgow.

April, 1963.

Acknowledgments.

I should like to thank Professor J.N. Davidson for providing the facilities for this research and to express my most sincere gratitude to Dr. R.M.S. Smellie for his advice, encouragement and patience as a supervisor.

I am also indebted to Dr. H.M. Keir for frequent discussions and to Dr. R.Y. Thomson and the other members of this department for their advice so freely given. In addition, it is a pleasure to thank Mrs. I. Fergusson for typing and Miss H. Moss, Mr. C. McLeod and Mr. G. Russel who rendered expert and valuable technical assistance from time to time.

I must also acknowledge with thanks the receipt of a Medical Research Council Scholarship during the early part of this work, and subsequently grants from the Jane Coffin Childs Memorial Fund for Medical Research and the British Empire Cancer Campaign.

Abbreviations

The following abbreviations will be used in this thesis:

RNA	ribonucleic acid
DNA	deoxyribonucleic acid
AdR	deoxyadenosine
GdR	deoxyguanosine
CdR	deoxycytidine
TdR	thymidine
IdR	deoxyinosine
dAMP	deoxyadenosine 5'-monophosphate
dGMP	deoxyguanosine 5'-monophosphate
dCMP	deoxycytidine 5'-monophosphate
TMP	thymidine 5'-monophosphate
dIMP	deoxyinosine 5'-monophosphate
dATP	deoxyadenosine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
TTP	thymidine 5'-triphosphate
dITP	deoxyinosine 5'-triphosphate
AMP	adenosine 5'-monophosphate
GMP	guanosine 5'-monophosphate
CMP	cytidine 5'-monophosphate
UMP	uridine 5'-monophosphate

ADP	adenosine 5'-diphosphate
GDP	guanosine 5'-diphosphate
CDP	cytidine 5'-diphosphate
UDP	uridine 5'-diphosphate
ATP	adenosine 5'-triphosphate
GTP	guanosine 5'-triphosphate
CTP	cytidine 5'-triphosphate
UTP	uridine 5'-triphosphate
^{32}P-TMP	thymidine 5'-monophosphate, labelled with a radioactive phosphorus atom.
^{32}P-TTP	thymidine 5'-triphosphate, labelled with radioactive phosphorus in the α phosphate group.
^{32}P-dCMP	cytidine 5'-monophosphate labelled with radioactive phosphorus in the phosphate group.
^{3}H-TdR	thymidine labelled with tritium
CEP	2-cyanoethyl phosphate
DCC	dicyclohexylcarbodiimide
NADH	reduced nicotinamide adenine dinucleotide
NAD^+	nicotinamide adenine dinucleotide
P_i	inorganic phosphorus
DEAE-Cellulose	diethylaminoethylcellulose
CM-cellulose	carboxymethylcellulose
tris	tris (hydroxymethyl) aminomethane
EDTA	ethylenediamine tetraacetic acid

Oligonucleotides are represented by the following symbols:

d- denotes the deoxyribo- compounds

pX, pXpY, pXpYpZ represent mono-, di-, and trinucleotides with 5'-phosphoryl terminal groups.

Xp, XpYp, XpYpZp represent mono-, di- and trinucleotides with 3'-phosphoryl terminal groups.

Pu denotes a purine base.

Py denotes a pyrimidine base.

Contents.

SECTION I

I N T R O D U C T I O N

Section I Introduction

It is now well established that nucleic acids are high molecular weight substances found in plant and animal cells and in viruses where they exist in association with protein as nucleoprotein. Two distinct types of nucleic acid are known : RNA and DNA.

I. I Structure of the nucleic acids.

(a) RNA.

RNA is composed of the nucleoside 5'-monophosphates of adenine, guanine, cytosine and uracil, which are linked together by 3'-5' phosphodiester bonds. The mode of linkage was determined by studies on the alkaline and enzymic hydrolysis of RNA (Cohn, 1950, 1951; Cohn and Volkin, 1953; Whitfeld, Heppel and Markham, 1955). Early studies on RNA led to the conclusion that it was a tetranucleotide, since the harsh methods of extraction employed gave rise to fragments of this order of molecular weight, and since the four nucleotides appeared to be present in approximately equimolar proportions. This theory had to be abandoned, however, when more precise methods became available for the analysis of the base composition of the nucleic acids. Such methods indicated that the molar proportions of bases vary very widely in

RNAs from different sources. Eventually it was concluded that RNA was a much larger molecule than had hitherto been suspected, although the exact molecular weight is still far from clear. This is due partly to the fact that there are several types of RNA within the cell, ranging from sRNA, which has a molecular weight of 10,000 to 20,000 (Zamecnik, Stephenson and Hecht, 1958) to highly polymerised types with molecular weights of 10^6 or more. There are also difficulties associated with the actual measurement of molecular weight, since the diffusion constant and sedimentation coefficient vary considerably with the ionic strength of the solvent (Jordan, 1962).

The secondary structure of RNA has not yet been fully elucidated, and probably varies with the different types of RNA. The larger molecules are thought to have small helical regions, involving up to half the nucleotides (Doty, 1961). sRNA is thought to be in the form of a double helix with the chains joined at one end (Spencer, Fuller, Wilkins and Brown, 1962).

(b) DNA.

DNA differs chemically from RNA in containing thymine instead of uracil and 2-deoxy-D-ribose instead

of D-ribose, and, in addition to the four major bases, certain others occur, usually in relatively small amounts. These unusual bases include 5-methylcytosine, which was first discovered in calf thymus DNA and was later found in small amounts in DNA from mammalian, fish and insect sources, and in larger quantities in wheat germ DNA (Wyatt, 1950, 1951). 5-Hydroxymethylcytosine has been found in the T-even bacteriophages, where it replaces cytosine (Wyatt and Cohn, 1952). 6-N-methylaminopurine has been discovered in the DNA of certain strains of E.coli (Dunn and Smith, 1958).

Like RNA, DNA was initially obtained in a degraded form because of the harsh extraction methods used. Later, when milder methods were employed, it became apparent that DNA is a very large molecule, and, with the aid of physical methods, such as light scattering and ultracentrifugation, it has been possible to determine its molecular weight. Estimates varying between 2 and 16×10^6 have been made, and it is possible that even molecules of this size are portions of a still larger molecule which has been degraded by mechanical shear or other processes (Hershey and Bungi, 1960). Values of over 100×10^6 have been quoted for the DNA of the T2

bacteriophage (Rubenstein, Thomas and Hershey, 1961), and it is thought that this represents one DNA molecule per bacteriophage.

The DNA molecule is built up from deoxyribonucleotide units joined together in a manner similar to that found in RNA. Unlike RNA, however, DNA exhibits certain regularities in its base composition. DNAs from widely different sources have been examined, and the vast majority exhibit the following regularities :

(i) Adenine + Guanine = Cytosine + Thymine

(ii) Adenine = Thymine

(iii) Guanine = Cytosine

These relationships were first pointed out by Chargaff (1950, 1951). Although unfractionated DNA obeys these rules, Bendich, Pahl, Korngold, Rosenkranz and Fresco (1958) have fractionated DNA chromatographically and found that the base composition of the fractions deviates appreciably from these rules.

The secondary structure of DNA has been investigated and elucidated mainly with the aid of X-ray diffraction methods and stereochemical models. X-ray studies were initiated by Astbury and Bell (1938) and

later, Pauling and Corey (1953a and b) proposed a structure which they considered compatible with their own and with Astbury and Bell's data. This structure consisted of three intertwined polynucleotide chains. However, certain features of this structure were untenable, and Watson and Crick (1953) suggested that DNA consists of two helical polynucleotide chains wound round a common axis, with the purine and pyrimidine bases on the inside and the phosphate groups on the outside. Wilkins (1957) in further X-ray studies corroborated the Watson-Crick hypothesis, with only minor modifications. The structure is stabilised by hydrogen bonding between the bases on each of the chains. When Watson and Crick constructed molecular models, they found that stereochemically the only type of hydrogen bonding which would fit was adenine bonded to thymine and cytosine to guanine. This fits in admirably with Chargaff's observations on the base composition of DNA.

Fig. 1a shows how the nucleotide units are linked together to form DNA, and Fig. 1b a three dimensional representation of the structure of DNA.

DNA in aqueous solution can undergo structural alterations usually referred to as denaturation. This

Fig. 1 (a).

Schematic representation of a portion of a DNA molecule.

Fig. 1(a).

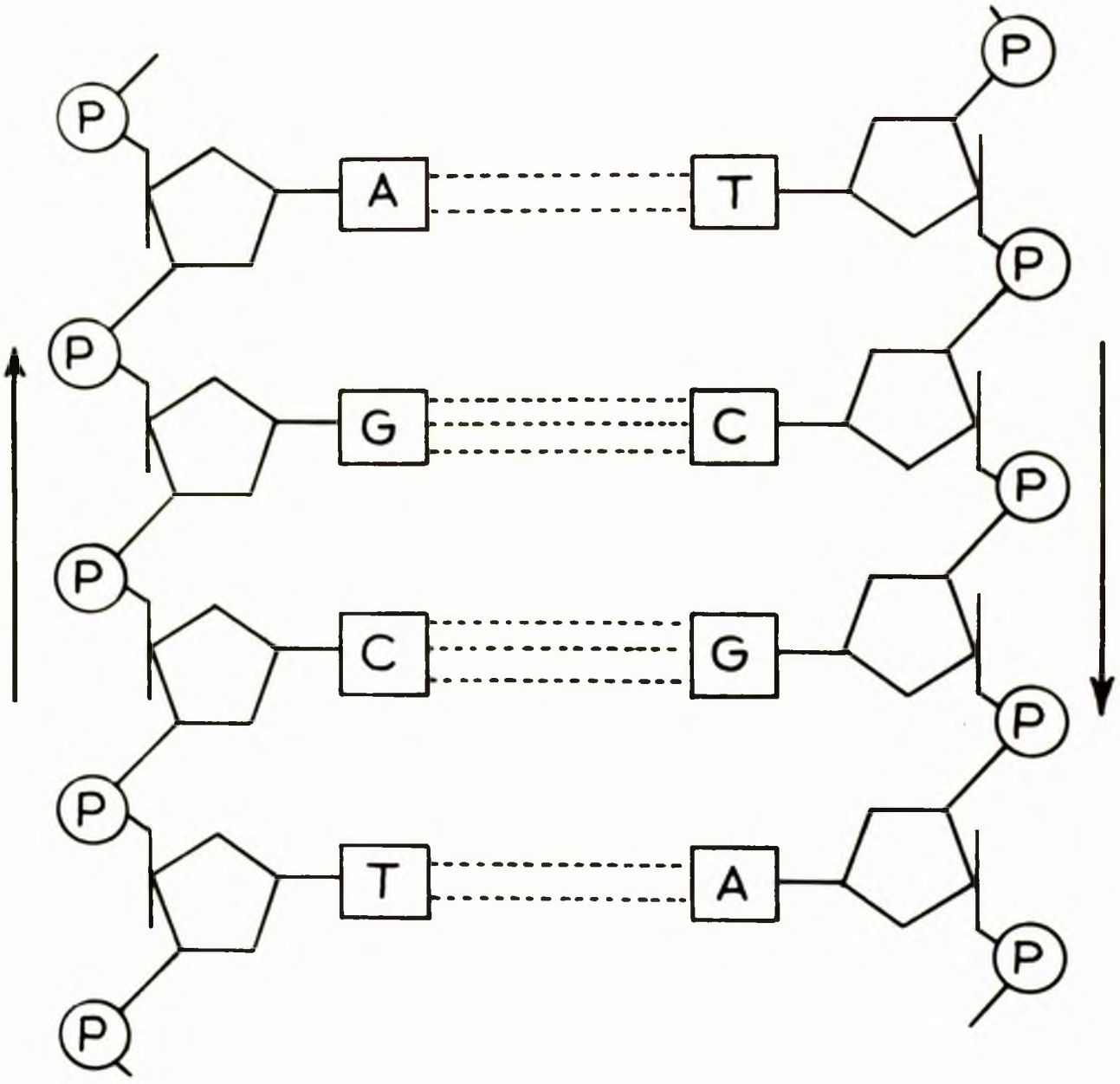
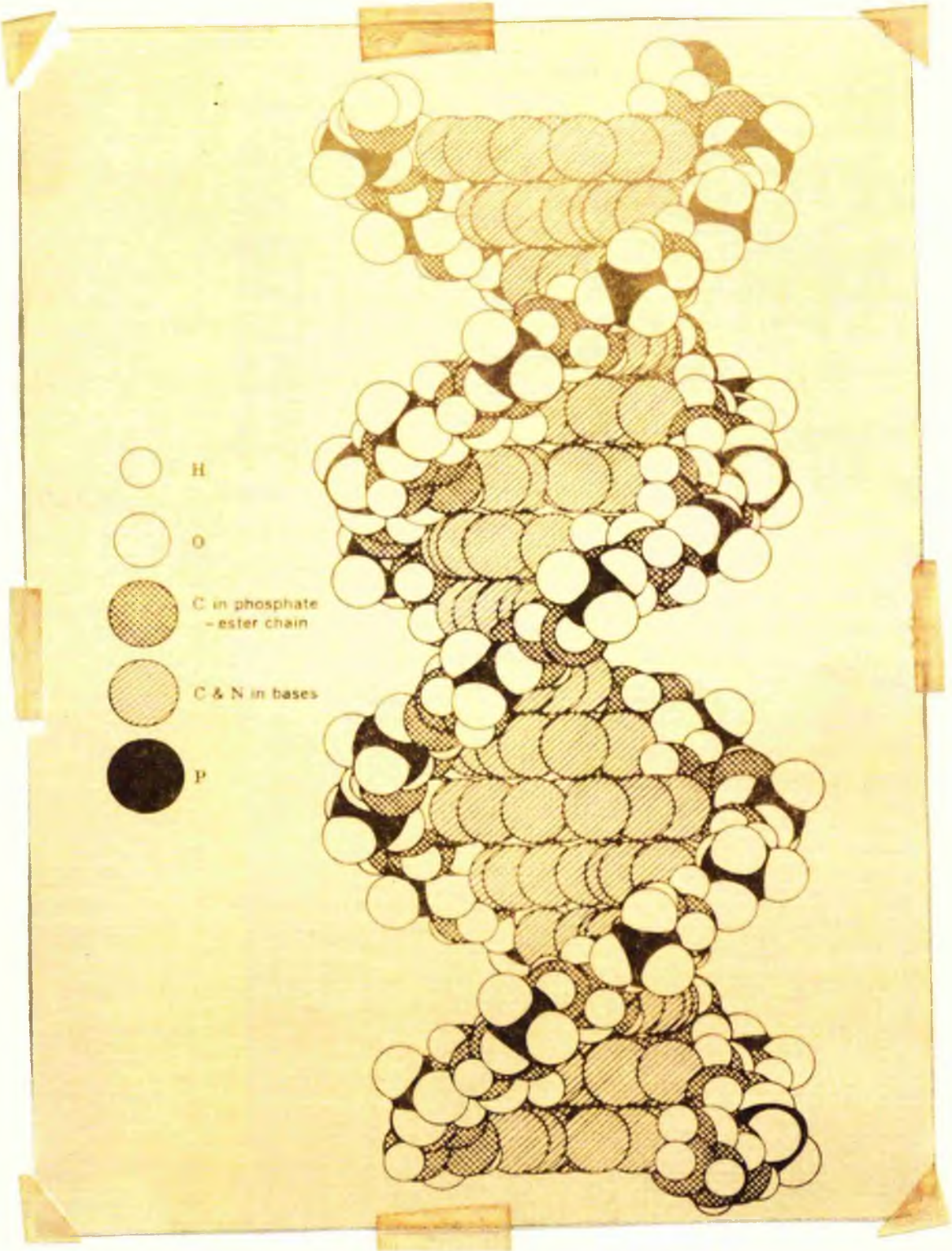


Fig. 1 (b).

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).

Fig. 1(b).



term is used to denote the collapse of the rigid helical structure into a random coil. Factors which govern the denaturation of DNA include temperature, pH, ionic strength and dielectric constant of the medium, and denaturation may also be brought about by reagents such as urea which are known to favour the dissociation of hydrogen bonds.

The process of denaturation can be followed and measured by several physical methods, which include the increase in ultraviolet absorption observed on denaturation (Thomas, 1954; Lawley, 1956), decrease in viscosity (Rice and Doty, 1957) and alterations in light scattering (Alexander and Stacey, 1955) and sedimentation (Reichmann, Bunce and Doty, 1953) characteristics.

The exact changes in the DNA molecule produced by denaturation are a matter of some dispute. It has been suggested (Doty, Marmur, Eigner and Schildkraut, 1960) that denatured DNA consists of single stranded material, since sedimentation and diffusion measurements suggested that the molecular weight was halved. The light scattering data of Rice and Doty (1957) and of Cavalieri, Deutsch and Rosenberg (1961) indicate, however, that the molecular weight of DNA is unchanged

on denaturation. Thus, it seems that there is some strand separation on DNA denaturation, but this may not be complete. It is possible to observe the partial renaturation of DNA on returning the solution to its initial state, but the molecule probably does not go back to its native form, and the degree of renaturation depends on the conditions used for denaturation.

I. 2 Biosynthesis of the polynucleotides.

(a) Synthesis of the ribonucleoside 5'-monophosphates.

The complete pathway for the biosynthesis of the purines has been elucidated, mainly by the work of Buchanan and his colleagues, and has recently been reviewed (Buchanan, 1960). It has been shown that IMP can be synthesised from simple molecules, and that it can subsequently undergo reactions which convert it to AMP and GMP. The complete synthesis is summarised in Fig. 2.

Much is also known of the synthesis of the pyrimidine mononucleotides. This too proceeds from simple precursors and details can be found in the review by Crosbie (1960). The first pyrimidine to be formed is orotidine 5'-phosphate, which can be decarboxylated

Fig. 2 (a).

The enzymic synthesis of inosinic acid de novo.

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

PRPP 5-phosphoribosylpyrophosphate.

GAR glycinamide ribonucleotide.

FGAR formylglycinamide ribonucleotide.

AIR 5-amino-4-imidazole ribonucleotide.

SAICAR 5-amino-4-imidazole (N-succinylocarboxamide)ribonucleotide.

AICAR 5-amino-4-imidazole carboxamide ribonucleotide.

FAICAR 5-formamido-4-imidazole carboxamide ribonucleotide.

Fig. 2(a)

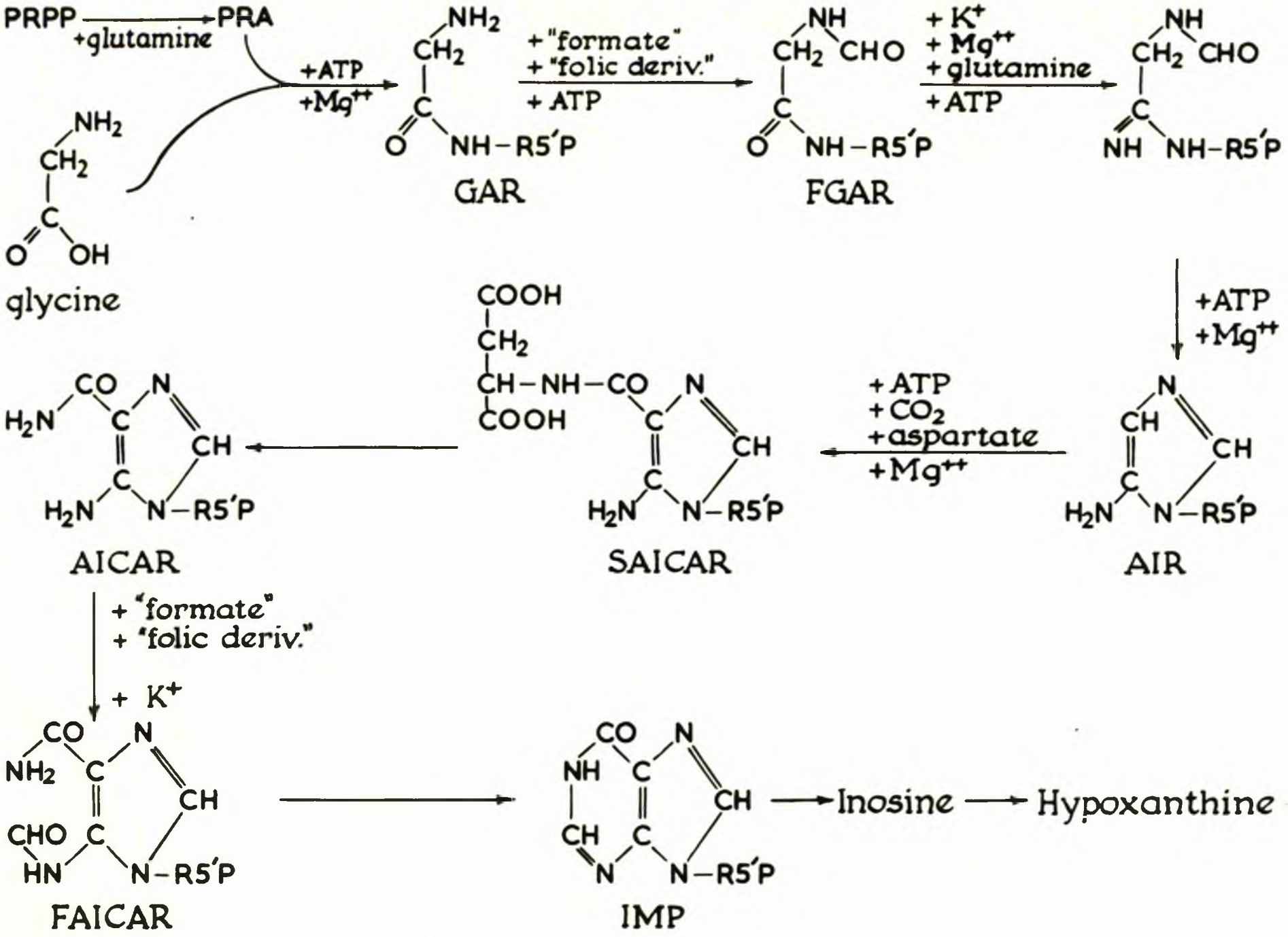
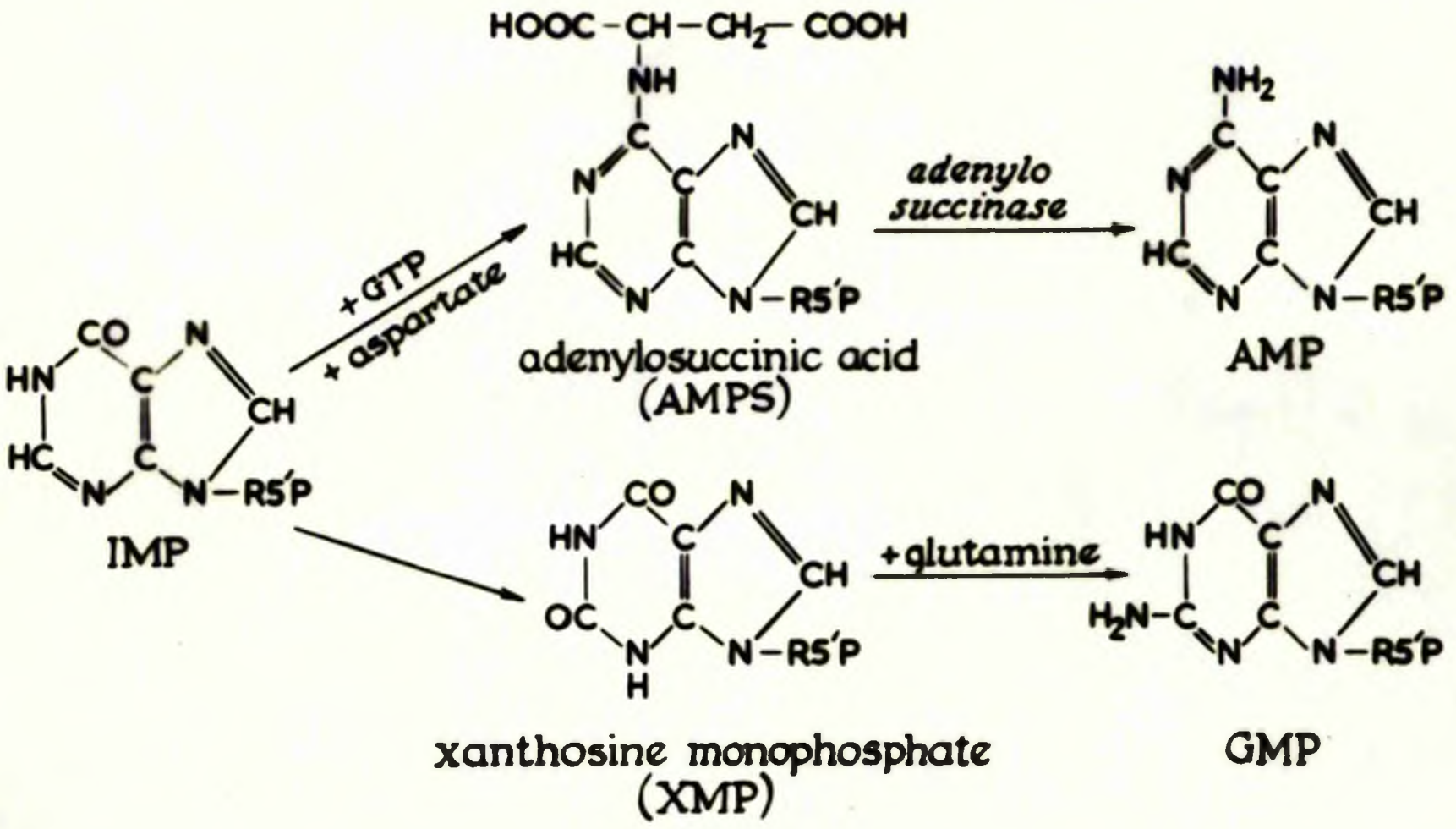

PRPP
+glutamine
PRA
+ATP
+Mg++
GAR
+"formate"
+"folic deriv."
+ ATP
FGAR
+ K+
+ Mg++
+glutamine
+ATP
glycine
+ATP
+Mg++
+ATP
+CO2
+aspartate
+Mg++
AICAR
SAICAR
AIR
+ "formate"
+ "folic deriv."
+ K+
FAICAR
IMP
Inosine
Hypoxanthine

Fig. 2 (b).

Enzymic synthesis of adenylic and guanylic acids from inosinic acid.

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

Fig. 2(b).


HOOC-CH-CH2-COOH
NH
adenylo
succinase
NH2
+GTP
+aspartate
adenylosuccinic acid
(AMPS)
AMP
IMP
+glutamine
xanthosine monophosphate
(XMP)
GMP

to give UMP. UMP and CMP are interconvertible through several systems at the mono-, di-, and triphosphate levels. Uracil appears to be the precursor of the thymine ring also, and the conversion has been shown to take place at the nucleoside and nucleoside monophosphate levels. The biosynthesis of UMP is illustrated in Fig. 3.

(b) Synthesis of the deoxyribonucleoside 5'-monophosphates.

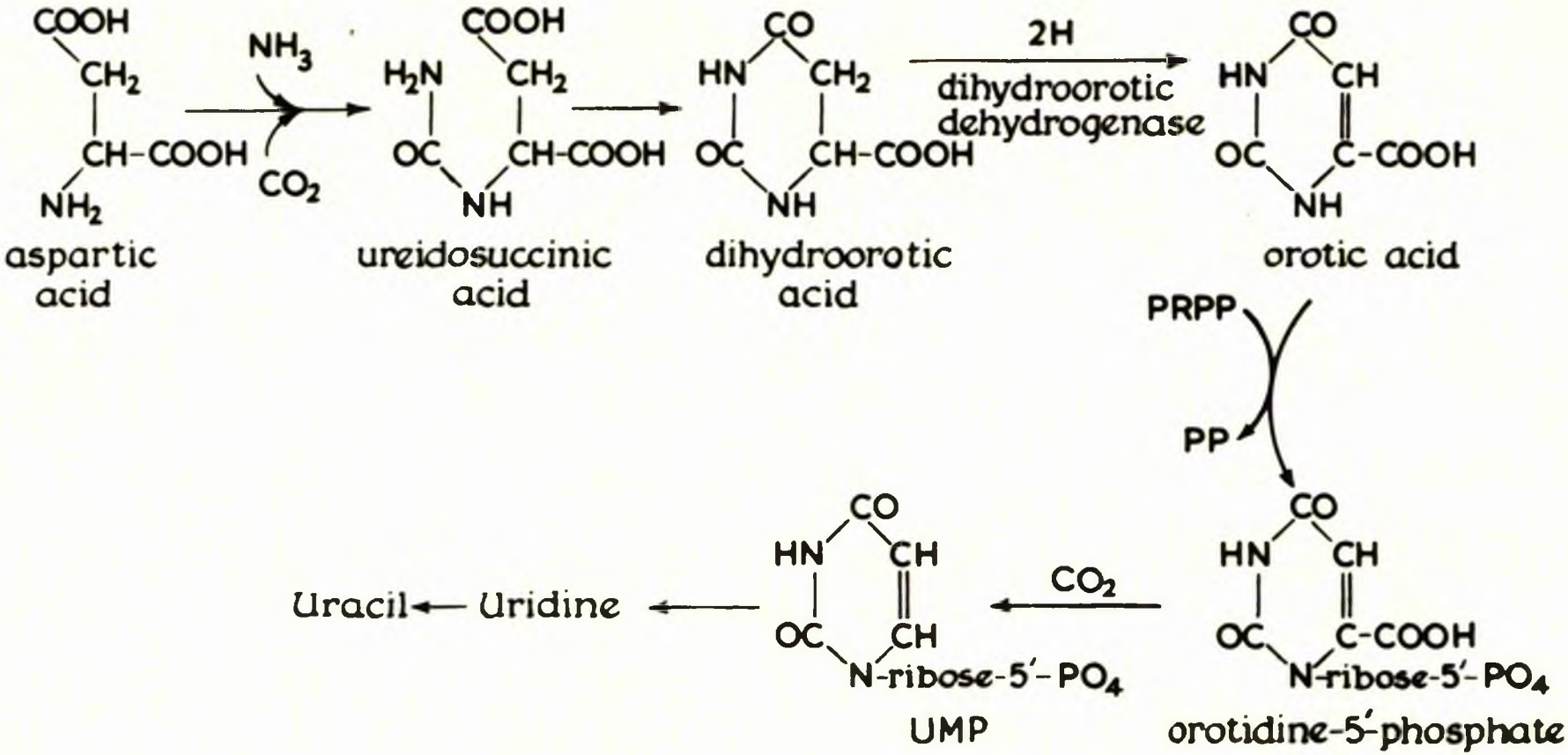
It is now generally believed that the deoxyribonucleotides are synthesised by the reduction of the corresponding ribonucleotide, without breakage of the glycosidic linkage. This was suggested by the in vivo experiments of Reichard and Estborn (1951) who found that cytidine was incorporated into DNA, and was confirmed by Rose and Schweigert (1953) who showed that labelled CMP was converted to dCMP and subsequently incorporated into DNA. These in vivo experiments were confirmed by work on soluble enzymes from avian, mammalian and bacterial sources. Reichard (1961) has recently shown that extracts of chick embryo form deoxycytidine phosphates from cytidine phosphates and deoxyguanosine phosphates from guanosine phosphates. The reaction is stimulated by the addition of ATP and is thought to proceed via the

Fig. 3.

The pathway of de novo synthesis of UMP.

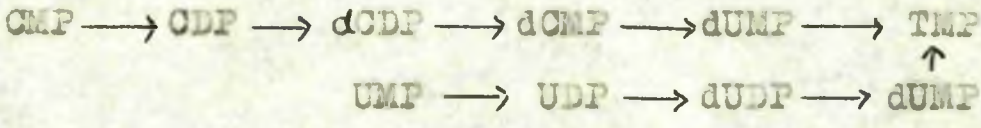
(From Davidson, J.N., 'The Biochemistry of the Nucleic Acids', 4th Ed., Methuen and Co.Ltd., London, 1960).

Fig. 3.


aspartic acid
ureidosuccinic acid
dihydroorotic acid
orotic acid
2H
dihydroorotic dehydrogenase
PRPP
PP
orotidine-5'-phosphate
UMP
Uridine
Uracil

ribonucleoside diphosphates as shown by Reichard, Baldesten and Rutberg (1961) in extracts of E. coli. The exact mechanism for the interconversion is unknown, although it is suspected that more than one enzyme is involved.

Thymidine presents a rather different situation from the other deoxyribonucleosides, in that the corresponding ribonucleoside does not participate in normal metabolic reactions. It can be produced by the reactions shown in the following scheme, which illustrates some of the ways in which the pyrimidine ribonucleotides can be converted to the deoxyribonucleotides.



(Canellakis, 1962).

(c) Synthesis of nucleoside diphosphates and triphosphates.

The known enzyme systems for the synthesis of polynucleotides require the nucleoside diphosphates or triphosphates, and thus it is important to consider the enzymes which convert the mononucleotides to these compounds. These enzymes are called nucleoside monophosphate kinases and nucleoside diphosphate kinases.

Enzymes which phosphorylate the ribonucleotides are known to be present in many sources, e.g. in yeast (Lieberman, Kornberg and Simms, 1955) and liver (Kornberg Lieberman and Simms, 1954).

The phosphorylation of the deoxyribonucleotides is carried out by enzymes which are known to be widely distributed. Maley (1958) has extensively purified an enzyme from A. vinelandii which catalyses the phosphorylation of dCMP by ATP. Canellakis and Mantsavinos (1958) have shown that soluble enzymes of rat liver, in the presence of ATP, can phosphorylate dAMP, dGMP, dCMP and TMP to the triphosphate level. There appears to be an interesting distinction between the enzymes phosphorylating TMP and others, since the activity of the latter alters only slightly under varied conditions of growth (Canellakis, Jaffe, Mantsavinos and Krakow, 1959), while the relative activities of the former vary from very low levels in normal rat liver to high levels in regenerating rat liver (Bollum and Potter, 1959). This suggests that the appearance of the enzymes converting TMP to TTP might be responsible for the initiation of DNA synthesis in liver regeneration, since thymine appears in the cell mainly in the form of thymidine and requires to

be phosphorylated before DNA synthesis can take place.

The mechanism of phosphorylation has been studied by Bianchi, Butler, Crathorn and Shooter (1961), who suggested that TMP is pyrophosphorylated directly to TTP. However, the investigations of Weissman, Smellie and Paul (1960) and Grav and Smellie (1962) suggest that TDP is an intermediate in the reaction sequence.

(d) Synthesis of RNA.

Three main types of enzyme system that catalyse the synthesis of polyribonucleotides have been discovered. The first uses nucleoside 5'-diphosphates with the elimination of inorganic phosphate; the second, using ribonucleoside 5'-triphosphates, adds a limited number of nucleotides to existing polynucleotide chains; the third, which also uses 5'-triphosphates, incorporates nucleotide residues into internucleotides linkage in polyribonucleotides.

The first of these enzymes, polynucleotide phosphorylase, was discovered in 1955 by Grunberg-Manago and Ochoa in extracts of A. vinelandii. On partial purification it was found that the enzyme catalyses the formation of high molecular weight polynucleotides. It

has been shown by chemical and enzymic studies that the synthetic polynucleotides yield products which are chemically indistinguishable from RNA (Singer, Heppel, Ochoa and Mii, 1959). The enzyme seems to be widely distributed in bacteria (Grunberg-Manago, Ortiz and Ochoa, 1956; Littauer and Kornberg, 1957; Brummond, Staehlin and Ochoa, 1957). Its occurrence has also been demonstrated in spinach leaves (Brummond, Staehlin and Ochoa, 1957). The evidence for its presence in animal tissues is somewhat tenuous. It has been reported to be present in Ascaris lumbricoides (Entner and Gonzalez, 1959), atypical epithelium of the rat (Yagi, Ozawa and Konogi, 1959) and in nuclei from guinea pig liver (Hilmoe and Heppel, 1957). The importance and even the existence of polynucleotide phosphorylase in animal cells is in some doubt, but it would seem to have some importance in bacteria, since it is so widely distributed and since it has been found that there is a relationship between the activity of the enzyme and the rate of RNA synthesis in synchronous cultures of A. agilis (Levin and Grunberg-Manago, 1960).

Enzyme systems from many sources are known to add nucleotide residues on to existing polynucleotide chains. The initial observation was made by Heidelberger,

Harbers, Leibman, Takagi and Potter (1956), who showed that ^{32}P-labelled AMP was incorporated into the RNA of rat liver cytoplasm. Later, Harbers and Heidelberger(1959) showed that RNA so labelled had a preferential linkage of the AMP to CMP. Several other biological systems have revealed similar reactions (Paterson and Lepage, 1957; Canellakis, 1957a, 1959; Edmonds and Abrams, 1957; Herbert, 1958; Preiss and Berg, 1960; Hurwitz, Bresler and Kaye, 1959). Zamecnik and his colleagues (Hoagland, 1960) have shown that such terminal addition reactions are an obligatory prelude to amino acid attachment in protein synthesis. Such an addition of a small number of nucleotide units to the end of an RNA chain cannot be regarded as polynucleotide biosynthesis, but it is an allied reaction.

In contrast to these reactions, Canellakis (1957b), using a rat liver system, showed that a UMP unit could be incorporated into a non-terminal position. Such incorporation would seem to be indicative of more extensive polynucleotide biosynthesis. Chung and Mahler (1959) found that preparations from chick embryo promote the incorporation of AMP, from ATP, into non-terminal positions. Subsequently it was found that this incorporation could be

considerably enchanced by GTP, CTP and UTP (Chung, Mahler and Enrione, 1960). Enzymes of this type, which incorporate a mononucleotide unit from a ribonucleoside 5'-triphosphate in the presence of the other three ribonucleoside 5'-triphosphates, are termed RNA nucleotidyltransferases (RNA polymerases). Such enzyme systems have been found in ascites tumour cells (Burdon and Smellie, 1961), pigeon liver microsomes (Straus and Goldwasser, 1961) and M. lysodeikticus (Nakamoto and Weiss, 1962). While these systems show a requirement for added RNA for incorporation, other systems are dependent on DNA. Such systems have been found in pea seedlings (Huang, Maheshwari and Bonner, 1960), HeLa cells and mouse fibroblasts (Goldberg, 1961), L. arabinosus and A. vinelandii (Ochoa, Burma, Kröger and Weill, 1961, M. lysodeikticus (Weiss and Nakamoto, 1961a), rat liver (Weiss, 1960) and E. coli (Stevens, 1961; Hurwitz, Bresler and Diringer, 1960; Furth, Hurwitz and Goldman, 1961).

DNA seems to play an important part in these reactions, since the base ratios of the product closely resemble those of the added DNA (Weiss and Nakamoto, 1961a), and the nearest neighbour sequences of the added DNA and the product resemble one another very closely

(Weiss and Nakamoto, 1961b). The importance of this system is probably that it provides a mechanism for the transfer of information from DNA to RNA.

(e) Synthesis of DNA.

In contrast to the several systems described for the synthesis of polyribonucleotides, only the reaction catalysed by the enzyme DNA nucleotidyltransferase (DNA polymerase) is known for the formation of polydeoxyribonucleotides, although Krakow, Coutsogeorgopoulos and Canellakis (1962) have described an enzyme which catalyses the incorporation of deoxyribonucleotides or ribonucleotides into the terminal positions of DNA.

DNA nucleotidyltransferase, which catalyses the incorporation of deoxyribonucleotides from the 5'-triphosphates of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine into DNA, was first described by Lehman, Bessman, Simms and Kornberg (1958) in extracts of E. coli. They purified the enzyme several thousand-fold and showed that for activity all four deoxyribonucleoside triphosphates, highly polymerised DNA and Mg^{++} are required. They also found that the newly synthesised DNA was produced in amounts that exceeded the amount of

DNA added by 10-fold. Analysis of the digests of enzymically synthesised DNA showed that it contained the 3'-5' phosphodiester linkage characteristic of DNA isolated from natural sources (Bessman, Lehman, Simms and Kornberg, 1958). The synthesis of DNA is accompanied by the release of pyrophosphate. The synthetic material cannot be distinguished from native DNA by physical techniques such as ultracentrifugation, viscosity and ultraviolet hypochromicity (Lehman, 1959). The ratio of the number of adenine-thymine pairs to the number of guanine-cytosine pairs is the same in the synthetic and in the primer DNA, even when the concentrations of substrates vary widely, and when the amount of DNA synthesised varies from 2% to 1,000% of the primer (Lehman, 1959). Further evidence for the similarity between the newly synthesised DNA and the primer is obtained from studies of the frequencies of the nearest neighbour base sequences. Josse, Kaiser and Kornberg (1961) have shown that different DNAs direct the synthesis of products which have unique and non-random patterns of the 16 nearest neighbour frequencies. Further, the synthetic DNA has the same nearest neighbour frequencies whether the primer is native DNA or enzymically prepared DNA containing only traces of the original native

DNA. The pattern of the nearest neighbour frequencies in every case involves both base-pairing of the adenine to the thymine and of the cytosine to the guanine, between sister strands of the DNA, and opposite polarity of the two strands, as proposed by Watson and Crick. This evidence clearly indicates that the DNA nucleotidyl-transferase provides a suitable mechanism for DNA replication.

Enzyme systems of this type have been found in other sources. Bollum (1958, 1960) has shown it to be present in regenerating rat liver and calf thymus extracts. The enzyme from calf thymus has been purified about 40-fold and shows a requirement for all four deoxyribonucleoside triphosphates and for Mg^{++}, which is similar to the E. coli enzyme. The enzyme has also been purified from ascites tumour cells (Keir, Binnie and Smellie, 1962; Keir, 1962).

One of the most important aspects of the DNA nucleotidyltransferase system is the type of primer required, since this obviously relates to the mechanism whereby DNA is replicated in vivo. When DNA is heated to 100° for 10 minutes, it becomes twice as effective as a primer for the E. coli enzyme (Lehman, 1959). Under Bollum's (1960) conditions, unheated DNA is less than 1%

as effective as heated DNA. Keir, Binnie and Smellie (1962) found that thermally denatured DNA is about seven times as effective as primer as is native DNA. It is interesting to note that after the infection of E. coli with T2 bacteriophage, the DNA nucleotidyltransferase which is produced has several characteristics which differ from those of the normal E. coli enzyme. Thus, denatured DNA primes the T2 transferase 10 times as effectively as does unheated DNA (Aposhian and Kornberg, 1962).

Treatment with minute amounts of pancreatic DNase will also increase the activity of DNA as primer for E. coli and ascites DNA nucleotidyltransferases (Lehman, 1959; Keir, Binnie and Smellie, 1962).

This mechanism for the replication of DNA fits well with the concept, which is now generally accepted, that DNA is the carrier of genetic information, since it provides a means whereby the exact sequence of bases along the DNA chain can be copied. The two strands of DNA are complementary and each strand forms a template on which a structure identical with the initial complementary strand can be built up. Meselson and Stahl (1958) provided some direct proof for this mechanism

by labelling the DNA of E. coli with heavy nitrogen to such an extent that it could be differentiated from non-labelled DNA by ultracentrifugation in a caesium chloride gradient. They showed that when the labelled cells were allowed to grow for one generation time in a medium containing ^{14}N, only hybrid molecules whose density was that expected for a half labelled molecule were produced. Subsequent generations contained only hybrid and completely unlabelled molecules. This work is consistent with the hypothesis that single strands of DNA remain intact but become physically separated during the replication process, and is inconsistent with the two alternatives, i.e. that the double helix remains completely intact throughout the replication process, or that the strands are fragmented and pieces of the original DNA dispersed throughout subsequent generations.

Various mechanisms for the unwinding of the double helix have been postulated (Platt, 1955; Levinthal and Crane, 1956), but it is not certain which, if any, is correct.

I. 3 Catabolism of the nucleic acids.

(a) General.

The enzymes that hydrolyse the internucleotide

bonds of both DNA and RNA are known as phosphodiesterases, and this term can equally be applied to enzymes which hydrolyse DNA or RNA specifically. Most of the enzymes known to hydrolyse both RNA and DNA are exonucleases, i.e. the enzyme hydrolyses mononucleotide units successively from one end of the nucleotide chain. These contrast with DNase and RNase, which are endonucleases, i.e. the enzyme attacks internucleotide linkages within the molecule. Laskowski (1959) has proposed that nucleolytic enzymes should be classified on the basis of four criteria:

I Susceptible substrate, i.e. RNA or DNA or both.
II Type of attack, i.e. exonuclease or endonuclease.
III Products, i.e. 3'-phosphate terminated or 5'-phosphate terminated.
IV Preferential linkage, i.e. specific purine or pyrimidine for point of attack.

(b) Enzymes hydrolysing both RNA and DNA.

Enzymes which hydrolyse both RNA and DNA are known to be fairly widely distributed. The first to be discovered was that in snake venom, which was first demonstrated by Uzawa (1932) and later Gulland and

Jackson (1938) showed that ribonucleoside 5'-phosphates were produced by the action of this enzyme on RNA. Several methods have been described for the purification of venom diesterase (Privat de Garilhe and Laskowski, 1955; Hurst and Butler, 1951; Koerner and Sinsheimer, 1957; Boman and Kaletta, 1957; Djork and Boman, 1959; Razzell and Khorana, 1959; Laskowski, 1959) and probably one of the most reliable is described by Laskowski and his colleagues (Felix, Potter and Laskowski, 1960; Williams, Sung and Laskowski, 1962; Sulkowski and Laskowski, 1962). The enzyme requires Mg^{++} or Ca^{++} for activity and is inhibited by high NaCl and substrate concentrations (Williams, Sung and Laskowski, 1962). Venom diesterase is capable of acting on a range of compounds varying from highly polymerised DNA to dinucleotides. Boman and Kaletta (1956, 1957) first showed that the enzyme could hydrolyse thymus DNA, and their observations were confirmed by Williams, Sung and Laskowski (1962) who demonstrated that purified diesterase quantitatively degrades intact thymus DNA to mononucleotides. At the opposite end of the scale, Razzell and Khorana (1959a) showed that pTpT (for explanation of this notation see list of abbreviations) was rapidly hydrolysed

by venom diesterase. The presence in the substrate of a free 5'-phosphomonoester end group greatly accelerates the reaction, and the 5'-phosphoryl terminal oligonucleotides are hydrolysed more rapidly than the corresponding 5'-hydroxyl compounds (Privat de Garilhe and Laskowski, 1956; Razzell and Khorana, 1959a). The enzyme will also hydrolyse polyribonucleotides and polydeoxyribonucleotides bearing a 3'-phosphate group at a very slow rate, but the mechanism of this hydrolysis is not clear (Volkin and Cohn, 1953; Turner and Khorana, 1959).

Kinetic studies on polyribonucleotides (Singer, Hilmoe and Heppel, 1958) and on synthetic deoxyribooligonucleotides (Razzell and Khorana, 1959b) bearing 3'-hydroxyl groups have indicated that hydrolysis begins from the 3'-hydroxyl end and results in the successive release of 5'-phosphate units.

Other phosphodiesterases which hydrolyse RNA and DNA to their constituent nucleotides are also known. For instance, Carter (1951) described an enzyme in extracts of intestinal mucosa that can form deoxyribonucleoside 5'-phosphates from DNA.

Another group of phosphodiesterases is typified by the enzyme found in spleen which splits polyribonucleo-

tides and polydeoxyribonucleotides to form nucleoside 3'-phosphates. This enzyme readily attacks 5'-hydroxyl terminated ribo- and deoxyribooligonucleotides in a stepwise fashion from the 5'-hydroxyl end and releases nucleoside 3'-phosphates (Razzell and Khorana, 1958). Using the synthetic substrate p-nitrophenyl thymidine 3'-phosphate, Razzell (1961a) has shown that enzymes of this type are widely distributed in animal tissues.

Enzymes having a predominantly endonucleolytic action which break down both RNA and DNA are also known. Sung and Laskowski (1962) have purified a nuclease from mung bean sprouts which hydrolyses RNA and DNA at the same rate. This enzyme preferentially attacks the pA-pX linkage, and the products of the reaction are terminated in 5'-phosphoryl and 3'-hydroxyl end groups. It can also hydrolyse some dinucleotides to mononucleotides, d-pApA being the most susceptible. Another enzyme, from A. agilis, which hydrolyses DNA and RNA to oligonucleotides with 5'-phosphomonoester end groups has been described by Stevens and Hilmoe (1960).

A different type of nuclease has been obtained from M. pyogenes. This enzyme, known as micrococcal nuclease, hydrolyses DNA and RNA to 3'-phosphoryl terminal

oligonucleotides and has been shown by Williams, Sung and Laskowski (1962) and Alexander, Heppel and Hurwitz (1961) to have a predominantly endonucleolytic action. However, the relative activities towards RNA and DNA change during the course of the purification and it is possible that the preparations contain two different enzymes (Pochon and Privat de Garilhe, 1960; Alexander, Heppel and Hurwitz, 1961). It is of particular interest that this preparation attacks heat denatured DNA much more rapidly than native DNA (Dirksen and Dekker, 1960).

(c) Enzymes specific for RNA (ribonucleases).

Enzymes which hydrolyse RNA, but not DNA, have been shown to be very widely distributed. Most of these enzymes catalyse the reaction in which the phosphodiester bond between the 3'- and the 5'- positions of the ribose moieties in the RNA chain are cleaved, with the formation of oligonucleotides terminating in 2', 3' cyclic phosphate derivatives. The terminal groups are split off as free mononucleotide cyclic phosphates which are then hydrolysed to give the corresponding nucleoside 3'-phosphates (Brown and Todd, 1955; Schmidt, 1955; Markham, 1957). Enzymes of this type have been isolated from plant, animal and

bacterial sources. Almost all possess a high degree of heat stability. They vary, however, in their pH optima and in specificity towards particular internucleotide bonds. Much of the work on ribonuclease has been concerned with those enzymes found in pancreas, which have been highly purified. These enzymes have a specificity for those phosphodiester bonds that involve the nucleosides of cytosine and uracil (Brown and Todd, 1955). The complete amino acid sequence of bovine pancreatic ribonuclease has been determined (Hirs, Moore and Stein, 1960).

(d) Enzymes specific for DNA (deoxyribonucleases).

The DNases are usually classified into two groups: the DNase I type of enzyme which has an alkaline pH optimum and gives rise to 5'-phosphoryl terminal oligonucleotides and the DNase II type which has an acid optimal pH and produces 3'-phosphoryl terminal oligonucleotides. Not all the known DNases can be put into one or other of these classifications, however, and the two terms are coming to be associated more with the nature of the products of the reaction and less with pH optimum.

The classical enzyme of the DNase I type is the

crystalline enzyme from bovine pancreas (Kunitz, 1948). The pH optimum of this enzyme is around 7.0 (Kunitz, 1950), it has a requirement for a divalent cation, Mg^{++}, Mn^{++} , or Co^{++} (Laskowski and Seidel, 1945; McCarty, 1946; Greenstein, Carter and Chalkley, 1947) and Ca^{++} has a powerful synergistic effect in the presence of Mg^{++} (Wiberg, 1958). DNase I is inhibited by cations which remove the activating anions, e.g. citrate, arsenate, fluoride or versene (McCarty, 1946; Gilbert, Overend and Webb, 1951; Feinstein and Green, 1956).

Alterations to the structure of the substrate DNA make it less susceptible to the action of DNase I. Thus, heat denaturation reduces the sensitivity of the DNA to attack, as measured by methyl green binding (Kurnick, 1954b) or release of protons (Dirksen and Dekker, 1958). The removal of some of the purine or pyrimidine bases (Tamm and Chargaff, 1951; Takemura, 1959) also decreases the hydrolytic rate.

The products of the action of pancreatice DNase I are mononucleotides and oligonucleotides varying in chain length from 2 to 8 units (Sinsheimer and Koerner, 1951, 1952a and b; Potter, Brown and Laskowski, 1955; Sinsheimer, 1954; Privat de Garilhe and Laskowski, 1955).

Evidence from many sources points to the fact that the d-pPu-pPy linkage is preferentially attacked, that a sequence of purines is resistant to the enzyme and that the absence of dCMP may hinder hydrolysis (Laskowski, 1961).

There is evidence for the presence in many other tissues of enzymes with properties similar to DNase I. Thus, Shack (1957) has demonstrated DNase activity at low salt concentrations and with a Mg^{++} requirement in various mouse tissues. However, there is no evidence and indeed it seems unlikely that all the enzymes with properties similar to DNase I are identical proteins or even have a similar mode of action.

An enzyme (streptodornase) resembling pancreatic DNase I has been obtained from the streptococcal group of bacteria. It has a very similar Mg^{++} requirement and pH optimum to the pancreatic enzyme (Tillet, Sherry and Christensen, 1958; McCarty, 1948) and forms oligonucleotides terminated by 5'-monoesterified phosphate. Stone and Burton (1961) have shown that streptodornase requires the synergistic action of two divalent cations, e.g. Ca^{++} and Mg^{++} for optimal activity. Potter and Laskowski (1959) have tentatively concluded that the enzyme

preferentially attacks the d-pPy-pPu bond. In other studies, Georgatsos, Unterholzner and Laskowski (1962) have shown that deoxyguanosine predominates at the 5'-phosphate terminal end of the oligonucleotides formed from DNA by streptodornase, whereas at the 3'-hydroxyl end there is a more random distribution of the bases.

The studies of Lehman and his colleagues on the DNases of E. coli have made a very important contribution to the understanding of this group of enzymes. Three distinct enzymes which degrade DNA have been demonstrated. The first of these (Lehman, 1960) is an exonucleolytic phosphodiesterase which hydrolyses heated DNA at a much more rapid rate than native DNA, and proceeds in a stepwise fashion from the 3'-hydroxyl end of the chain, producing deoxyribonucleoside 5'-monophosphates until the terminal dinucleotide is reached, when cleavage ceases. The enzyme cannot hydrolyse free dinucleotides. It has an optimal pH of 9.2 to 9.8, and requires Mg^{++}.

The second enzyme is an endonuclease which degrades native DNA seven times as rapidly as heat denatured DNA. It gives rise to a limit digest in which the average chain length of the oligonucleotides is seven. In crude extracts the enzyme is bound to inhibitory RNA, which is

removed in the process of purification (Lehman, Roussos and Pratt, 1962a). While RNAs from many sources can inhibit this enzyme, the most potent of those tested was the sRNA from E. coli. Polynucleotides, synthesised by polynucleotide phosphorylase do not inhibit. This enzyme is readily distinguishable from most other DNases which are not affected by RNA (Lehman, Roussos and Pratt, 1962b).

The third nuclease from E. coli, which has been described only briefly (Lehman, 1962), attacks DNA in the presence of RNA and preparations of the enzyme purified 2,000-fold with respect to nuclease activity are enriched to an equal extent with DNA nucleotidyltransferase activity. Native DNA is hydrolysed at about 5 times the rate of heat denatured DNA, and the attack on the synthetic dAT copolymer is exonucleolytic, with a quantitative release of 5'-nucleotides.

The enzyme of the DNase II type which has been studied most extensively is that obtained from spleen. It was first described by Catcheside and Holmes (1947) and a similar enzyme was later demonstrated in thymus gland by Maver and Greco (1949). Many methods have been described for the purification of the enzyme from spleen,

but the procedure most commonly employed is that described by Sinsheimer and Koerner (1957). DNase II activity has now been shown to be present in many tissues (Shack, 1957).

It is not possible to define the pH optimum of the DNase II type of enzyme with accuracy, since this has been found to be dependent on the method of estimation, on the cations present and on the ionic strength of the medium (Oth, Fredericq and Hacha, 1958; Koerner and Sinsheimer, 1957). Splenic DNase II has a very small requirement for Mg^{++} (of the order of 1mM), and there is usually sufficient Mg^{++} in the substrate to satisfy this requirement (Laskowski, 1961). For optimal activity a monovalent cation at the concentration of 0.2 to 0.3M is required (Koerner and Sinsheimer, 1957). The products of hydrolysis are terminated in 3'-phosphate groups and the digest contains mono- di- and oligonucleotides (Laurila and Laskowski, 1957). It has not yet been determined unequivocally whether any linkage in DNA is preferentially attacked by DNase II, but Laurila and Laskowski (1957) have suggested that the Pyp-Pup linkage is particularly susceptible.

(e) Breakdown of the mononucleotides.

There are many enzymes present in plant, animal

and bacterial sources which are capable of removing phosphate from the ribo- and deoxyribonucleoside 5'- and 3'-monophosphates. However, many of these are relatively non-specific phosphatases which will break down a wide variety of phosphate esters. Enzymes specific for the hydrolysis of 5'- and 3'- mononucleotides are also known. Reis (1934, 1937, 1938) showed that 5'-nucleotidase was widespread in mammalian tissues, and the enzyme has been studied particularly in snake venom and bull semen. In both cases the enzyme can hydrolyse the ribo- and the deoxyribonucleotides. A different 5'-nucleotidase has been demonstrated in microsomes which hydrolyses 5'-AMP 10 times more rapidly than 5'-dAMP (Segal and Brenner, 1960).

Enzymes which specifically dephosphorylate 3'-ribonucleotides have been found in rye grass, barley and Taka Diastase (Shuster and Kaplan, 1955). These enzymes show only slight activity towards 3'-deoxyribonucleotides (Cunningham, 1958).

The breakdown of nucleosides to the constituent bases and sugar phosphates or free sugars is catalysed by enzymes known as nucleoside phosphorylases or nucleoside hydrolases. Kalckar (1945) first recognised the

phosphorolytic mechanism of nucleoside cleavage. Since then enzymes have been found in a wide variety of sources which can catalyse the formation of a free base and a sugar phosphate from nucleosides (Friedkin and Kalckar, 1961), and it seems certain that purine nucleoside phosphorylases differ from the pyrimidine nucleoside phosphorylases. Nucleoside hydrolases have so far been demonstrated in yeast (Heppel and Hilmoe, 1952; Carter, 1951) and in L. pentosus (Lampden and Wang, 1952).

Further degradation of the bases and nucleosides takes place under the influence of specific enzymes. Adenine and guanine are deaminated by adenase and guanase to give hypoxanthine and xanthine respectively. Adenase does not seem to occur very widely in the tissues of higher animals, although adenosine deaminase does, and it is possible that deamination takes place at the nucleoside level (Schmidt, 1955). Guanase, however, is found in the tissues of many higher animals. Both xanthine and hypoxanthine are degraded by xanthine oxidase to uric acid. This, according to species, may or may not be converted to allantoin by the enzyme uricase. Some fish, amphibia and more primitive organisms are capable of degrading allantoin to allantoic acid which may be further degraded to ammonia and urea.

Uracil and thymine are broken down to give alanine and β-amino isobutyric acid respectively. This proceeds by reduction of the pyrimidine ring to the dihydro derivative, ring opening to give the ureido-acid and removal of ammonia and CO_2. (Schulman, 1954; Canellakis, 1957c; Fink, Cline, Henderson and Fink, 1956).

1. 4 The control of DNA biosynthesis.

It is particularly important that the mechanism of biosynthesis of DNA should be closely controlled, since the preservation of the genetic material of the cell is of prime importance in the processes of cellular proliferation and function. It is well known that there is little or no renewal of DNA except in preparation for cell division and control of the synthetic mechanism presumably lies in the availability in the correct form and at the correct time and place of the various substrates.

Various enzymic differences have been demonstrated between proliferating and non-proliferating tissues and it is possible that at least some of these constitute a means whereby control can be exercised. These differences have been shown at various stages of the biosynthesis of DNA from the synthesis of the bases to the final polymerisation reaction.

Several feedback mechanisms are known to be concerned with the reactions involved in the synthesis of nucleic acids. Both AMP and GMP can inhibit the first specific enzyme in the purine biosynthetic pathway, phosphoribosylpyrophosphate aminotransferase (Wyngaarden and Ashton, 1959). Further, the intracellular pools of adenine and guanine derivatives are maintained at constant levels, both in absolute amounts and in relation to one another, since they can mutually inhibit the interconversion of adenine and guanine derivatives (Magasanik and Karibian, 1959). In a similar fashion, CMP has a controlling influence in pyrimidine nucleotide biosynthesis by its ability to inhibit aspartic carbamyl transferase, the first specific enzyme in the pathway for the biosynthesis of the pyrimidines (Yates and Pardee, 1956).

At a higher level, Reichard, Canellakis and Canellakis (1960) have shown that in chick embryo the conversion of CDP to dCDP is inhibited by very low concentrations of dGTP, dATP and TTP. Munch-Petersen (1960) has reported that dATP inhibits the reduction of GMP to dGMP in ascites tumour cells. Potter (1962a) has shown that TTP exerts a negative feedback on the TdR kinase.

The activity of certain enzymes involved in DNA biosynthesis differs in proliferating and non-proliferating tissues. The variation in the activity of the kinases phosphorylating TdR to TTP has already been described (see section I.2(c)). Weissman, Smellie and Paul (1960) found that in regerating rat liver the kinases associated with the phosphorylation of TdR to TTP appear sequentially and suggested that this could be due to enzyme induction and that the enzymes converting TdR to TTP are specifically concerned with the processes relating to cell division.

The enzyme dCMP deaminase, which gives rise to dUMP, from which TMP is formed by TMP synthetase, has been widely studied in normal tissues, tumours and cultured cells. Scarano (1958) and Maley and Maley (1959) found that the activity of this enzyme varies from low, but measurable, amounts in normal tissues to high levels in regenerating rat liver and tissues undergoing active growth. Potter (1962b) has reviewed the occurrence of this enzyme in normal tissues and in various hepatomas, and has shown that the activity of the enzyme in the tumours varies from very low levels, comparable to those found in normal tissues, to the very high level found in the Novikoff hepatoma. It is not clear, therefore,

whether or not this enzyme plays an important part in the regulation of DNA biosynthesis. Maley and Maley (1960) have also shown that the activity of the TMP synthetase, which converts dUMP to TMP, shows variations analagous with those found with dCMP deaminase.

Control over DNA biosynthesis could also be exerted by the rapid catabolism of DNA precursors in non-proliferating tissues. Stevens and Stocken (1960) have shown that foetal rat liver cannot degrade uracil, but as the age of the animal increases the activity of the degradative enzymes increases. In the Dunning and Novikoff hepatomas the catabolic enzymes are lacking (Potter, Pitot, Ono and Morris, 1960).

In spite of these possible control mechanisms, the final control of DNA biosynthesis must lie in the presence and activity of the DNA nucleotidyltransferase. The activity of the transferase has been shown to be highest in tissues which are proliferating, such as bone marrow and thymus, and lowest in non-proliferating tissues such as liver, brain and kidney (Smellie, 1961). Gray, Weissman, Richards, Bell, Keir, Smellie and Davidson (1960) showed that the DNA nucleotidyltransferase of Ehrlich ascites tumour cells was less effective in the presence

of extracts of liver and kidney and of calf serum, but that the enzyme was not affected by extracts of tissues such as muscle and brain.

It was therefore the purpose of the present study to investigate the factors causing this inhbition, since it seemed that they could be of importance in the control of DNA biosynthesis.

SECTION II

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

Section II. Experimental.

2. 1 DNA nucleotidyltransferase.

(a) Preparation of enzyme from calf thymus glands.

An extract with DNA nucleotidyltransferase activity was prepared from calf thymus glands by the method of Bollum (1960).

Calf thymus glands were obtained immediately after slaughter and packed in ice for transport to the laboratory. All subsequent operations were carried out at 0-4°. The glands were cut into small pieces and washed with 0.15M NaCl in a gauze bag. Using a Waring Blendor, portions of the glands were homogenised with 3 vol. of buffered sucrose medium, for 60 sec. at alternate 15 sec. periods of low and high speed. The homogenisation medium consisted of 1 l. of 0.10M tris, pH 8.1, and 24 ml. 1M KCl, diluted to 4 l. with water. After the homogenate had been centrifuged at 15,000 x g in a Servall refrigerated centrifuge, the supernatant solution was centrifuged at 105,000 x g in a Spinco Model L ultracentrifuge. 2 ml. aliquots of the supernatant solution were dispensed into small test tubes, and these were stored at -20° and thawed when required. It was found that only a little of the activity was lost on storage of the extract for a year.

(b) Assay of activity.

DNA nucleotidyltransferase was assayed by measuring the incorporation of ^{32}P-TMP, from TdR-^{32}P-P-P into DNA by the method of Bollum (1959), modified slightly. The details of the media used for the assays will be found in the results section with the individual experiments. Incubation was carried out in 4 x ½" rimless test tubes, sealed with "parafilm", in a water bath at 37°. At the end of the incubation period, the tubes were either stored in ice, if they were to be assayed at once, or frozen in an ethanol/solid CO_2 mixture and placed in a deep freeze at -20°, if they were to be stored before assay. 0.05 ml. portions of the incubation medium were pipetted on to numbered discs of Whatman No.1 filter paper, 1 in. in diameter. The discs were dropped into a beaker containing ice-cold 5% (w/v) trichloracetic acid (15 ml./disc), and 10 min. after the last disc had been added, the TCA was poured off and replaced by an equal volume of fresh acid. This washing procedure was carried out three times and the discs allowed to remain in each batch of TCA for 10 min. After the last acid wash had been decanted, the discs were washed with 95% ethanol and allowed to dry. They were then counted in a Nuclear Chicago windowless gas flow

counter in the Geiger region. Usually each estimation was carried out in duplicate and 3 discs plated from each incubation tube.

2. 2 Preparation and fractionation of tissue extracts.

(a) Crude extracts.

Male albino rats, weighing from 180 to 220 g., from the departmental colony were used. They were anaesthetised with ether and the required tissue removed after severing the hepatic artery and superior vena cava, and allowing the blood to drain. Immediately after removal the tissue was chilled on ice, cut into small pieces with scissors, weighed and homogenised in 8 vol. cold 0.01M tris, pH 7.5, in a cooled Potter-type homogeniser (Potter and Elvehjem, 1936). After the initial suspension of the tissue, about five passes of the homogeniser pestle were found to give an extract relatively free from whole cells, as judged from microscopic examination of wet smears, stained with 1% crystal violet in 0.1M citric acid. The homogenate was centrifuged in a Servall refrigerated centrifuge at 27,000 x g for 1 hr., and the slightly cloudy supernatant solution decanted. This constituted the crude extract.

(b) Preparation of fractions from crude extracts.

Crude extracts of liver were fractionated with acid, ammonium sulphate and acetone, both for the purposes of purification and for the examination of relative inhibitory and phosphatase activities.

Acid fractionation was carried out on crude liver extracts as follows : The pH was adjusted to the required value with 0.1N acetic acid, at 0^{o} with stirring, and the solution centrifuged at 7,700 x g for 8 min. The precipitate was dissolved in 0.01M tris, pH 7.5, and the procedure repeated with the supernatant solution to give as many fractions as required. After the final precipitation, the pH of the supernatant solution was adjusted to the initial pH with 0.5N KOH.

Ammonium sulphate fractionation was carried out on the redissolved acid precipitate. Solid ammonium sulphate was added to the required concentration, and, after allowing the solution to stand for 20 min. at 0^{o}, it was centrifuged at 7,700 x g for 7 min. The precipitate was redissolved in 0.01M tris, pH 7.5, and dialysed overnight against 7 l. 0.01M tris, pH 7.5

(c) Procedure adopted for the purification of the inhibitory factor from rat liver.

All operations were carried out at 0-4°. The pH of the crude liver extract (prepared as in section 2.1a), which was usually around 7.2, was measured and adjusted, with constant stirring, to 4.5 with 0.1N acetic acid. The solution was centrifuged at once in the Servall centrifuge at 7,700 x g for 8 min. The precipitate, cream in colour, was suspended in a volume of 0.01M tris, pH 7.5, equal to the volume of the original crude extract. After stirring for 1 to 2 hr., a cloudy solution, with no obvious large particles, was obtained, whose protein concentration was about 5 mg./ml.

This solution was fractionated with a saturated solution of ammonium sulphate adjusted to pH 7.5 with ammonium hydroxide. A volume of saturated ammonium sulphate such that the final concentration of ammonium sulphate was equivalent to 0.4 saturation was added slowly with stirring, and the solution allowed to stand for 20 min. and centrifuged at 7,700 x g for 8 min. in the Servall centrifuge. The precipitate was discarded and the supernatant solution taken to 0.6 saturation with saturated ammonium sulphate (pH 7.5). After standing for 20 min.,

the solution was centrifuged at 7,700 x g for 20 min., and the supernatant solution decanted. The precipitate was dissolved in 30 ml. 0.01M tris, pH 7.5. This solution had a protein concentration of approximately 3.5 mg./ml. The ammonium sulphate concentration of this solution was measured using Nessler's reagent, and the concentration adjusted to 80 mg./ml. with solid ammonium sulphate, so that the ensuing acetone fractionation would take place at a standard salt concentration, since this was found to affect the precipitation. The ammonium sulphate fraction was divided into 2 x 15 ml. portions in 50 ml. centrifuge tubes, previously cooled to -15° in a bath of ethylene glycol. 2.5 ml. of 1M tris, pH 6.5, and 10.5 ml. acetone (36.9% v/v) were added to each tube. After stirring, the solutions were allowed to stand for 5 min., and were centrifuged at 700 x g for 15 min. at -15°. After centrifugation, the supernatant solutions were decanted into cooled 50 ml. tubes, and a further 4.5 ml. acetone at -15° (45.4% v/v) added. The solutions were stirred, allowed to stand and centrifuged as before. After centrifugation the precipitate was present as a thin

layer at the interface of two solutions. The layers of liquid were removed successively by tilting the tube very carefully, leaving the interfacial protein adhering to the tube. The protein was dissolved in 10 ml. of 0.01M tris, pH 7.5. The solution, which usually contained about 2 mg. protein/ml., was dialysed for 16 hr. against 7 l. of 0.01M tris, pH 7.5, at the end of which it was ready for use. The whole fractionation was carried out on one day. If the material was to be stored it was dispensed in 1 ml. portions in small test tubes and kept at -20°. It was found to be stable for up to three weeks. After this, the activity usually started to decline.

The inhibitory activity of the fractions was measured by adding them to DNA nucleotidyltransferase assays. One unit of inhibitory activity was defined as the amount of protein which, when added to a DNA nucleotidyltransferase assay, caused an inhibition of the incorporation of ^{32}P-TMP into DNA of 30%. Usually an amount of inhibitory protein corresponding to one unit was added to the assays, since it was found that inhibition of the transferase with respect to protein concentration was only linear up to about 40% inhibition, after which the rate of increase of inhibition, with respect to protein concentration started to decline.

2. 3 Nucleic acids.

(a) The preparation and denaturation of DNA.

Preparations of DNA from both Landschutz ascites carcinoma cells and calf thymus glands were used during the course of these investigations. Ascites tumour cell DNA was used almost exclusively as a primer in the assay of the DNA nucleotidyltransferase, and, unless stated otherwise, the term "DNA" implies ascites tumour cell DNA.

Extracts of Landschutz ascites tumour cells were prepared by the method of Keir, Binnie and Smellie (1962) and, from the sediment obtained by the centrifugation of such extracts at 105,000 x g for 1 hr., DNA was prepared by the method of Kay, Simmons and Dounce (1952). DNA was also prepared from calf thymus glands by this method. The DNA obtained was usually dissolved in water to give a final concentration of 2 mg./ml.

Thermally denatured DNA was prepared by heating a solution (containing 2 mg./ml. in water) in a boiling water bath for 10 min., and then cooling rapidly by placing in an ice-bath.

(b) Purification of commercial yeast RNA.

Yeast RNA was prepared by a modification of the method

described by Kirby (1956). 2 g. commercial RNA (free acid) were suspended in water and 0.1N NaOH added until the pH was approximately neutral, at which point most of the RNA had dissolved. The solution (100 ml.) was made 2% with respect to potassium acetate and 2 vol. absolute ethanol added. The heavy, flocculent precipitate obtained was centrifuged down at 600 x g for 10 min., and then washed with 100 ml. 2% potassium acetate and 200 ml. absolute ethanol. The precipitate was then dissolved in 50 ml. water, made 2% with respect to potassium acetate and 100 ml. ethanol added. The precipitate was then centrifuged down at 400 x g for 3 min. This latter procedure was repeated twice more and the precipitate washed with ethanol, ethanol/ether (3/1) and ether and dried in air.

30 mg. of this precipitate were dissolved in 10 ml. 0.01M tris, pH 7.5, and dialysed overnight against 2 l. 0.01M tris, pH 7.5. The volume was adjusted so that the final concentration was 2 mg./ml. This solution gave only a very small amount of acid-soluble material when precipitated with perchloric acid, as measured by the absorption at 260 mμ.

2. 4 Chemical preparation of nucleotides.

(a) ^{32}P-TTP. (Modified from Smith and Khorana (1958))

TTP, labelled with ^{32}P in the proximal phosphate group, was synthesised in three stages:-

1. Cyanoethylphosphate (^{32}P-CEP) was prepared from ^{32}P-labelled phosphoric acid and cyanoethanol.
2. ^{32}P-TMP was prepared from ^{32}P-CEP and TdR.
3. ^{32}P-TTP was prepared from ^{32}P-TMP and unlabelled phosphoric acid.

1. Preparation of ^{32}P-CEP.

Labelled phosphoric acid was prepared by mixing 50 mC of radioactive sodium orthophosphate (PBS1, obtained from the Radiochemical Centre, Amersham) with 1 mmole of unlabelled phosphoric acid in dilute aqueous solution. This solution was then taken to dryness in vacuo at 40° in a flash evaporator, the labelled phosphoric acid dissolved in 10 ml. of anhydrous pyridine (prepared by storing over calcium hydride) and 1 ml. of cyanoethanol added. The solution was evaporated to an oil at 40° in vacuo, the residue redissolved in a further 10 ml. of anhydrous pyridine and the evaporation repeated.

The residue was dissolved in 5 ml. anhydrous pyridine

and 2.1 g. of dicyclohexylcarbodiimide (DCC) added. The reaction flask was stoppered securely and left overnight at room temperature. After adding 5 ml. of water to stop the reaction, the flask was heated in a boiling water bath for 30 min. The mixture was taken to dryness at 40° in vacuo, 10 ml. water and 10 ml. saturated barium hydroxide added and, after 5 min. at room temperature, the pH of the solution was adjusted to 7.5 with glacial acetic acid. The solution was filtered to remove dicyclohexylurea and barium phosphate, the precipitate washed with water and the washings added to the bulk solution. The ^{32}P-CEP was precipitated from the aqueous solution by adding 2 vol. ethanol, stirring and leaving at 0° for 1 hr. The CEP crystals were collected by centrifugation and the ethanol discarded. The crystals were redissolved in 5 ml. water with the aid of a minimum volume of acetic acid. The solution was reneutralised with barium hydroxide and a trace of insoluble barium phosphate was removed by centrifugation. After reprecipitating the CEP with 2 vol. ethanol the crystals were collected in a pre-weighed test tube and washed with ethanol, acetone and ether and allowed to dry at room temperature. The weight of CEP was measured, and the yield calculated. This was of the order of 60-70%.

The CEP thus prepared is in the form of the barium salt, and must be converted to the free acid in order to dissolve it readily in pyridine for the synthesis of TMP. This was done by dissolving the crystals in 10 ml. water, using acetic acid to assist the solution, and passing through a Dowex-50-H^+ column (about 10 ml.). The column was washed with water until most of the activity was in the effluent, which contained the free acid of CEP and a little acetic acid. The solution was taken to dryness at 40° in vacuo to remove the acetic acid, and the residue was dissolved in pyridine.

2. ^{32}P-TMP.

2 mmoles of thymidine were dissolved in anhydrous pyridine and 1 mmole of ^{32}P-CEP was added. The solution was concentrated to an oil at 40° in vacuo, 10 ml. anhydrous pyridine added and the solution again taken to an oil. This process was repeated with a further 10 ml. pyridine and the residue was finally dissolved in 5 ml. pyridine. 620 mg. DCC were added, the flask was securely stoppered and left for 18 hr. at room temperature. 5 ml. water were added to stop the reaction and, after 30 min. at room temperature, 10 ml. of concentrated ammonium hydroxide (0.880) were added and the solution heated to 60° for 1 hr.

The mixture was then taken to dryness in vacuo at 40°, 10 ml. of water added to the residue and the resulting solution filtered to remove dicyclohexylurea. The residue was washed carefully, the washings combined with the original filtrate and a sample was chromatographed on Whatman No.1 filter paper in the isobutyric acid/ammonia/EDTA solvent for 18 hr. This chromatogram showed the presence of thymidine, TMP-3' and TMP-5'. The TMP-3' was not required.

The amount of TMP-5' present was calculated by eluting the ultraviolet absorbing regions of the paper chromatogram with water, measuring their extinctions at 267 mμ (pH 1) and estimating the proportion of TMP-5' to the total ultraviolet absorption. The percentages of the total absorption were of the following order:- TdR 60%, TMP-3' 10%, TMP-5' 30%. This resulted in a yield of 300 μmoles of TMP-5'.

The main part of the TMP was separated from the TdR by column chromatography. The filtrate was applied to a Dowex-1-Cl^- column, TdR, which was not absorbed, was washed from the column with water and then TMP was eluted with 0.05N HCl. Since the TMP-3' was found to have no effect on the subsequent reaction, no attempt was made to remove it.

After determination of the amount of TMP from its extinction at 267 mμ, the TMP was taken to dryness at 40° in vacuo.

3. ^{32}P-TTP.

The following reaction mixture was set up :-

per 100 μmoles ^{32}P-TMP (free acid)
1.2 ml. tri-n-butylamine
6.0 ml. pyridine
0.2 ml. 85% phosphoric acid
3.0 g. DCC

The reactants were dissolved in the order shown, the flask stoppered securely and left at room temperature for 48 hr. The reaction was stopped by the addition of 1 vol. water. The solution was filtered, to remove the precipitate of dicyclohexylurea, and the flask and the precipitate thoroughly washed with water. The washings were added to the filtrate, which was then extracted with several small portions of ether to remove pyridine. The ether washes were in turn washed with a small volume of water and the water wash added to the main aqueous solution. The ether extracts were discarded and the aqueous solution, which contained the TdR nucleotide, was taken to dryness at 40° in vacuo.

The residue was dissolved in water and passed through a Dowex-50-Na^+ column to remove tri-n-butylamine and traces of pyridine. The column was washed with water until all the material was removed, the effluent and the water wash combined and absorbed on to a Dowex-1-Cl^- column at pH 6-7. The column was then washed with water until all traces of ultraviolet absorbing material were removed. A gradient was applied to the column with 0.2M LiCl in 0.1N HCl in the reservoir and 0.01N HCl in the mixing vessel. The volume in the mixing vessel was determined by the amount of material on the column. For up to 100 µmoles nucleotide, 1,000 ml. were used, for 500 µmoles, 1,500 ml. and for up to 1,000 µmoles, 2,000 ml. were used. The flow rate was 1.5 - 2.0 ml./min., fractions were collected at 10-15 min. intervals and the extinction at 267 mµ determined. The largest peak, the third to emerge was the TTP. The tubes containing the TTP were pooled and the amount of TTP estimated by its ultraviolet absorption. The TTP was absorbed on to a charcoal column (for preparation see end of this section) at pH 1, the charcoal washed with water until no Cl^- ions were detectable and then with 0.01M sodium bicarbonate until the ultraviolet absorbing material began to come off (usually about 10 column volumes).

The column was allowed to drain and was washed with two separate column volumes of water, the column being allowed to drain each time. These effluents contained a little TTP, but, due to the presence of bicarbonate, they were discarded. Elution of the column was continued with 0.14M ammonium hydroxide/70% ethanol, until the extinction fell below 0.5 units/ml. The amount of TTP in the eluate, which should be at least 90% of the amount applied, was estimated. The TTP, in ethanol/ammonia, was taken to dryness at 25° in vacuo to remove the ethanol/ammonia. This was dissolved in water and the TTP was converted to the Na^+ salt by passing through a column of Dowex-50-Na^+. It was then lyophilised and dissolved in water to give a concentration of 10 µmoles/ml.

The TTP was chromatographed in the isobutyric acid/ammonia/water solvent, and the chromatogram examined for the presence of TMP, TDP and inorganic phosphate. The specific activity of the ^{32}P-TTP prepared by this method varied between 10 and 20 x 10^6 counts/min./µmole, when assayed in a windowless gas flow counter. The overall yield was 20 - 30%.

Preparation of charcoal.

Charcoal was prepared from animal charcoal, supplied

by British Charcoals and MacDonalds Ltd. It was ground and sieved to the appropriate mesh size (40 - 60 mesh) and then left for about 48 hr. in 5N HCl with occasional stirring. The HCl was removed by washing with water and decantation, which also served to remove small particles. The charcoal was washed extensively with ethanol by decantation (about 100 ml./100 g.), until no coloured material was removed, and then with water. The charcoal was packed into a column and washed with 0.14M ammonium hydroxide/70% ethanol until the extinction of the effluent fell below 0.05/ml. at 260 mμ. The column was washed with water and finally with 0.1N HCl.

(b) dIMP.

dIMP was synthesised by the deamination of dAMP, using a method described by Birnie (1959) for the deamination of cytidylic acid, which was adapted from the method described by Wyatt and Cohn (1953) for the deamination of 5-hydroxymethyl cytosine.

0.4 ml. of glacial acetic acid was added to a solution of 50 mg. dAMP in 2 ml. 2M sodium nitrite. The mixture was allowed to stand at room temperature for 24 hr. and then taken to dryness in vacuo. 0.4 ml. barium chloride (350 mg./ml.) was added to the residue dissolved in

distilled water, the mixture was chilled in ice and the barium salt of dIMP was precipitated by the addition of 14 ml. ethanol. The mixture was centrifuged at 700 x g for 20 min. and the precipitate washed 5 times with 10 ml. portions of a 5:1 mixture of ethanol and water. The salt was finally washed with ethanol and ether and dried in air. The barium salt was converted to the sodium salt by dissolving it in a minimum volume of 0.1N HCl and adding one equivalent of sodium sulphate. The precipitate of barium sulphate was removed by centrifugation and the pH of the supernatant solution adjusted to 7.0.

The identity of the dIMP was confirmed chromatographically and by its absorption spectrum at acid and alkaline pH values.

2. 5 Estimations.

(a) Protein.

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951), using bovine serum albumin as standard.

(b) Phosphate.

Inorganic phosphate was determined by the method of Allen (1940) or that of Griswold, Humoller and McIntyre(1951).

The choice of method was governed by the amount of phosphate present. The Griswold method was used for amounts of phosphorus up to 5 µg. and for larger amounts than this, the Allen method was used.

(c) Ammonium salts.

Ammonium salts were estimated using a commercial Nessler's reagent (B.D.H.). The solution to be estimated was made up to a total volume of 7 ml. with water and 1 ml. Nessler's reagent added. After allowing the material to stand for a few minutes, the colour intensity was determined in a Unicam S.P. 600 spectrophotometer at 490 mµ. This procedure was satisfactory for samples containing between 0.1 and 0.005 mg. nitrogen.

(d) Spectrophotometric measurements.

A Beckman D.U. spectrophotometer was used for the measurement of the ultraviolet absorption of nucleic acid derivatives. For the measurement of the deoxyribonucleosides and deoxyribonucleoside monophosphates the values shown in Table 12 were used. For the estimation of the oligonucleotide concentration in a digest, or fractionated digest, of DNA an assumed value of 1 extinction unit at 260 mµ./40 µg. nucleotides was used.

2. 6 Preparation of enzymes.

(a) Splenic DNase II.

This was kindly supplied by Dr. H.M. Keir. The method of preparation was that of Koerner and Sinsheimer (1957). The final product, after chromatography on Celite 545, contained 1.1×10^6 units and was completely free from the phosphodiesterase and phosphomonoesterase activities described by the authors.

(b) Snake venom diesterase.

This was prepared from *Crotalus adamanteus* venom by a combination of methods described by Laskowski and his colleagues (Felix, Potter and Laskowski, 1960; Williams, Sung and Laskowski, 1961; Sulkowski and Laskowski, 1962).

Step 1. Acetone fractionation.

2 g. venom were dissolved in 120 ml. of cold water, stirred for 30 min. at 0° and filtered on a Buchner funnel with Whatman No.1 filter paper. The clear solution was placed in an ice bath and 80 ml. cold 0.5M acetate buffer, pH 4.0, and 145 ml. acetone (-20°) added to give a concentration of 42% (v/v). The mixture was stirred for 30 min., centrifuged at 7,700 x g in a Servall refrigerated centrifuge for 15 min. and the yellow precipitate discarded.

The supernatant solution was stirred in a bath at -15° for 2 hr., and the precipitate removed by centrifuging at 600 x g at -15° for 20 min. The supernatant solution was transferred to the bath at -15° and 55 ml. cold acetone added with stirring. The mixture was stirred for 1 hr., centrifuged at 600 x g for 20 min. at -15°, and the precipitate dissolved in 20 ml. water.

Step 2. Ethanol fractionation.

Water and 1M acetate buffer, pH 6.0, were added to give an extinction at 280 mμ of 10, and a final molarity of acetate buffer of 0.1. 0.5 vol. ethanol was added and the mixture centrifuged at 600 x g for 20 min., all at 0°. The precipitate was discarded, an additional 1.5 vol. of ethanol added to the supernatant solution, at -15°, and the mixture centrifuged at 600 x g for 20 min. at -15°.

Step 3. Ethanol fractionation.

The precipitate from the previous step was dissolved in 0.1M tris buffer, pH 8.9, and the volume adjusted to give an extinction at 280 mμ. of 10. The fractionation was repeated as in the previous step.

Step 4. Acetone fractionation.

The precipitate from the previous step was dissolved

in water to give an extinction at 280 mμ of 11, and 0.1 vol. 2M sodium acetate buffer, pH 4.0, was added. The solution was cooled in an ice bath, treated with acetone at -20° to give a concentration of 44% and transferred to a -15° bath for 30 min. The mixture was centrifuged at 600 x g for 20 min. at -15° and the precipitate discarded. The supernatant solution was treated with acetone to give a total concentration of 50%. The mixture was allowed to stand at -15° for 1 hr. and was then centrifuged at 600 x g for 20 min. at -15°. The precipitate was dissolved in a small volume of 0.05M acetate buffer, pH 6.0, and dialysed overnight against 2 l. of the same buffer.

Step 5. Chromatography on CM-cellulose.

The solution from the previous step was fractionated on a column of CM-cellulose, 1 x 8 cm., previously adjusted to the same buffer. The column was washed with 0.05M acetate buffer, pH 6.0, and then with 0.2M acetate buffer, pH 6.0, which removed the active fraction.

Step 6. Chromatography on DEAE-cellulose.

The material from the previous step was lyophilised and dissolved in 1 ml. 0.01M tris buffer, pH 9.0, and dialysed against 1 l. of the same buffer overnight.

The dialysed solution was placed on a column of DEAE-cellulose 1 x 5 cm. The column was washed with 0.01M tris buffer, pH 8.9 which removed most of the activity, without removing the residual phosphomonoesterase activity.

The final yield of the enzyme was 0.088 extinction units at 280 mμ, which compares with the quoted yield of 1.04 units from 10 times the amount of enzyme. No assays of activity during the purification procedure could be carried out since such small amounts of enzyme were involved, but the final solution was found to have a very high activity against the synthetic substrate, p-nitrophenol thymidine 5'-phosphate.

2. 7 Enzyme assays.

(a) DNase.

The medium for the DNase assays varied according to the type of experiment. The volume was usually somewhere between 0.3 and 0.8 ml. and incubation was carried out in 15 ml. conical centrifuge tubes. The method consists of measuring the ultraviolet absorption of the acid-soluble products, and is based on that described by Lehman, Bessman, Simms and Kornberg (1958). At the end of the incubation time 0.2 ml. 2 mg./ml. bovine serum albumin was

added. This was used as a carrier, instead of DNA, since it was found that the results obtained with both carriers were identical. 0.5 ml. ice-cold 1N $HClO_4$ was added and, after allowing the solutions to stand for 5 min., the tubes were centrifuged at 600 x g for 5 min. at 0° and the supernatant solution carefully and thoroughly decanted into tubes containing 2 ml. water. The extinction of the solutions at 260 mμ was determined in a Beckman D.U. spectrophotometer.

(b) Phosphatase.

Phosphatase activity was determined by the release of inorganic phosphate from the appropriate substrate. The constituents of the incubation medium varied from experiment to experiment, but the volume was usually around 0.4 ml. Incubation was carried out in 15 ml. conical centrifuge tubes. After incubation, 0.25 ml. 4N $HClO_4$ was added, the solutions were allowed to stand for 5 min. at 0° and the precipitate was removed by centrifuging at 600 x g for 5 min. at 0°. Inorganic phosphate was then determined either by the method of Allen (1940) or of Griswold, Humoller and McIntyre (1951)(see Section 2.5b), according to the amount of inorganic phosphate present.

Phosphatase activity was occasionally quoted in terms

of units, one unit being equivalent to 1 μmole of inorganic phosphate released.

2. 8 Paper chromatographic techniques.

Several solvents were used for the separation of nucleosides and nucleotides in fractions obtained from DEAE-cellulose columns.

Solvent (i) 100 ml. isobutyric acid, 55.8 ml. distilled water, 4.2 ml. 35% (w/v) ammonia solution and 1.6 ml. of 0.1M EDTA. This was originally 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 for 16 to 18 hr. as a descending chromatogram. This solvent separates the deoxyribonucleosides or the deoxyribonucleoside monphosphates satisfactorily, but the slowest nucleoside overlaps with the fastest monophosphate.

Solvent (ii) 60 ml. 1M ammonium acetate, containing 0.01M EDTA, 140 ml. 90% ethanol (Paladini and Leloir, 1952). A 16 hr. descending run was used for this solvent. It separates completely the deoxyribonucleosides, which have a high R_f value, from the deoxyribonucleoside monophosphates, which have a low R_f value.

Solvent (iii) 130 ml. isopropanol, 37 ml. water, 33 ml. conc. HCl (Wyatt, 1951). Descending chromatograms were run in this solvent for 16 hr. It decomposes the purine deoxyribonucleosides and deoxyribonucleoside monophosphates to the free bases, but it was found useful for identifying the bases present.

Solvent (iv) 172 ml. butanol, 18 ml. water, 10 ml. 0.88 ammonia (MacNutt, 1952). This was generally employed as a second solvent, following chromatography in (iii). This second solvent was run for 16 hr., in an ascending fashion, with the solvent in the bottom of the tank. The combination had the disadvantage of hydrolysing the purine bases, as described in (iii). However, it was found useful for separating the pyrimidine deoxyribonucleosides from the monophosphates, which do not move in the second solvent.

Solvent (v) 24 ml. isopropanol, 18 ml. water and 80 ml. saturated ammonium sulphate (Markham and Smith, 1952). This was used as a second solvent after chromatography in a slightly modified version of solvent (ii) - 75 ml. 95% ethanol and 30 ml. 1M ammonium acetate, adjusted to pH 7.5. This combination of solvents was described by Felix,

Potter and Laskowski (1960) and Sulkowski and Laskowski (1962). Whatman 3 mm. paper was used. Both solvents were used in the descending direction. The development time in the first solvent was 18 hr. and in the second 12 hr. This combination of solvents separated all the deoxyribonucleosides and deoxyribonucleoside monophosphates. By increasing the development time, it is also possible to separate the dinucleotides.

Spots were located by scanning the paper in a Hanovia Chromatolite. When it was desired to examine the absorption properties of a spot, it was cut out of the paper, cut into small pieces, and the pieces placed in 2 - 3 ml. water or 0.01N HCl. After allowing the tubes to stand, usually overnight, the paper was centrifuged down, the supernatant solution decanted and diluted for examination in a spectrophotometer.

2. 9 The use of DEAE-cellulose for the fractionation of oligonucleotides.

Commercial DEAE-cellulose was separated on wire sieves to give a preparation of 60 - 80 mesh. The cellulose was then washed using a modification of the method described by Staehlin (1961). It was first washed with 0.5N NaOH, and then with water in a large beaker. The cellulose was

allowed to settle partially, and the supernatant liquid decanted to remove the fine particles. It was washed three times in this way and was then transferred to a Buchner funnel and washed with water until the pH of the effluent solution was neutral. The sediment was then washed with 95% ethanol and water three times. The cellulose was resuspended in 0.5N NaOH, and the decantation and washing with water on the Buchner funnel repeated as before. Finally, the cellulose was washed with a large volume of the starting buffer and suspended in it.

After packing the washed cellulose into a column 2 x 40 cm., with a small amount of pressure, it was washed with a few hundred ml. of the starting buffer. A small amount of glass wool was put on to the top of the column to prevent the top particles being disturbed and the digest of DNA applied to the column. After all the digest had been applied, a gradient elution was commenced. Two different buffers were used during the course of the studies: ammonium bicarbonate and ammonium formate. The concentrations of buffer varied from experiment to experiment and will be described in the results section. A two chamber closed system was used. Fractions were collected at 13 min. intervals and the extinction of these

read at 260 mμ in a Beckman spectrophotometer.

At the end of a run, the contents of the tubes were pooled, according to the peaks of ultraviolet absorption, and the fractions lyophilised. It was found that most of the ammonium bicarbonate could be removed by lyophilisation, but that the ammonium formate was not so easily removed. The method used for the removal of the ammonium formate was rapid dialysis, with vigorous stirring. This was normally carried on for about 20 min., and involved only small losses of ultraviolet absorbing material, but complete removal of the ammonium formate, as judged by the lack of colour produced with Nessler's reagent. Oligonucleotide fractions containing ammonium bicarbonate were desalted using charcoal (prepared by the method used in the synthesis of ^{32}P-TTP). 1 ml. of solution (containing approx. 2 mg. oligonucleotide) was made 0.5N with respect to formic acid and 40 mg. charcoal added. The solution was allowed to stand for about 2 hr. and, at the end of this time, the extinction of the supernatant solution at 260 mμ was measured, and usually found to be small. The suspension was centrifuged and the supernatant solution decanted and discarded. The charcoal was then washed with 10 ml. solution containing 60% ethanol, 1% concentrated ammonia

and 39% water. The charcoal was centrifuged down and the volume of the supernatant solution reduced by evaporation.

2. 10 The treatment of oligonucleotide fractions with bacterial alkaline phosphatase.

Certain oligonucleotide fractions were treated with bacterial alkaline phosphatase to remove the terminal phosphate. The oligonucleotide fractions were incubated together with the bacterial alkaline phosphatase (in the presence of 0.01M tris, pH 8.0), and, after the incubation (2 hr.), 2 ml. of chloroform were added and the tubes shaken for 1 hr. at room temperature. The two layers were separated by centrifugation, and the upper layer removed with a Pasteur pipette.

2. 11 Preparation of calcium phosphate gel.

(Keilin and Hartree, 1938)

150 ml. calcium chloride solution (132 g. $CaCl_2.6H_2O$/l) were diluted to 1,600 ml. with water and shaken with 150 ml. trisodium phosphate solution (152g. $Na_3PO_4.12\ H_2O$/l.). The mixture was brought to pH 7.4 with dilute acetic acid and the precipitate washed three times by decantation with large volumes of water (15 - 20 l.). The precipitate was finally washed and centrifuged. The yield was 9.1 g. dry weight. It was suspended in distilled water and

allowed to stand for about a month. After removing the clear water layer, the gel was well shaken and the dry weight/ml. determined. It was then ready for use.

2. 12 Buffer solutions.

Tris In all cases, the term "tris" refers to a buffer made by adjusting the pH of a solution of 2-amino-2-(Hydroxy-methyl)-propane-1:3-diol to the required value with HCl.

Phosphate Phosphate buffer was prepared by adding M/15 KH_2PO_4 to M/15 $Na_2HPO_4.2H_2O$ until the desired pH was reached.

Glycine/NaOH 0.1N NaOH was added to 0.1M glycine in 0.1N NaCl until the desired pH was reached.

2. 13 Separation of subcellular fractions from rat liver.

Rat liver was fractionated according to the methods of Appelmans, Wattiaux and de Duve (1955) and de Duve, Pressman, Wattiaux and Appelmans (1955).

Rats were killed by a blow on the head and bled, the livers taken out quickly and immersed in a beaker containing ice-cold medium (0.25M sucrose with 0.01M EDTA). After allowing the tissue to become chilled, it was cut up with scissors and homogenised in a Potter-type homogeniser,

using one pass of the pestle, in 3 vol. of medium. The resulting homogenate was centrifuged in a refrigerated centrifuge at 1,000 x g for 10 min. The sediment was rehomogenised in the same volume of medium and centrifuged at 600 x g for 10 min. The sediment was redispersed using the homogeniser and the final volume made up to 4 times the original weight of the liver. This was the nuclear fraction. The supernatant solutions were combined and made up to a final volume which was equal to 10 times the weight of liver. Estimations were carried out separately on the nuclear and cytoplasmic fractions and the sum of these values was considered to be equal to the whole homogenate.

The cytoplasmic extract was fractionated further using the Spinco Model L preparative ultracentrifuge, with the No. 40 rotor. Two mitochondrial and one microsomal fractions were prepared. 80 ml. of cytoplasmic extract were centrifuged at 6,500 x g for 5 min., and the precipitate was collected in two tubes, and washed twice with 2 x 10 ml. medium, at 6,500 x g each time. After centrifugation, the pellet consisted of two layers, a light brown layer, with a cream layer on top. The upper layer was removed with the supernatant solution. The precipitate

after washing was suspended to give a final volume of 16 ml. This was the mitochondrial fraction. The combined supernatant fluids and washings from the mitochondria were then centrifuged at 25,000 x g for 10 min., the precipitate collected in two tubes and washed twice with 2 x 10 ml. medium. The precipitate was resuspended in a total volume of 8 ml. This was the lysosomal fraction, which consisted of a small dark brown precipitate, with a pink "fluffy" layer on top. The latter layer was removed in the washing procedure. The combined supernatant solutions and washings were centrifuged at 100,000 x g for 30 min., the precipitates collected in two tubes and washed twice with 2 x 10 ml. of medium. The precipitate, which was red and gelatinous, was dispersed to give a final volume of 16 ml.

In the first experiment, nuclei, mitochondria, lysosomes and microsomes were disrupted by dialysing against distilled water. 2 ml. of each fraction were dialysed overnight against distilled water. After dialysis, the volumes of the solutions were measured, and the precipitates which had formed were dispersed by means of a small Potter-type homogeniser. In the second experiment, the particles were disrupted by subjecting them to ultrasonic vibration, using a Mullard ultrasonic

drill (50 W. : 20 kc) for 2 min.

The protein concentration of these fractions was determined by a slight modification of the Lowry method. Aliquots of the suspensions of disrupted particles were incubated overnight at 37° with the amount of NaOH normally added in the NaOH/sodium potassium tartrate/ copper sulphate reagent, i.e. 0.5 ml. 1N NaOH, in a total volume of 0.55 ml. The other constituents of the reagent were then added and the estimation continued in the normal way. This method was adopted to ensure that the proteins of the disrupted particles were in solution before the estimation was carried out.

2. 14 Materials.

Deoxyribonucleosides and deoxyribonucleoside phosphates were obtained from Schwarz Bioresearch Inc., the Sigma Chemical Co. and Pabst Laboratories. Calf serum was purchased from Oxoid, Ltd., DEAE-cellulose from Eastman Kodak, CM-cellulose from Whatman, crystalline pancreatic DNase and bacterial alkaline phosphatase from Nutritional Biochemicals Corp., DEAE-sephadex from Pharmacia, Uppsala, Sweden, yeast RNA from British Drug Houses, Ltd., bovine serum albumin from Armour Pharmaceutical Co., and *Crotalus adamanteus* venom from the Sigma Chemical Co.

SECTION III

R E S U L T S

Section III. Results.

3. 1 Characteristics of calf thymus DNA nucleotidyl-transferase.

Some properties of the calf thymus DNA nucleotidyl-transferase have been described by Bollum (1960), but since his system differed, in some respects, from that employed in the present experiments, it was important to characterise the enzyme in crude thymus extracts.

The incorporation of ^{32}P-TMP into DNA is linear over at least 1 hr., as shown in Fig. 4, and in subsequent experiments incubation was carried out for 1 hr. The effect of $MgCl_2$ concentration on the reaction is illustrated in Fig. 5, which shows an optimal $MgCl_2$ concentration of 6.5 µmoles/ml., a value that agrees with Bollum's (1960) figure of 5 - 8 µmoles/ml. In later experiments it was decided to increase the concentration of deoxyribonucleoside triphosphates, in an attempt to minimise the possible effects of phosphatases on the reaction, and it was found that the $MgCl_2$ concentration had to be raised to 16 µmoles/ml. when the nucleotide concentration was increased to 2 µmoles/ml.

The effect of DNA concentration on the incorporation of ^{32}P-TMP residues into DNA is shown in Fig. 6, which indicates that maximal activity is reached at about 50 - 75 µg.

Fig. 4.

The time course of the incorporation of ^{32}P-TMP into DNA by calf thymus DNA nucleotidyltransferase.

The medium contained 25 µmoles tris, pH 7.5, 0.75 mg. enzyme protein, 50 µg. denatured DNA, 1 µmole 2-mercaptoethanol, 2 µmoles $MgCl_2$, 0.05 µmole EDTA and 50 mµmoles each of dATP, dGTP, dCTP and ^{32}P-TTP in a total volume of 0.3 ml.

Fig. 4.

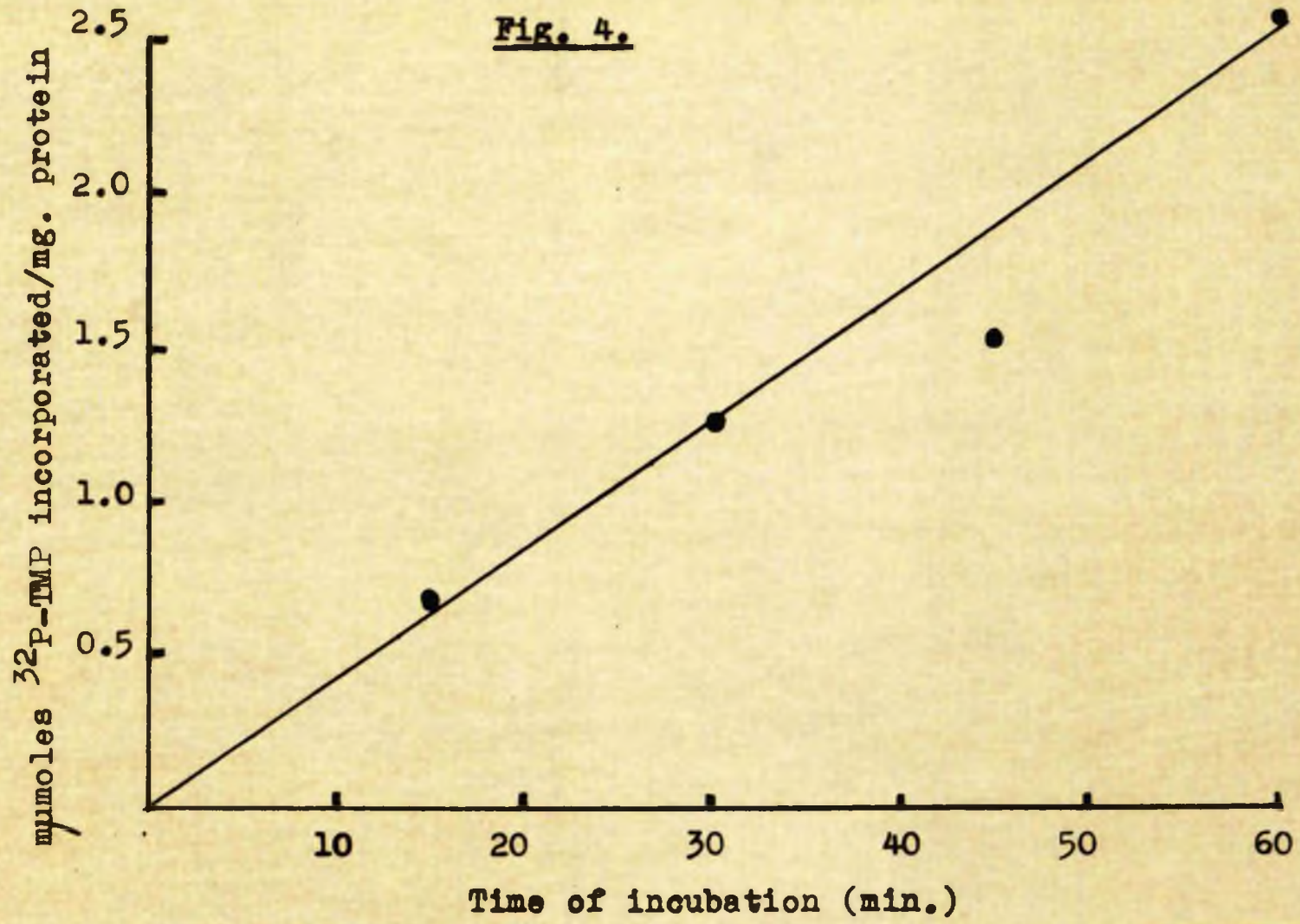

2.5
2.0
1.5
1.0
0.5
mumoles ^{32}P-TMP incorporated/mg. protein
10
20
30
40
50
60
Time of incubation (min.)

Fig. 5.

The effect of $MgCl_2$ concentration on the incorporation of ^{32}P-TMP into DNA by calf thymus DNA nucleotidyltransferase.

The assay medium contained 25 μmoles tris, pH 7.5, 0.75 mg. enzyme protein, 50 μg. denatured DNA, 1 μmole 2-mercaptoethanol and 50 mμmoles each of dATP, dGTP, dCTP and ^{32}P-TTP in a total volume of 0.3 ml. Incubation was for 1 hr. at 37°.

Fig. 5.

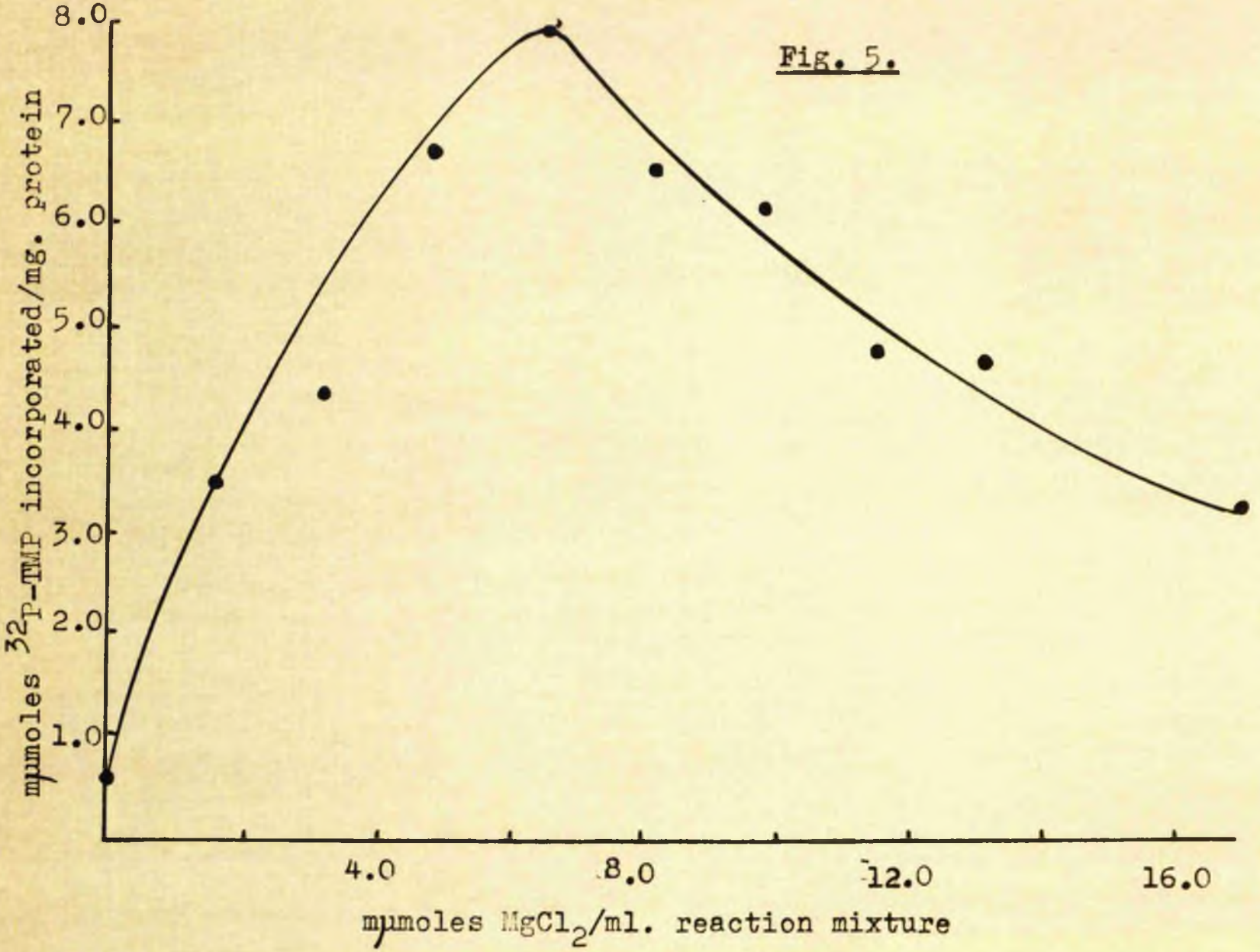
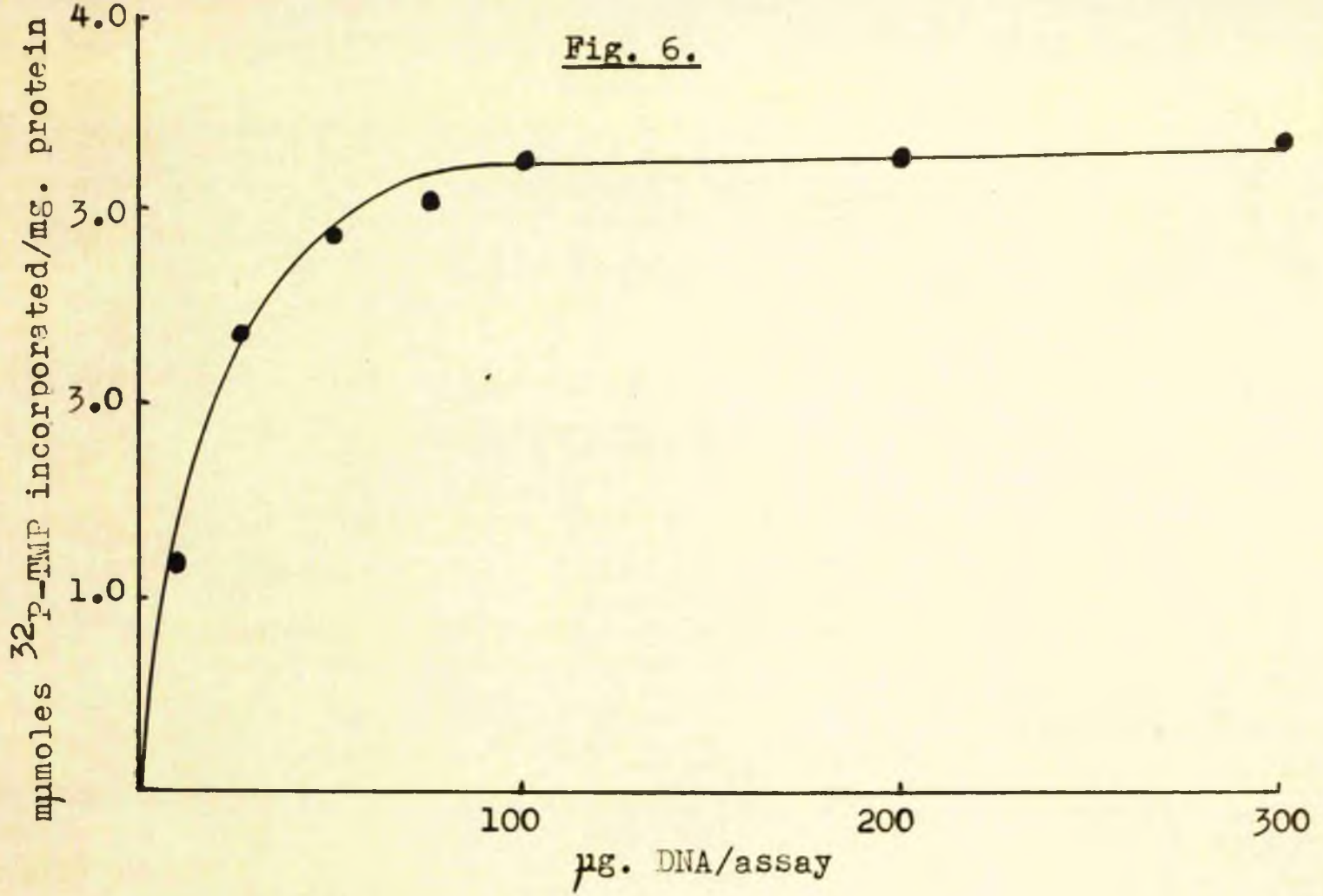

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
mμmoles ^{32}P-TMP incorporated/mg. protein
4.0
8.0
12.0
16.0
mμmoles $MgCl_2$/ml. reaction mixture

Fig. 6.

The effect of DNA concentration on the incorporation of ^{32}P-TMP into DNA by calf thymus DNA nucleotidyltransferase.

The medium contained 10 μmoles tris, pH 7.5, 0.75 mg. enzyme protein, 1 μmole 2-mercaptoethanol, 6 μmoles $MgCl_2$ and 125 mμmoles each of dATP, dGTP, dCTP and ^{32}P-TTP in a total volume of 0.38 ml. Incubation was for 1 hr. at 37°.

Fig. 6.


4.0
3.0
3.0
1.0
mμmoles 32P-TMP incorporated/mg. protein
100
200
300
μg. DNA/assay

DNA/assay. A level of 50 µg./assay was used for most of the subsequent experiments. Fig. 7 demonstrates the effect of increasing concentrations of deoxyribonucleoside 5'-triphosphates on the activity of the DNA nucleotidyltransferase. A concentration of 500 mµmoles/assay was used to give a slight excess, in the event of there being phosphatase activity present in fractions added to the assay.

Thymus DNA nucleotidyltransferase does not have an absolute requirement for 2-mercaptoethanol, but its inclusion in the reaction medium results in an increase of over 50% in the incorporation (illustrated in Fig. 8). 2-Mercaptoethanol was therefore added routinely to assays at the level of 1 µmole/assay. As with 2-mercaptoethanol, the addition of EDTA enhanced the reaction, and therefore EDTA was also added routinely to assays at the level of 0.05 µmole/assay (Fig. 9).

Hence, most of the subsequent assays were carried out with an assay mixture of the following composition, with only minor deviations: 6 µmoles $MgCl_2$, 50 µg. DNA, 125 mµmoles each of dATP, dGTP, dCTP and ^{32}P-TTP, 0.05 µmole EDTA, 1 µmole 2-mercaptoethanol and 10 µmoles tris buffer, pH 7.5.

Fig. 7.

The effect of deoxyribonucleoside triphosphate concentration on the incorporation of ^{32}P-TMP into DNA by calf thymus DNA nucleotidyltransferase.

The medium contained 25 μmoles tris, pH 7.5, 0.75 mg. enzyme protein, 1 μmole 2-mercaptoethanol, 2 μmoles $MgCl_2$ and 50 μg. denatured DNA in a total volume of 0.3 ml. Incubation was for 1 hr. at 37°.

Fig. 7.

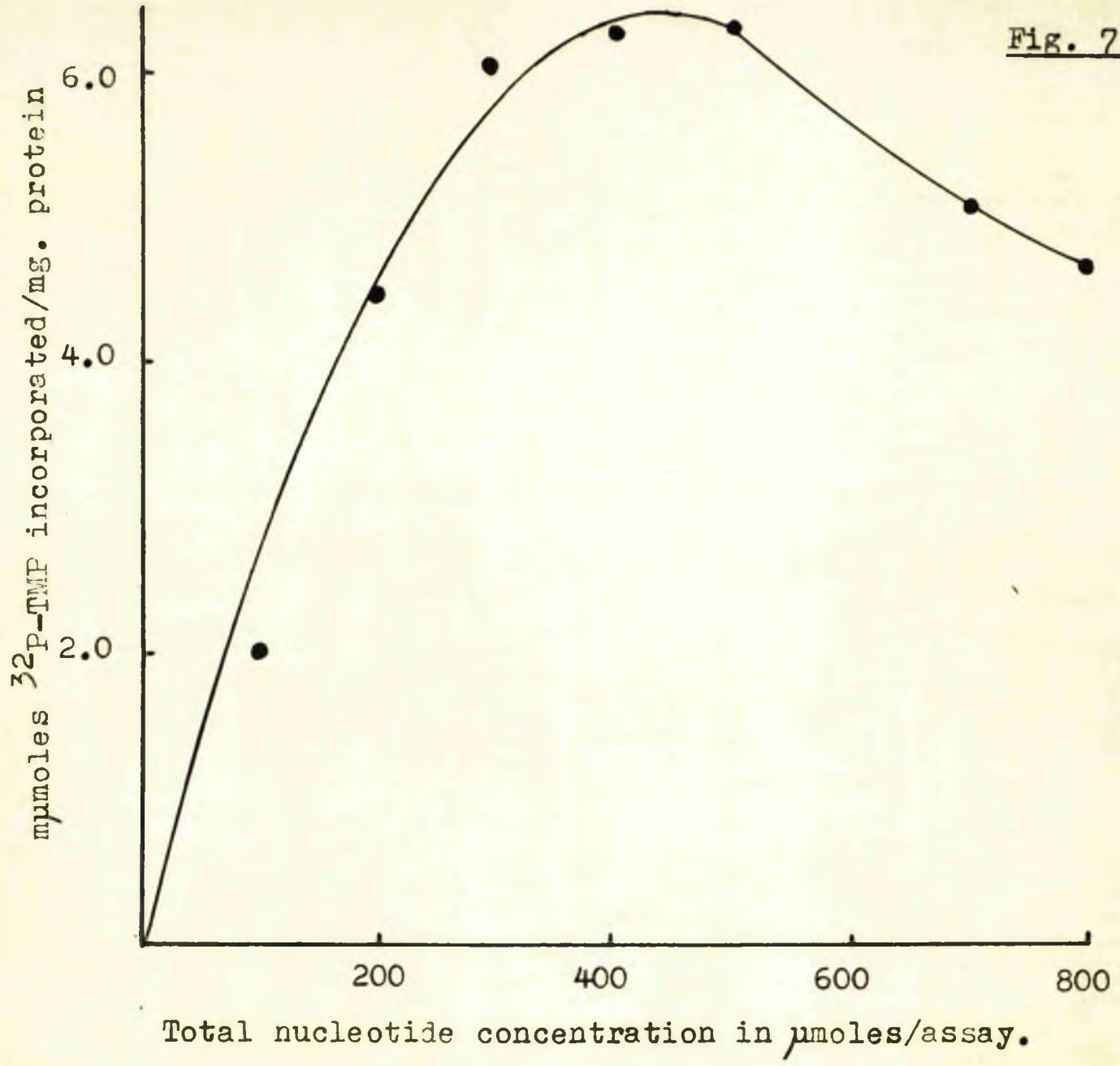

mμmoles ^{32}P-TMP incorporated/mg. protein
6.0
4.0
2.0
200
400
600
800
Total nucleotide concentration in μmoles/assay.

Fig. 8.

The effect of 2-mercaptoethanol concentration on the incorporation of ^{32}P-TMP into DNA by calf thymus DNA nucleotidyltransferase.

The medium contained 10 μmoles of tris, pH 7.5, 0.75 mg. enzyme protein, 6 μmoles $MgCl_2$, 50 μg. denatured DNA, 0.05 μmole EDTA and 125 mμmoles each of dATP, dGTP, dCTP and ^{32}P-TTP in a total volume of 0.28 ml. Incubation was for 1 hr. at 37°.

Fig. 8.

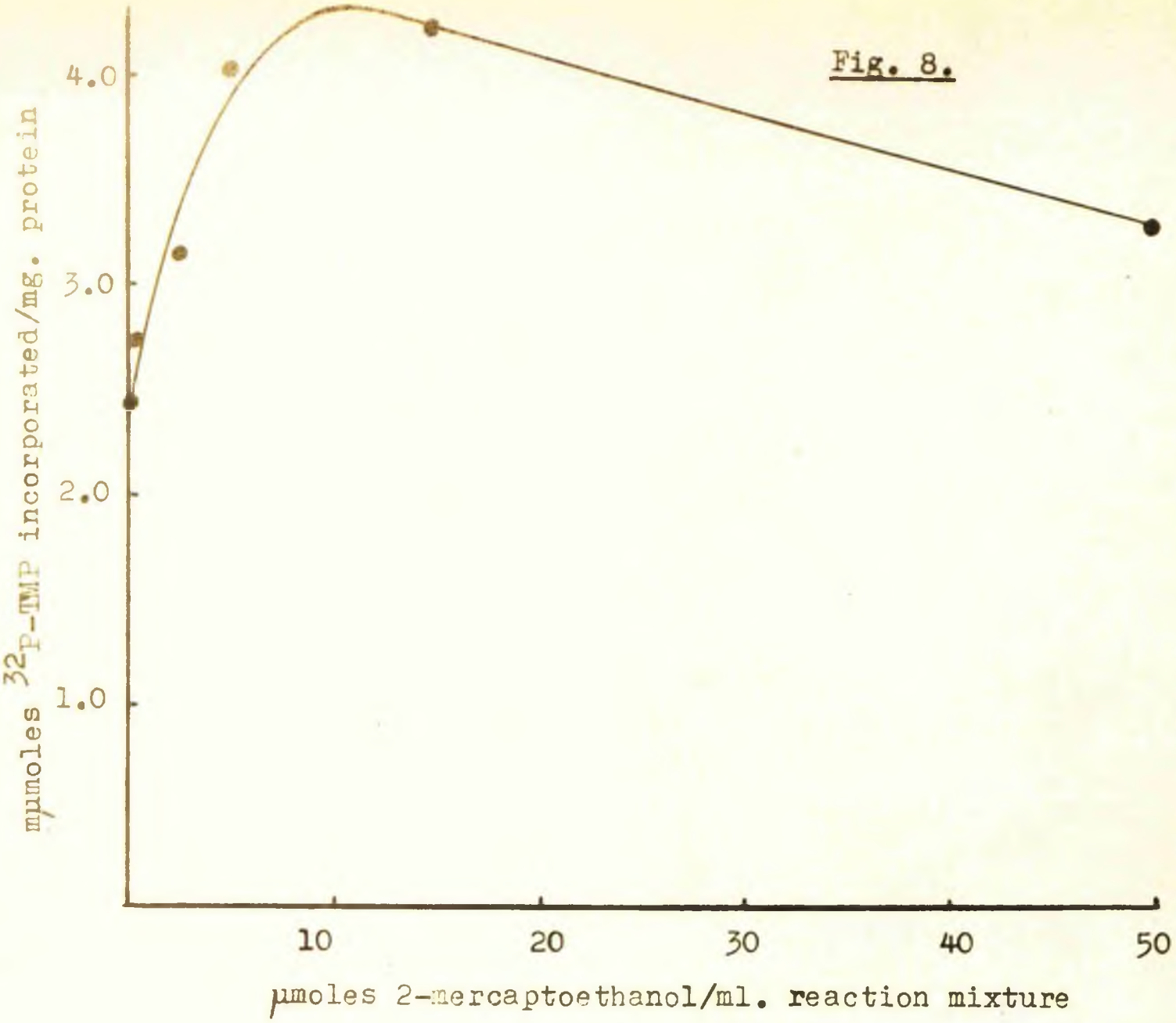

4.0
3.0
2.0
1.0
mμmoles ^{32}P-TMP incorporated/mg. protein
10
20
30
40
50
μmoles 2-mercaptoethanol/ml. reaction mixture

Fig. 9.

The effect of EDTA concentration on the incorporation of ^{32}P-TMP into DNA by DNA nucleotidyltransferase.

The medium contained 25 µmoles tris, pH 7.5, 0.75 mg. enzyme protein, 50 µg. denatured DNA, 1 µmole 2-mercaptoethanol, 2 µmoles $MgCl_2$ and 50 mµmoles each of dATP, dGTP, dCTP and ^{32}P-TTP in a total volume of 0.3 ml. Incubation was for 1 hr. at 37°.

Fig. 9.

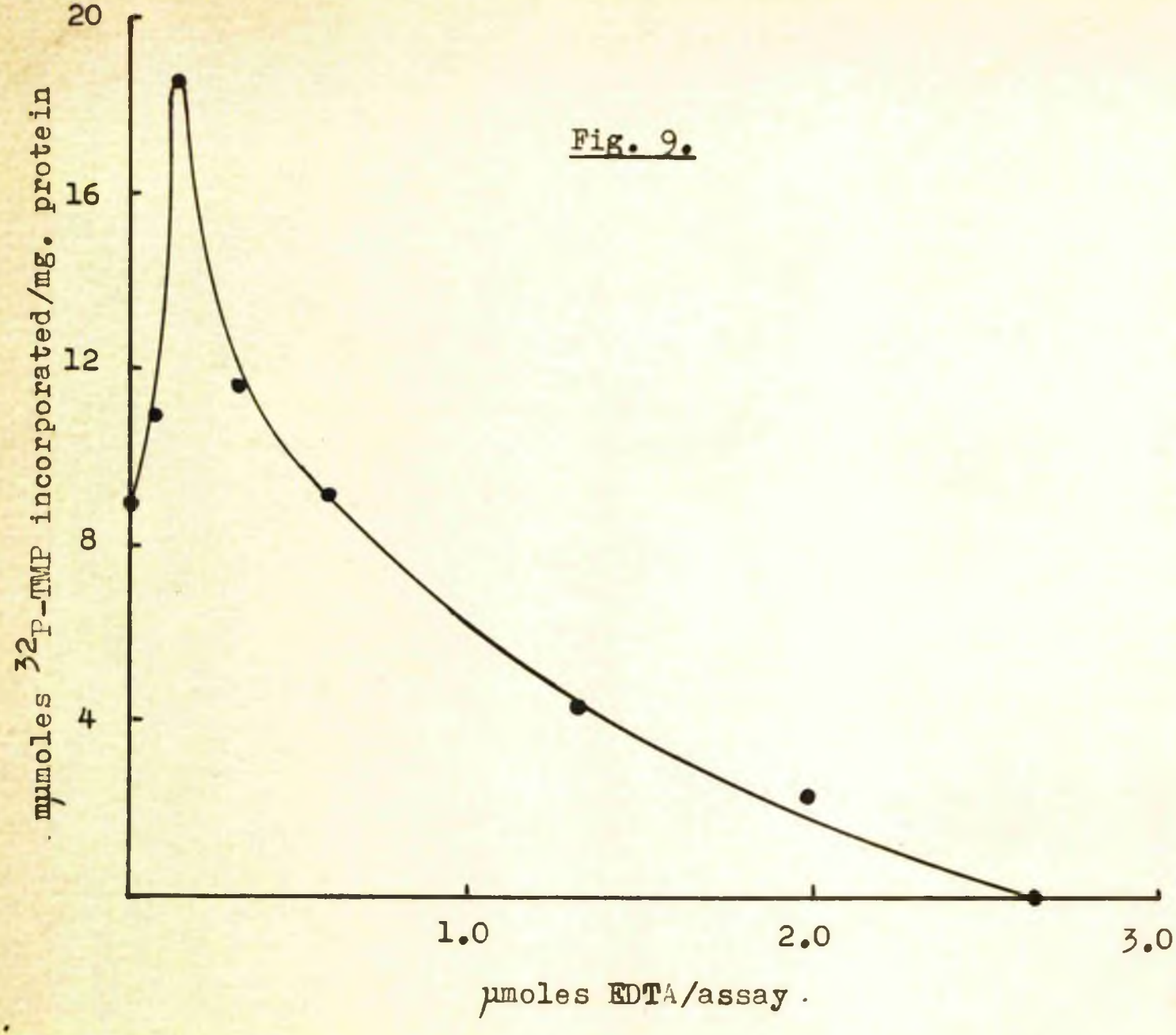
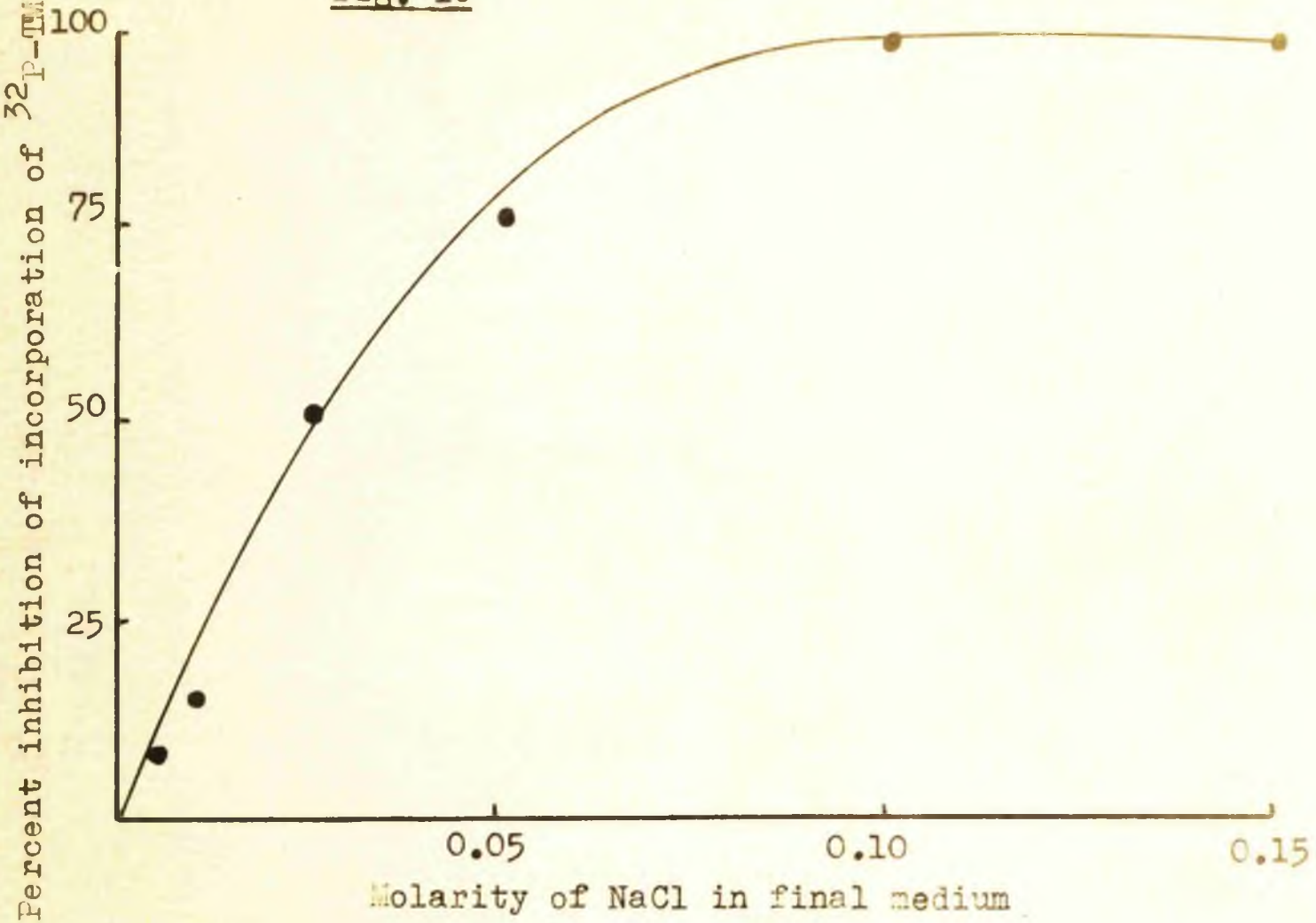

20
16
12
8
4
mμmoles ^{32}P-TMP incorporated/mg. protein
1.0
2.0
3.0
μmoles EDTA/assay.

Fig. 10.

The effect of NaCl concentration on the incorporation of ^{32}P-TMP into DNA by calf thymus DNA nucleotidyltransferase.

The medium contained 25 µmoles of tris, pH 7.5, 0.75 mg. of enzyme protein, 50 µg. denatured DNA, 1 µmole 2-mercaptoethanol, 0.05 µmole EDTA, 2 µmoles $MgCl_2$ and 50 mµmoles each of dATP, dGTP, dCTP and ^{32}P-TTP in a total volume of 0.3 ml. Incubation was for 1 hr. at 37°.

Fig. 10


Percent inhibition of incorporation of ^{32}P-TMP into DNA
100
75
50
25
0.05
0.10
0.15
Molarity of NaCl in final medium

3. 2 Observations on the effects of heating and dialysis on the inhibition of the DNA nucleotidyltransferase by calf serum and extracts of rat liver and kidney.

It was reported by Gray et al. (1960) that calf serum and extracts of a number of tissues inhibited the DNA nucleotidyltransferase from Ehrlich ascites tumour cells. These observations have been confirmed and further investigated using crude preparations of calf thymus DNA nucleotidyltransferase.

Calf serum was found to inhibit calf thymus DNA nucleotidyltransferase to an extent similar to that found by Gray et al. with the enzyme from ascites tumour cells. This is shown in Table 1 which also demonstrates that dialysis of calf serum removes about 80% of its inhibitory activity while, on the other hand, heating the serum to 100° for 5 min. did not alter its ability to inhibit the DNA nucleotidyltransferase. The residual inhibitory activity after dialysis was not removed by heating to 100°. It would seem, therefore, that the major part of the inhibitory activity of calf serum is due to a dialysable, heat-stable component.

Extracts of rat liver and kidney were also found to inhibit the DNA nucleotidyltransferase, confirming the earlier observations of Gray et al. Dialysis of such

Table 1.

The incorporation of ^{32}P-TMP into DNA by DNA nucleotidyltransferase in the presence of normal, heated and dialysed calf serum.

Additions	mμmoles ^{32}P-TMP incorporated/mg. protein	Per cent inhibition
Control i.e.no serum	1.45	-
Untreated serum	0.462	68.1
Serum heated to 100°	0.454	68.7
Dialysed serum	1.271	12.3
Dialysed serum heated to 100°	1.33	9.0

The incubation medium contained 0.075 ml. of the appropriate calf serum preparation, 25 μmoles tris, pH 7.5, 2 μmoles $MgCl_2$, 0.75 mg. enzyme protein, 50 μg. denatured DNA and 50 μmoles each of dATP, dGTP, dCTP and ^{32}P-TTP in a total volume of 0.2 ml. Incubation was for 1 hr. at 37°.

extracts did not alter their ability to inhibit the incorporation of ^{32}P-TMP into DNA. The effect of heating dialysed extracts of liver and kidney to various temperatures on their inhibitory activity is illustrated in Fig. 11. In both cases, the major portion of the inhibition was found to be due to a heat-labile component (or components), but a small amount of inhibitory activity remained after heating and dialysis, and this may be analagous to the inhibitory activity which remained in calf serum after heating and dialysis.

3. 3 Phosphatase activity in various fractions of rat liver extract.

One possible mechanism for the action of the inhibitor of the DNA nucleotidyltransferase found in extracts of rat liver would be hydrolysis of the deoxyribonucleoside triphosphates in the reaction medium. Since crude liver extracts contain appreciable phosphatase activity, fractionation experiments were performed to determine whether the phosphatase and inhibitory activities could be correlated. Table 2 shows the inhibitory and ATPase activities of fractions obtained from a crude liver extract by fractionation with acid. Although fractionation failed to give rise to any fraction with a relatively high

Fig. 11.

The effect of heating dialysed extracts of rat kidney (a) and liver (b) to various temperatures on their ability to inhibit the incorporation of ^{32}P-TMP into DNA.

25 ml. kidney or liver extract (17 and 14 mg.protein/ml. respectively) were dialysed against 2 l. of 0.01M tris at pH 7.5. Aliquots of these were heated to the specified temperature in a water bath for 5 min. and the precipitates that formed removed by centrifugation.

The DNA nucleotidyltransferase assay contained 25 µmoles tris, pH 7.5, 1 µmole 2-mercaptoethanol, 2 µmoles $MgCl_2$, 50 µg. denatured DNA, 0.05 µmole EDTA, 0.75 mg. enzyme protein, 0.75 ml. of the appropriate kidney or liver extract and 50 mµmoles each of dATP, dGTP, dCTP and ^{32}P-TTP in a total volume of 0.3 ml. Incubation was for 1 hr. at 37°.

Fig. 11.

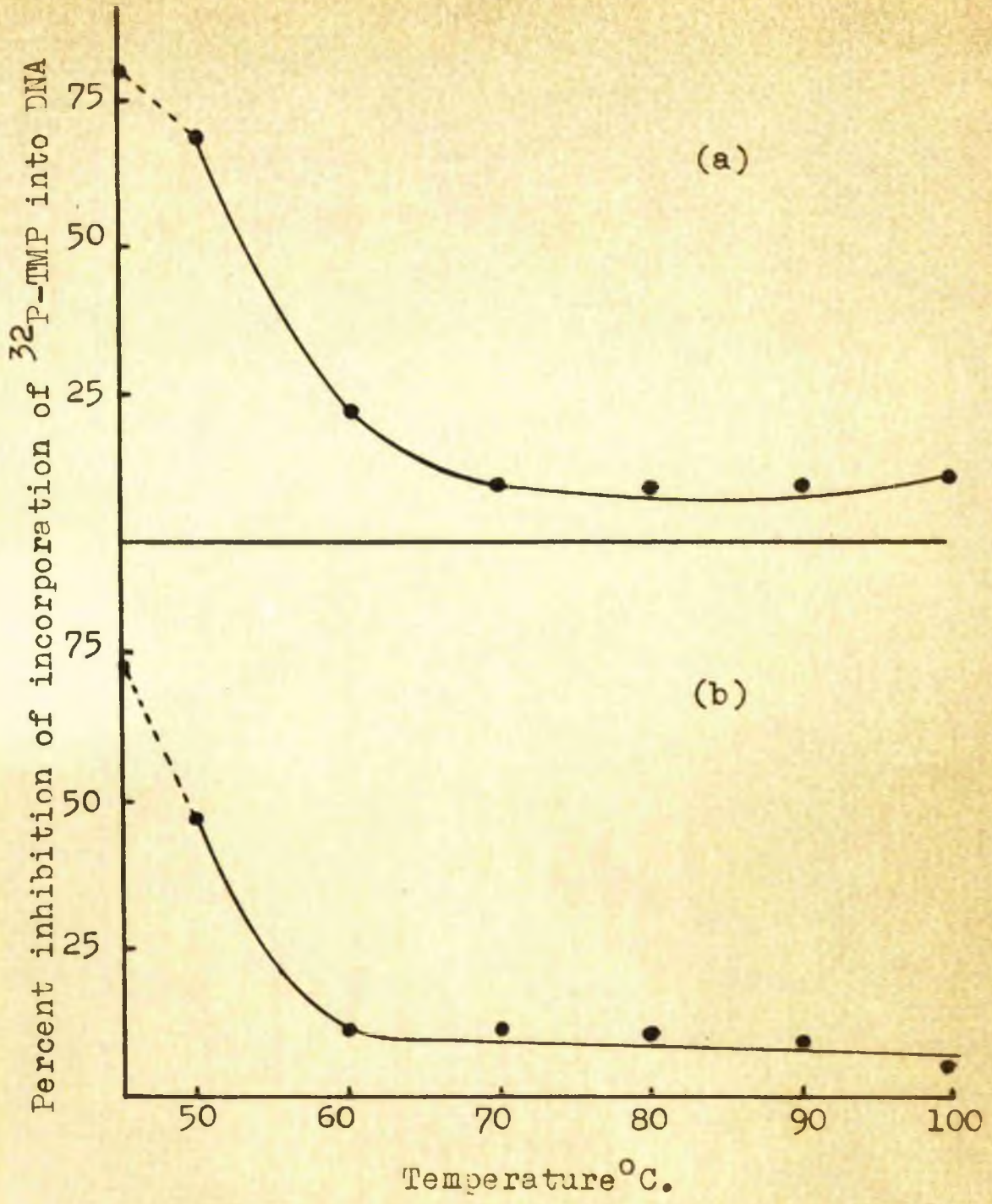

Percent inhibition of incorporation of ^{32}P-TMP into DNA
75
50
25
(a)
75
50
25
(b)
50
60
70
80
90
100
Temperature °C.

Table 2.

The medium for the DNA nucleotidyltransferase assays contained 10 μmoles tris, pH 7.5, 1 μmole 2-mercaptoethanol, 6 μmoles $MgCl_2$, 50 μg. denatured DNA, 0.75 mg. enzyme protein, 1.35 mg. protein from liver extract or acid fraction and 125 mμmoles each of dATP, dGTP, dCTP and ^{32}P-TTP in a total volume of 0.38 ml. Incubation was for 1 hr. at 37°.

The medium for the ATPase estimations contained 30 μmoles tris, pH 7.5, 3 μmoles 2-mercaptoethanol, 0.15 μmoles EDTA, 150 μg. denatured DNA, 4.05 mg. protein and 1.5 umoles ATP in a total volume of 0.74 ml. Incubation was for 1 hr. at 37°.

	Fraction precipitating between pH values.
AP I	7.1 - 6.75
AP II	6.75 - 6.2
AP III	6.2 - 5.5
AP IV	5.5 - 5.1
AP V	5.1 - 4.5
AS	Supernatant solution after last precipitation.

Definitions of units are in the Methods section.

Table 2.

Inhibitory and ATPase activities of fractions precipitated by acid from liver extract.

Fraction	mg.protein /fraction	Units inhibitory activity /mg. protein.	Units phosphatase /mg. protein	Total inhibitory units /fraction	Total phosphatase units /fraction	Rel. inhibitory activity	Rel. phosphatase activity
Liver extract	980	44.3	5.36	43,410	5,250	1	1
AP I	9.6	145.2	17.9	1,394	172	3.28	3.34
AP II	149.0	126.0	6.4	14,990	960	2.74	1.2
AP III	134.0	111.3	6.4	14,914	857	2.52	1.19
AP IV	88.8	86.3	5.2	7,660	457	1.95	0.96
AP V	27.2	51.7	3.7	1,410	101	1.16	0.69
AS	573.0	17.3	3.3	9,900	1,910	0.38	0.62
Total in fractions	981.6			50,268	5,457		

inhibitory activity and relatively low phosphatase activity, it can be seen that the removal of the precipitate formed at pH 4.5 leaves 35% of the phosphatase units, but only 10% of the inhibitory units in the supernatant solution. This indicates that the phosphatase and the inhibitory activities are not identical, and further evidence for this was obtained by additional fractionation. The results in Table 2 show that the recovery of inhibitory units was greater than 100%, and subsequently this was observed on almost every occasion when acid precipitation of a liver extract was carried out. These results suggest that there may be a factor present in the crude liver extract which partially masks the activity of the inhibitor. Acid precipitation seemed to provide a useful initial step in the purification, since some purification is achieved without loss of activity.

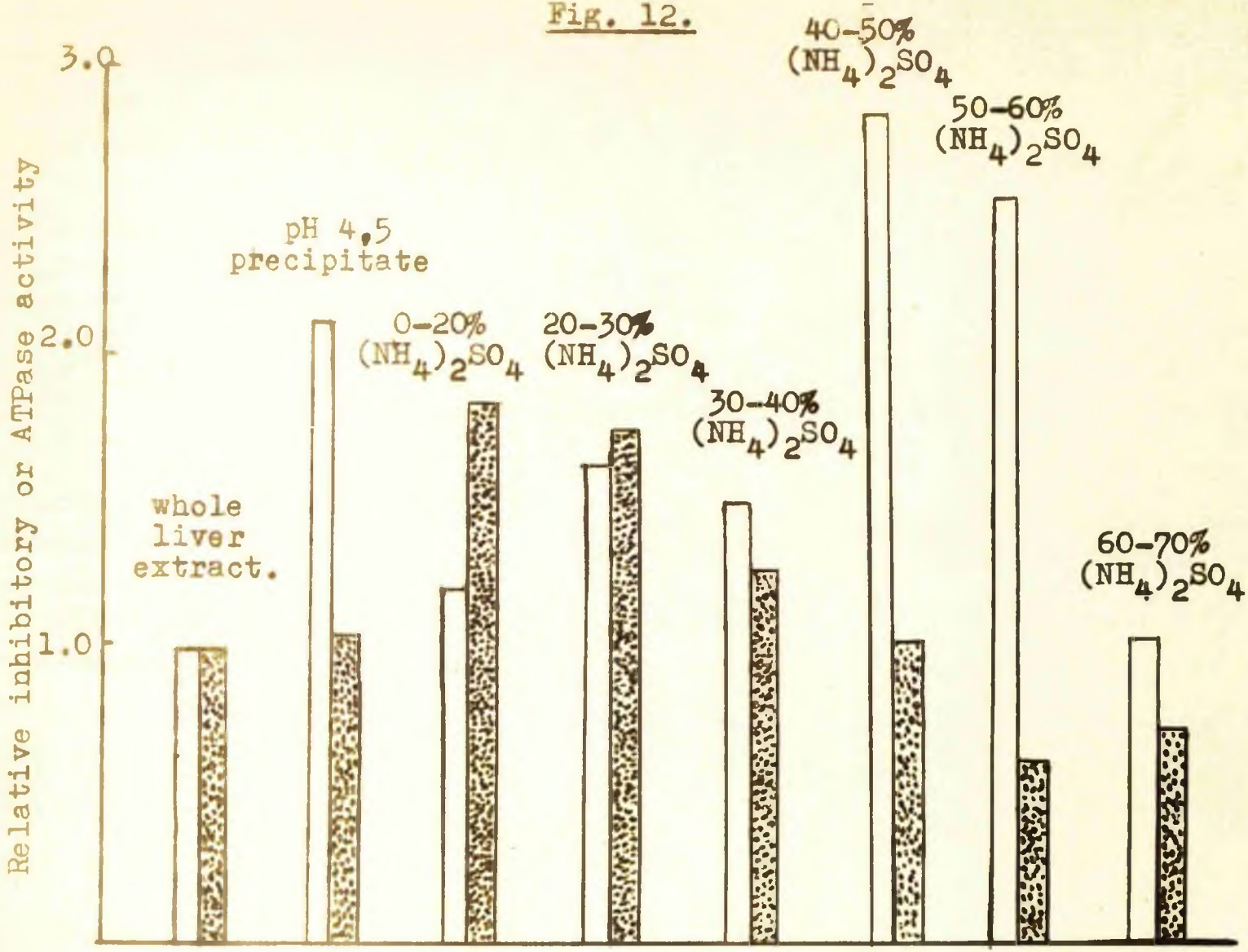
Further fractionation of the inhibitor preparation was carried out with ammonium sulphate, and the inhibitory and phosphatase activities measured in fractions prepared from the acid precipitate. From the results shown in Fig. 12, the distribution of the inhibitory and phosphatase activities seems to be unrelated, since the phosphatase activity tends to be precipitated at low concentrations of

Fig. 12.

Inhibitory and ATPase activities in fractions of rat liver extracts.

The media for the DNA nucleotidyltransferase and ATPase assays were as for Table 2.

Fig. 12.


Relative inhibitory or ATPase activity
3.0
2.0
1.0
whole liver extract.
pH 4.5 precipitate
0–20% $(NH_4)_2SO_4$
20–30% $(NH_4)_2SO_4$
30–40% $(NH_4)_2SO_4$
40–50% $(NH_4)_2SO_4$
50–60% $(NH_4)_2SO_4$
60–70% $(NH_4)_2SO_4$

ammonium sulphate, while the inhibitory activity is preferentially precipitated at higher concentrations of ammonium sulphate.

To eliminate the possibility that there might be enzymes present which hydrolysed the deoxyribonucleoside triphosphates specifically, but did not attack ATP, the ability of several fractions from rat liver to hydrolyse the deoxyribonucleoside 5'-triphosphates was examined. The results are shown in Table 3. The acetone fraction was precipitated directly from the ammonium sulphate fraction, without the refinements introduced later for the purification of the inhibitory factor. The hydrolysis of the 4 deoxyribonucleoside triphosphates appears to proceed at different rates, TTP being the least susceptible to attack by any of the fractions. The experiments using ATP as a substrate showed that the phosphatase activity increased in the fraction precipitated by acid, and decreased in the ammonium sulphate fraction, and a similar pattern was found with the deoxyribonucleoside triphosphates. In addition, the activity towards the deoxyribonucleoside triphosphates declined still further in the acetone precipitate, while the inhibitory activity increased. The final acetone fraction had 2.7 times the inhibitory activity and 0.4 times the phosphatase activity of the crude liver extract.

Table 3.

The medium for the inhibitory assays contained 10 μmoles tris, pH 7.5, 1 μmole 2-mercaptoethanol, 0.05 μmoles EDTA, 6 μmoles $MgCl_2$, 50 μg. denatured DNA, 0.75 mg. enzyme protein and 125 mμmoles each of dATP, dGTP, dCTP and ^{32}P-TTP and an amount of the appropriate inhibitory fraction, such that the final inhibition was about 30%. The total volume was 0.28 ml., and the incubation time was for 1 hr. at 37°.

The phosphatase assay medium contained 1.5 μmoles of the appropriate deoxyribonucleoside triphosphate, or an equimolar mixture of the 4 triphosphates, to a total amount of 1.5 μmoles, 30 μmoles tris, pH 7.5, 3 μmoles 2-mercaptoethanol, 18 μmoles $MgCl_2$, 150 μg. denatured DNA, 0.15 μmole EDTA and 0.45 mg. protein from the appropriate fraction in a total volume of 0.84 ml. Incubation time was for 1 hr. at 37°.

Table 3.

Comparison of the inhibitor and the deoxyribonucleoside triphosphatase activities of various fractions from rat liver.

Fraction	μmoles inorganic phosphate released/ μmole nucleotide.					Relative inhibitory activity.
	dATP	dGTP	dCTP	TTP	mixture 4 nucleotides.	
Liver extract	0.53	0.55	0.30	0.20	0.44	1
Acid ppt. pH 4.5	0.60	0.91	0.63	0.43	0.73	2.04
$(NH_4)_2SO_4$ 40-60%	0.41	0.60	0.35	0.25	0.48	2.45
Acetone fraction 37.5-47.4%	0.10	0.13	0.29	0.06	0.18	2.68

3. 4 The purification of the inhibitory factor from extracts of rat liver.

Extracts of rat liver were fractionated by the methods described in Section 2 to purify the inhibitory factor and the distribution of protein and inhibitory activity in the course of a typical fractionation is outlined in Table 4. The total purification usually varied between 8 and 15-fold, and the procedure resulted in the removal of 95% of the phosphatase activity. Further attempts were made to purify the acetone fraction, using columns of DEAE-cellulose, calcium phosphate gel and DEAE-Sephadex (A50), but it was found that although good fractionation of the protein was obtained and the activity could be confined to one or two of these fractions, drastic losses in activity occurred in all cases. In the experiments with DEAE-Sephadex, an opaque band appeared at the top of the column when the protein was absorbed, and this was taken as an indication of denaturation of the protein. The inhibitory activity is very sensitive to heat treatment, and also to surface denaturation, and it may be that absorption on to gel or cellulose results in extensive inactivation.

The ultraviolet spectra of the fractions are shown in Fig. 13. These indicate that the final acetone precipitation results in a shift of the absorption maximum

Table 4.

The DNA nucleotidyltransferase assay medium contained 10 μmoles tris, pH 7.5, 1 μmole 2-mercaptoethanol, 6 μmoles $MgCl_2$, 0.05 μmoles EDTA, 50 μg. denatured DNA, 0.75 mg. enzyme protein and 125 mμmoles each of dATP, dGTP, dCTP and ^{32}P-TTP in a total volume of 0.28 ml. Incubation was for 1 hr. at 37°.

Inhibitory units were as defined in the Methods Section.

Table 4.

The purification of the inhibitory factor from rat liver.

Fraction	Vol. ml.	Activity units /ml.	Total units	Protein mg. /ml.	Purity units /mg.	Yield %	Purification.
Crude extract	180	31.8	5,720	11.1	2.86	100	1
Ppt. pH 4.5	155	36.2	5,930	4.48	8.5	104	2.97
40-60% $(NH_4)_2SO_4$	34	72.0	2,440	3.34	21.4	42.8	7.5
36.9-45.4% acetone.	10	106.0	1,060	3.62	28.4	18.6	10.0

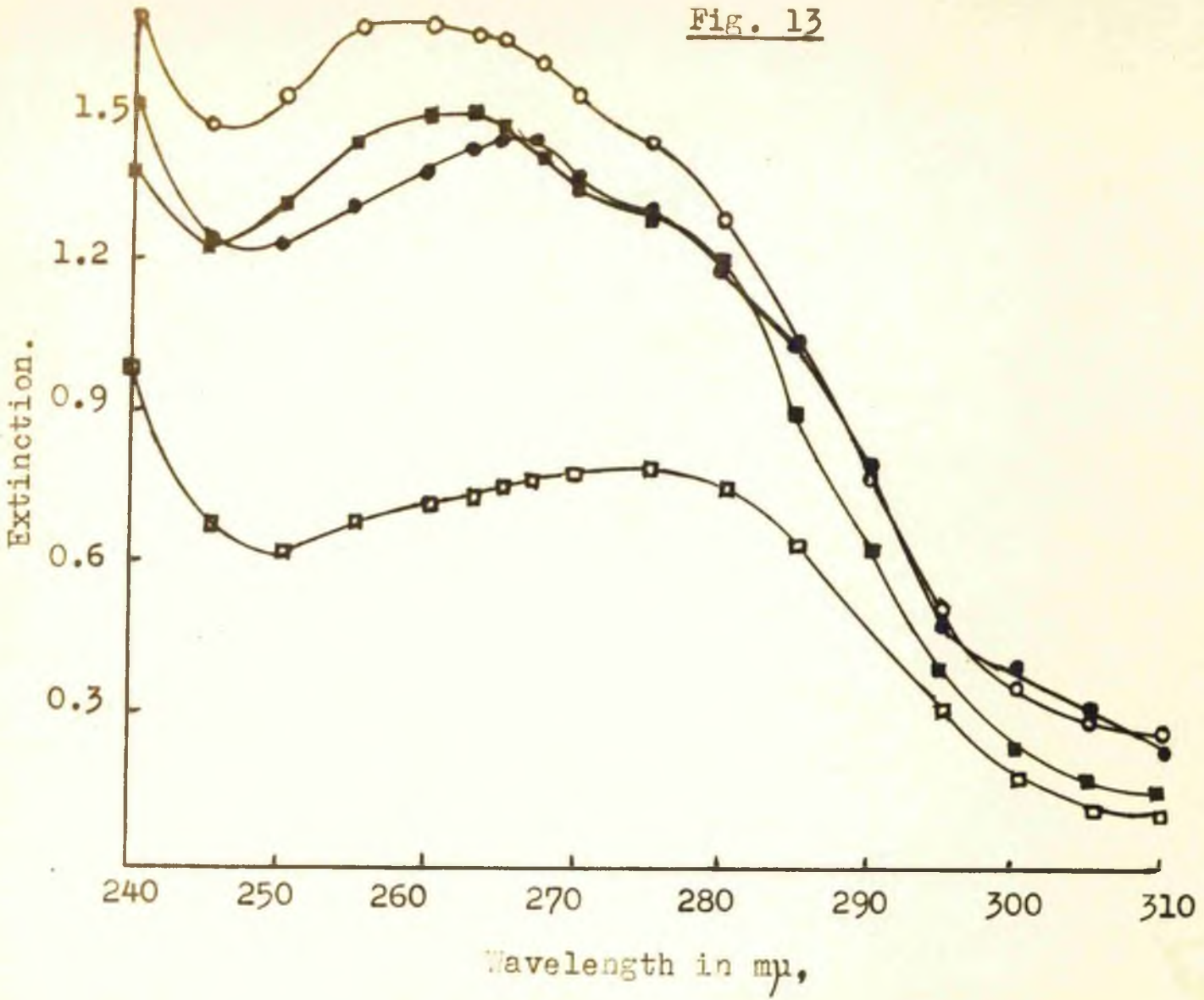
Fig. 13.

The ultraviolet spectra of the fractions obtained from rat liver extract during the purification procedure.

All protein concentrations were adjusted to 0.6 mg/ml.

Fraction	$\frac{\text{Extinction at 260 mu}}{\text{Extinction at 280 mu}}$
●——● Crude liver extract	0.88
o——o pH 4.5 precipitate	0.77
■——■ 40-60% $(NH_4)_2SO_4$	0.76
□——□ 36.9-45.4% acetone	1.07

Fig. 13


1.5
1.2
0.9
0.6
0.3
Extinction.
240
250
260
270
280
290
300
310
Wavelength in mμ.

from 260 to 273 mμ, and an increase in the ratio of the extinction at 260 mμ to that at 280 mμ; both observations suggest that the protein is being purified with the removal of contaminating nucleic acid.

In view of the difficulties associated with further purification, it was decided to investigate the mode of action of the inhibitory factor at the partially purified stage reached by precipitation with acid, ammonium sulphate and acetone.

3. 5 The mode of action of the purified inhibitory factor.

Since it had been demonstrated that the inhibitory factor did not act by destruction of the substrate deoxyribonucleoside triphosphates, i.e. by phosphatase action, there remained two possible sites for its action: the DNA primer and the DNA nucleotidyltransferase itself. Experiments were therefore carried out to determine whether prior incubation of either the DNA or the DNA nucleotidyltransferase with the purified inhibitor preparation affected the incorporation of ^{32}P-TMP into DNA.

Fig. 14 shows the effect of preincubating the thymus extract, containing the DNA nucleotidyltransferase, and the purified inhibitory factor alone and together. Although preincubation of the thymus enzyme or the inhibitory

Fig. 14.

The effect on the incorporation of ^{32}P-TMP into DNA of a prior incubation of DNA nucleotidyltransferase with the purified inhibitor preparation.

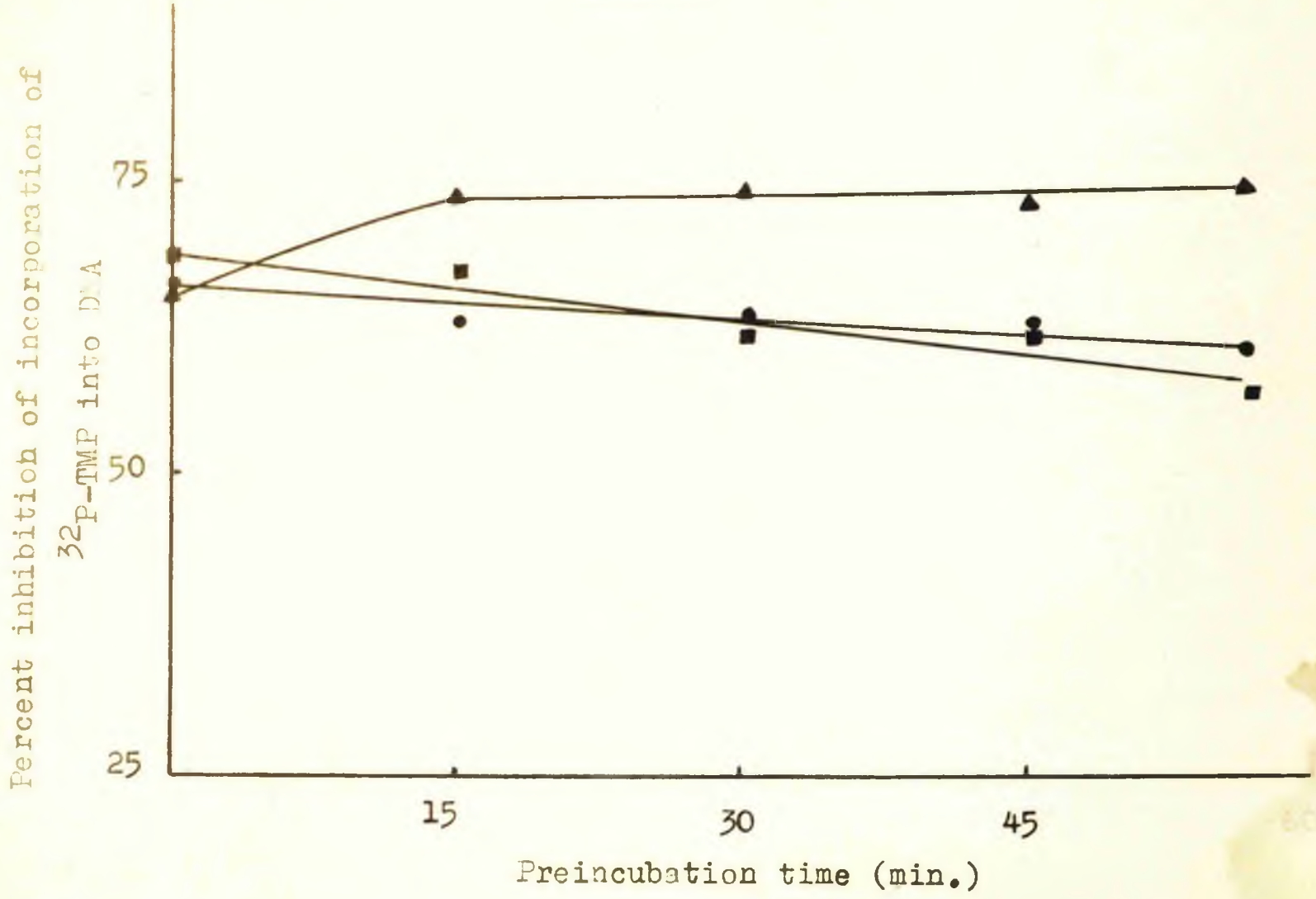
DNA nucleotidyltransferase and the purified inhibitor preparation were incubated separately and together, and at intervals of 0, 15, 30, 45 and 60 min. aliquots of each, corresponding to 0.75 mg. enzyme protein and 0.1 mg. inhibitor protein were removed and added to tubes which contained the following components of the DNA nucleotidyltransferase assay: 10 µmoles tris, pH 7.5, 0.05 µmoles EDTA, 6 µmoles $MgCl_2$, 50 µg. denatured DNA, 1 µmole 2-mercaptoethanol and 125 mµmoles each of dATP, dGTP, dCTP and ^{32}P-TTP. In addition, 0.75 mg. enzyme protein or 0.1 mg. inhibitory protein was added to those tubes which did not otherwise contain these. Incubation was for 1 hr. at 37°.

▲——▲ Thymus extract + inhibitory fraction preincubated.

■——■ Inhibitory fraction preincubated alone.

●——● Thymus extract preincubated alone.

Fig. 14


Percent inhibition of incorporation of 32P-TMP into DNA
75
50
25
15
30
45
Preincubation time (min.)

factor alone led to a slight fall in the level of inhibition, prior incubation of the thymus enzyme and the inhibitor preparation together caused a slight increase in inhibition over the first 15 min. of preincubation. This is a relatively small effect, however, and seemed unlikely to account for the action of the inhibitor on the DNA nucleotidyltransferase reaction. The effect of preincubating the purified inhibitor preparation and the primer DNA separately and together is shown in Fig. 15. It is clear that the prior incubation of DNA with the inhibitor gives rise to a progressive increase in the inhibition of the transferase reaction, so that after 60 min. preincubation the incorporation of ^{32}P-TMP is completely inhibited.

The most obvious explanation for this effect would be the destruction of the primer DNA by a DNase in the inhibitor preparation. In an experiment to test this possibility, the DNase activity of the purified inhibitor preparation was measured under conditions comparable with those employed in the preliminary incubations in the experiments shown in Figs. 14 and 15. From the results shown in Fig. 16, it can be seen that the inhibitor has a strong DNase activity. The amount of inhibitor used in the experiments shown in Figs. 14, 15 and 16 was about 6 times that required to give a 22% inhibition of the

Fig. 15.

The effect of prior incubation of DNA with the purified inhibitor preparation on the DNA nucleotidyltransferase reaction.

670 µg. denatured DNA, 134 µmoles tris, pH 7.5, 6.7 umoles 2-mercaptoethanol and 30 umoles $MgCl_2$ were incubated alone and together with 1.33 mg. of the purified inhibitor preparation. The volumes for these 3 preincubations were respectively 1.07, 2.07 and 1.0 ml. At intervals of 0, 15, 30, 45 and 60 min., 0.08 ml. aliquots were removed from the first, 0.155 from the second and 0.075 from the third. These aliquots were then added to DNA nucleotidyltransferase assays and, where necessary, the components not included in the preincubation medium were added so that the final concentrations were: 0.75 mg. thymus DNA nucleotidyltransferase, 50 µg. denatured DNA, 6 µmoles $MgCl_2$, 1 µmole 2-mercaptoethanol, 10 µmoles tris, pH 7.5, 0.05 µmole EDTA, 0.1 mg. inhibitory protein and 125 mµmoles each of dATP, dGTP, dCTP and ^{32}P-TTP. The total volume was 0.28 ml., and incubation was for 1 hr. at 37°.

▲——▲ Inhibitor preparation + DNA, tris, $MgCl_2$, 2-mercaptoethanol, EDTA preincubated.

■——■ Inhibitory preparation preincubated alone.

●——● DNA, tris, $MgCl_2$, 2-mercaptoethanol, EDTA preincubated.

Fig. 15.

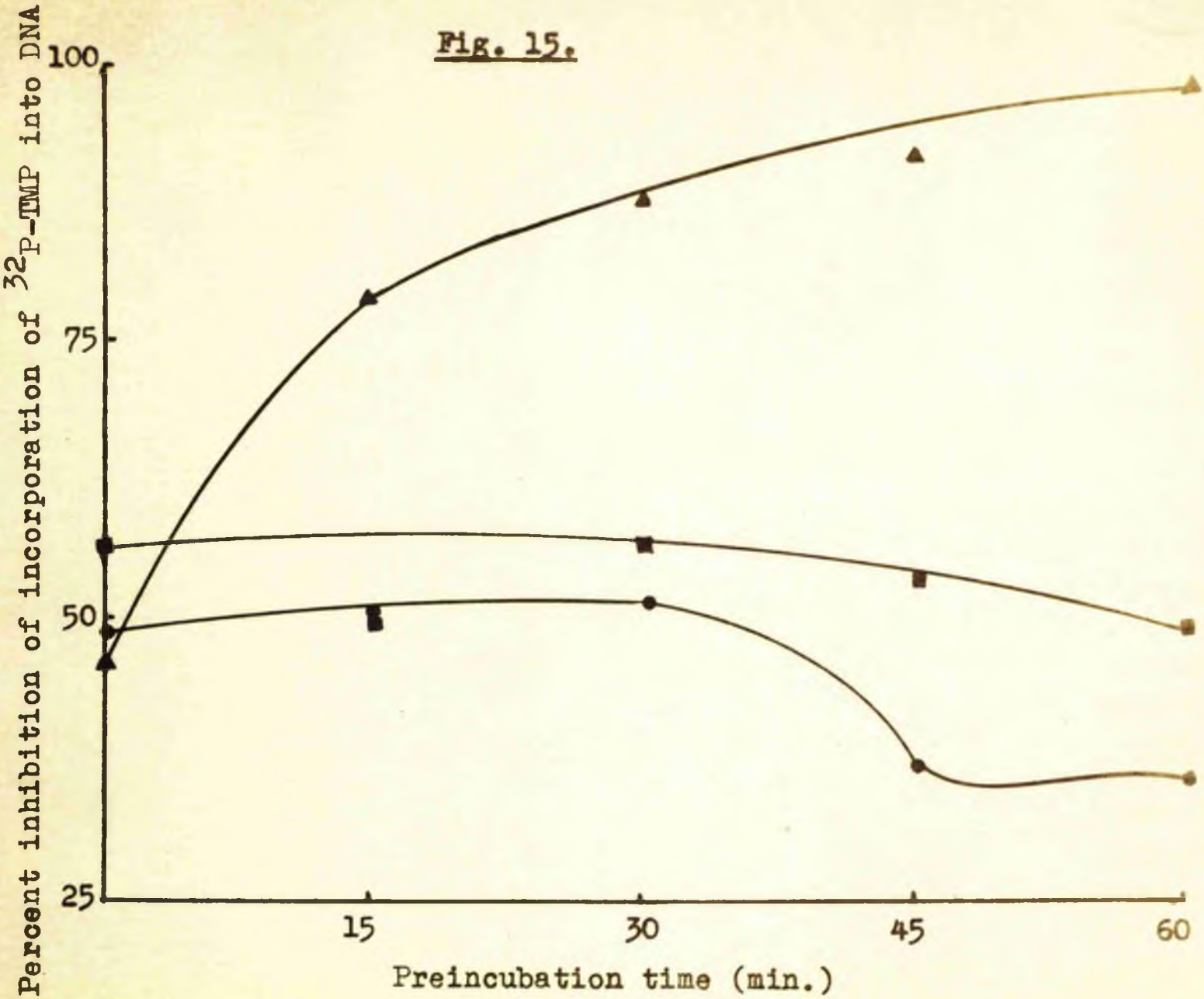
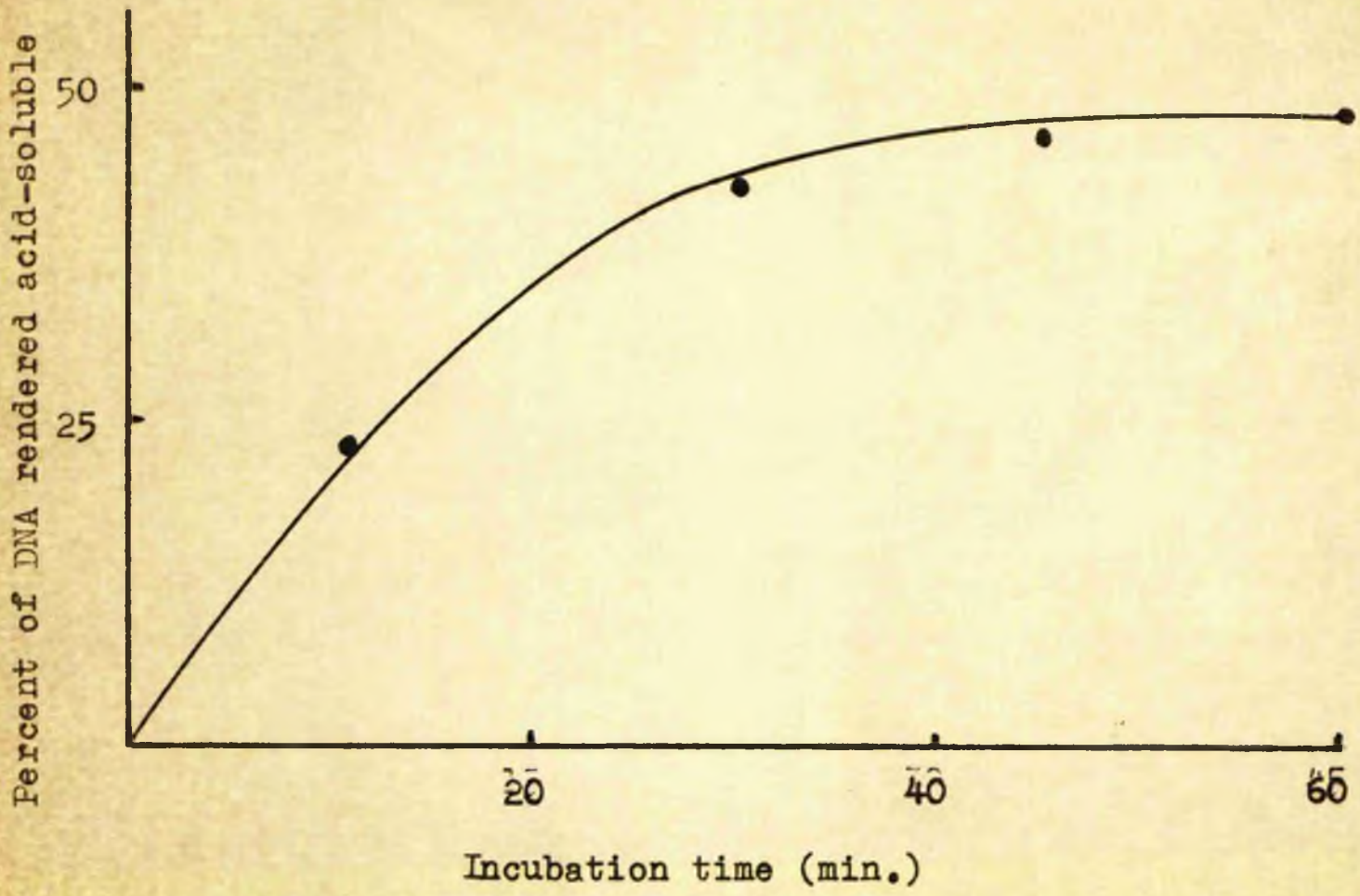

Percent inhibition of incorporation of ^{32}P-TMP into DNA
100
75
50
25
15
30
45
60
Preincubation time (min.)

Fig. 16.

The DNase activity of the purified inhibitor preparation.

The incubation medium contained 20 μmoles tris, pH 7.4, 2 μmoles 2-mercaptoethanol, 12 μmoles $MgCl_2$, 0.1 μmole EDTA, 12 μmoles $MgCl_2$, 100 μg. denatured DNA and 0.2 mg. inhibitor protein in a total volume of 0.56 ml. Incubation was at 37°.

Fig. 16.


Percent of DNA rendered acid-soluble
50
25
20
40
60
Incubation time (min.)

incorporation into DNA in a normal DNA nucleotidyltransferase assay.

Since the purified inhibitor preparation possessed DNase activity, experiments were carried out to determine the DNase activity of the inhibitor during the purification procedure, and a comparison of the DNase activity with the inhibitory activity is shown in Fig. 17. The two activities show similar increases during the purification and it seems likely that they are closely related.

Previous experiments (Fig. 11) had shown that the inhibitory activity was very heat-labile, and the sensitivity of the DNase to heat inactivation was therefore compared with that of the inhibitor. The results of such an experiment (Fig. 18)show that after heating to 45° for 5 min., 50% of the inhibitory and 70% of the DNase activity is lost. A small residual DNase activity appears to resist heating to 60°, while all inhibitory activity is lost at 50°. Nevertheless, there is a considerable similarity between the response of the DNase and the inhibitor to heating, and it may well be that the residual activity is due to a second, more heat-stable, nuclease present in the purified inhibitor preparation.

If the inhibitory activity is due to the presence of a nuclease in the preparation, the inhibition should be

Fig. 17.

The relative inhibitory and DNase activities during the purification of the inhibitory factor from rat liver.

The DNA nucleotidyltransferase assay medium contained 10 μmoles tris, pH 7.5, 1 μmole 2-mercaptoethanol, 6 μmoles $MgCl_2$, 0.05 μmole EDTA, 50 μg. denatured DNA, 0.75 mg. enzyme protein, an amount of the protein from each of the fractions, such that the final inhibition was around 30% and 125 mμmoles eech of dATP, dGTP, dCTP and ^{32}P-TTP. The total volume was 0.28 ml. and incubation was for 1 hr. at 37°.

The medium for the DNase assays contained 20 μmoles tris, pH 7.5, 2 μmoles 2-mercaptoethanol, 12 μmoles $MgCl_2$, 0.1 μmole EDTA, 100 μg. DNA, and twice the amount of inhibitory protein used for the assays of the inhibition of the DNA nucleotidyltransferase. The total volume was 0.31 ml., and incubation was for 1 hr. at 37°.

The open columns represent the inhibitory activity and the hatched ones the DNase activity.

Fig. 17.

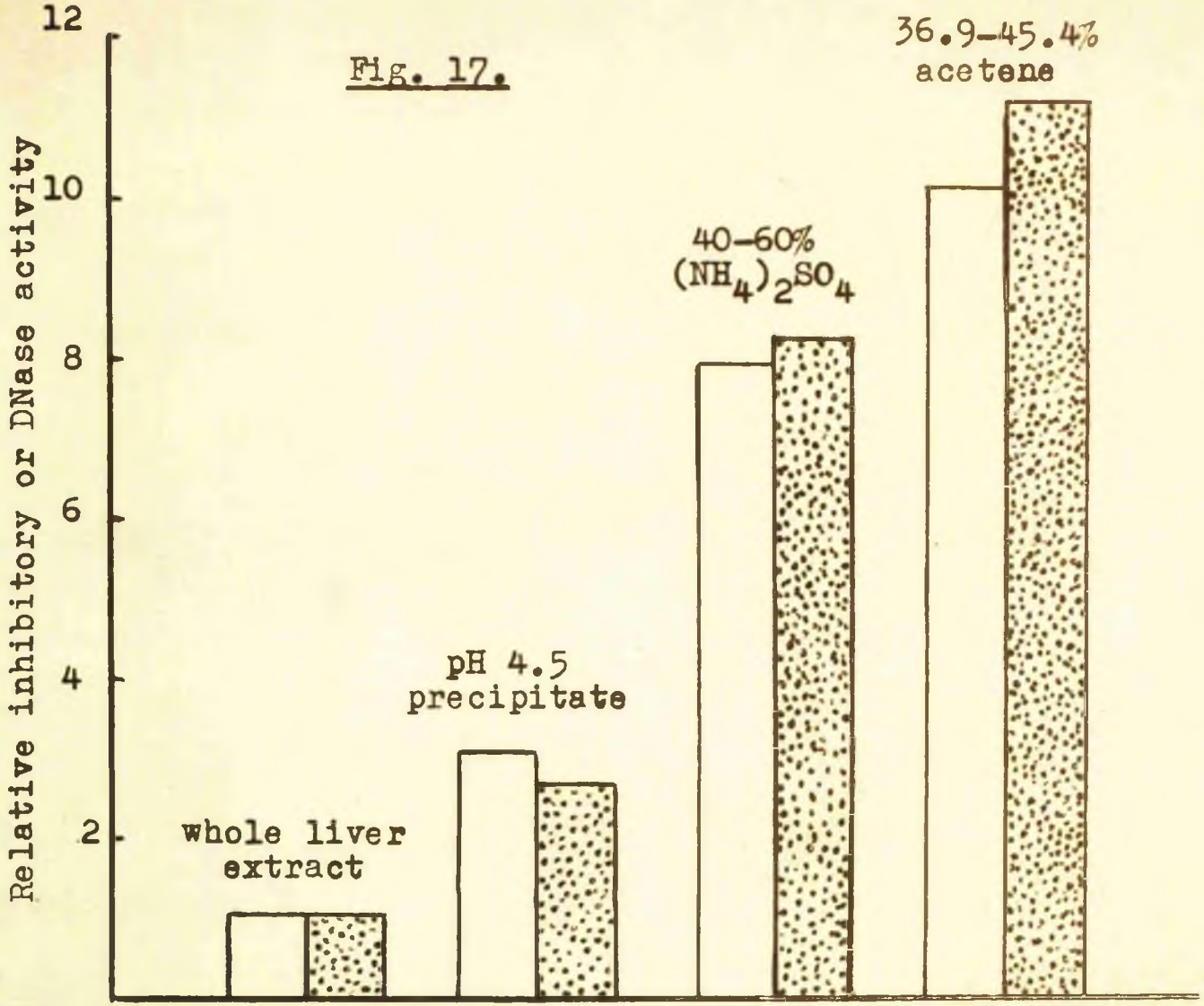

Relative inhibitory or DNase activity
12
10
8
6
4
2
whole liver extract
pH 4.5 precipitate
40-60% $(NH_4)_2SO_4$
36.9-45.4% acetone

Fig. 18.

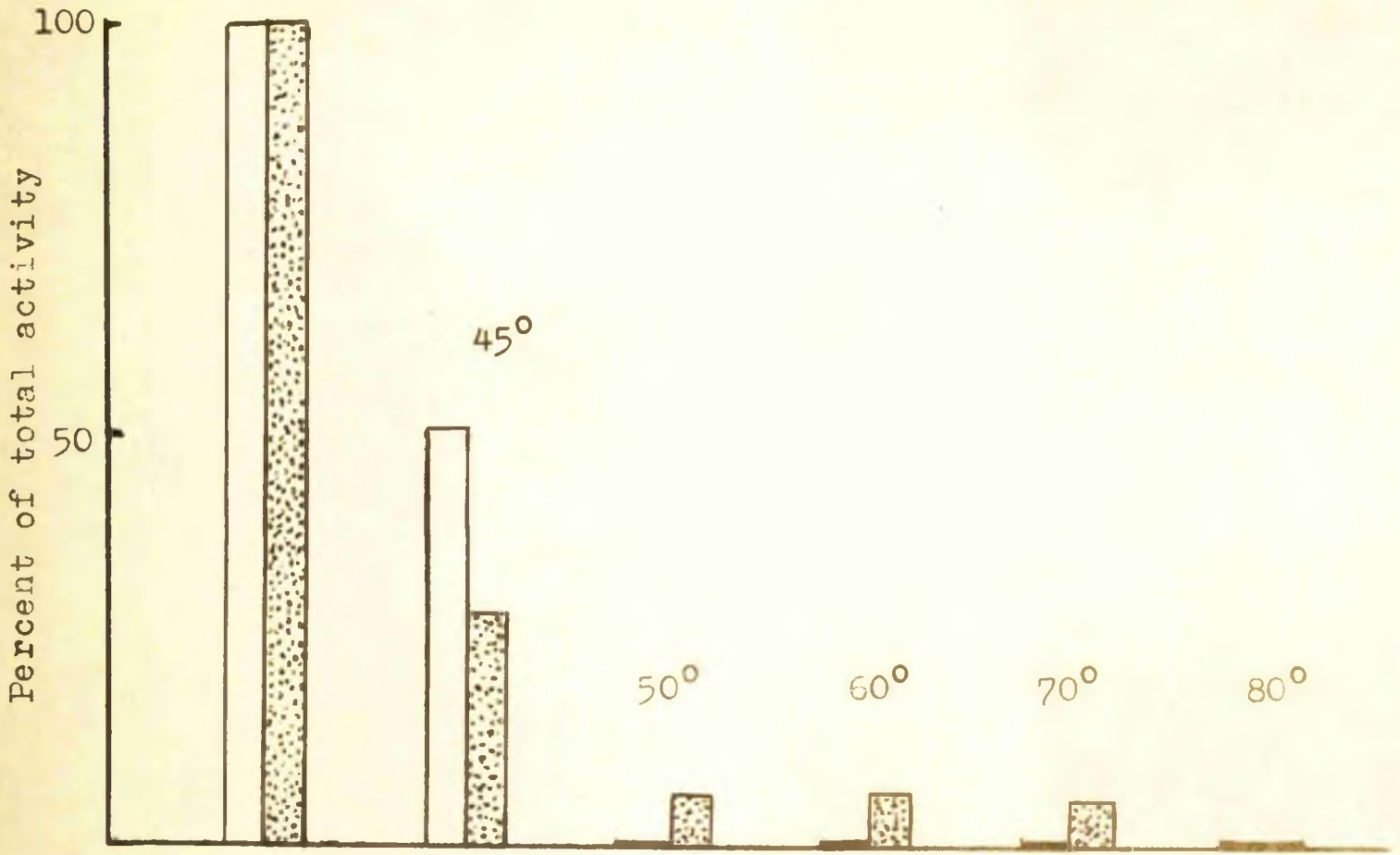
The effect of temperature on the inhibitory and DNase activities of the purified inhibitor preparation.

0.6 ml. portions of the inhibitory preparation (2 mg. protein/ml.) were heated to the specified temperature in a water bath for 5 min. and placed in an ice bath.

0.15 mg. of protein from the purified inhibitor preparation was used for the DNA nucleotidyltransferase assays and 0.3 mg. for the DNase assays. The other components of the incubation media were as for Fig. 17.

The open columns represent the inhibitory activity and the hatched ones the DNase activity.

Fig. 18.



decreased by increasing the DNA concentration in the DNA nucleotidyltransferase assay. The results of such an experiment are shown in Table 5. The inhibitory effect reaches a maximum at a low concentration of DNA, but the "protective" effect of adding additional DNA appears to reach a maximum at about 200 µg DNA/assay, and the addition of further DNA does not appear to cause any further lowering of the inhibitory activity. This suggests that there may be two factors involved in the inhibition: the DNase activity itself and some additional inhibitor, such as a product of the action of the nuclease. In order to investigate the latter possibility, the effect of adding the products of incubation of DNA with purified inhibitor preparation to DNA-nucleotidyltransferase assays, containing various amounts of DNA, were examined. In Fig. 19, the upper curve illustrates the effect of adding DNA previously incubated without any inhibitor, while the lower curve shows the effect of adding DNA previously treated with the inhibitor for 1 hr. If the products of the action of the inhibitor preparation did not affect the DNA nucleotidyltransferase system, the lower curve should reach a plateau when about 50 to 75 µg. DNA are added to the assay, and this plateau should coincide with the incorporation in the corresponding controls. In fact,

Table 5.

The effect of DNA concentration on the inhibition of DNA nucleotidyltransferase by a purified inhibitor preparation.

µg. DNA added/assay	Percent inhibition
10	52.3
25	69.8
50	63.0
75	55.0
100	51.7
200	21.5
250	20.1

The incubation medium contained 10 µmoles tris, pH 7.5, 1 µmole 2-mercaptoethanol, 6 µmoles $MgCl_2$, 0.05 µmole EDTA, 0.75 mg. enzyme protein, 125 mµmoles each of dATP, dGTP, dCTP and ^{32}P-TTP and 0.15 mg. inhibitory protein. The total volume was 0.3 ml. and incubation was for 1 hr. at 37°.

Fig. 19.

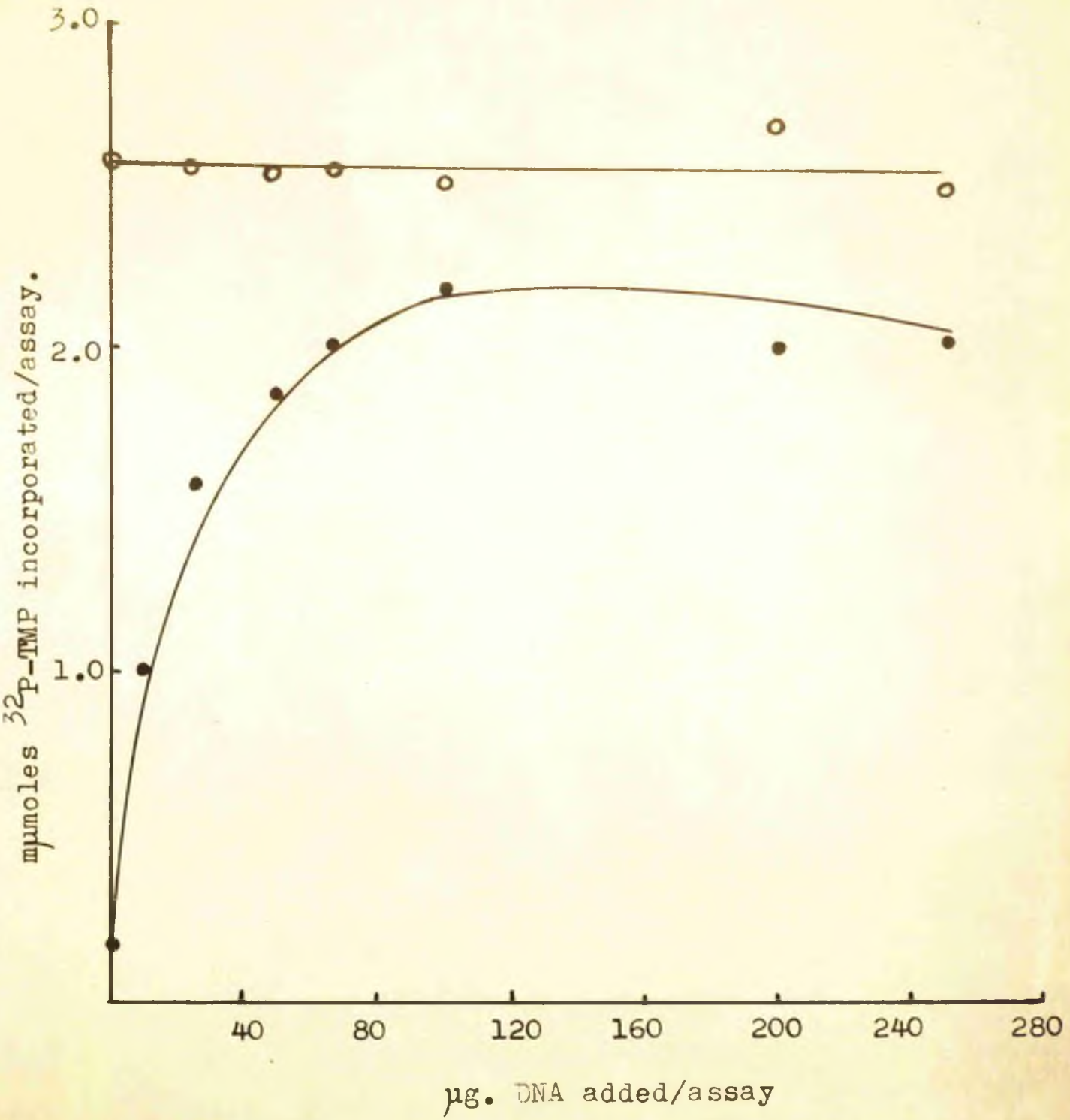
The effects of the products of incubation of DNA with the purified inhibitor preparation on the incorporation of ^{32}P-TMP into DNA at various DNA concentrations.

1.25 mg. denatured DNA, 250 μmoles tris, pH 7.5, 25 μmoles 2-mercaptoethanol, 1.25 μmoles EDTA and 150 μmoles $MgCl_2$ were incubated alone and together with 3.75 mg. protein from the purified inhibitor preparation added. At the end of 1 hr. incubation, the tubes were placed in a boiling water bath and 3.75 mg. enzyme protein added to the tube which did not already have this. The tubes were heated at 100° for 5 min. Aliquots from these tubes were then added to DNA nucleotidyltransferase assays in such a way that the reaction mixture finally contained 50 ug. denatured, preincubated (with or without the purified inhibitor preparation) DNA, 1 μmole 2-mercaptoethanol, 6 μmoles $MgCl_2$, 0.05 μmole EDTA, 10 μmoles tris, pH 7.5, 0.75 mg. enzyme protein, additional denatured DNA, as shown in Fig. and 125 mμmoles each of dATP, dGTP, dCTP and ^{32}P-TTP. The final volume was 0.3 ml. and incubation was for 1 hr. at 37°.

●——● DNA, 2-mercaptoethanol, $MgCl_2$, EDTA, tris and purified inhibitor preparation preincubated.

o——o DNA, 2-mercaptoethanol, $MgCl_2$, EDTA and tris preincubated, purified inhibitor preparation added while solution was at boiling point.

Fig. 19.


3.0
2.0
1.0
mμmoles ^{32}P-TMP incorporated/assay.
40
80
120
160
200
240
280
μg. DNA added/assay

a plateau is reached at about 80 µg/assay, but the final level of incorporation is approximately 20% lower than in the control, suggesting that some component of the inhibitor-treated DNA preparation interferes with the DNA nucleotidyltransferase assay.

Table 6 shows the effect of adding a purified inhibitor preparation to a normal assay which had been incubated for 1 hr. At this time the system had synthesised DNA corresponding to 3.96 mµmoles ^{32}P-TMP, but when the purified inhibitor was added to this, and the incubation carried out for a further hour, the level of incorporation was reduced to 2.85 mµmoles, i.e. instead of a further 2.35 mµmoles TMP being incorporated, 1.11 mµmoles of TMP were removed from DNA already synthesised. This indicates that the newly synthesised DNA is susceptible to the action of the nuclease in the purified inhibitor preparation.

In view of the possibility that the inhibition of the DNA nucleotidyltransferase by the inhibitor preparation was due entirely to its nucleolytic activity, the effects of adding pancreatic DNase I and splenic DNase II to DNA nucleotidyltransferase assays were determined. As can be seen from the results in Table 7, very high concentrations

Table 6.

The effect of adding a purified inhibitor preparation to a DNA nucleotidyltransferase assay after incubation for 1 hr.

Assay conditions	mμmoles ^{32}P-TMP incorporated/mg. protein
Normal incubation for 1 hr. in absence of inhibitor.	3.96
Incubation for 1 hr. in the presence of 0.1 mg. inhibitor.	1.36
Normal incubation for 2 hr. in absence of inhibitor.	6.31
Normal incubation for 1 hr., 0.1 mg. inhibitor protein added and incubation continued for 1 hr.	2.85

The incubation medium contained 10 μmoles tris, pH 7.5, 1 μmole 2-mercaptoethanol, 6 μmoles $MgCl_2$, 0.05 μmole EDTA, 0.75 mg. enzyme protein and 125 mμmoles each of dATP, dGTP, dCTP and ^{32}P-TTP in a total volume of 0.28 ml. Incubation was at 37°.

Table 7.

The DNA nucleotidyltransferase assay system consisted of 10 μmoles tris, pH 7.5, 1 μmole 2-mercaptoethanol, 0.05 μmole EDTA, 50 μg. denatured DNA, 6 μmoles $MgCl_2$, 0.75 mg. enzyme protein and 125 mμmoles each of dATP, dGTP, dCTP and ^{32}P-TTP.

The DNase assay consisted of 20 μmoles tris, pH 7.5, 2 μmoles 2-mercaptoethanol, 0.1 μmole EDTA, 100 μg. denatured DNA, 12 μmoles $MgCl_2$.

* Splenic DNase II units were as described by Koerner and Sinsheimer (1957).

Table 7.

The effect of the purified inhibitor preparation, pancreatic DNase I and splenic DNase II on the incorporation of ^{32}P-TMP into DNA.

Pancreatic DNase I

µg. crystalline enzyme/assay	Extinction at 260 mµ of acid-soluble products.	Percent inhibition.
525	0.636	89.6
105	0.461	6.5
52.5	0.381	0

Splenic DNase II

*units/assay	Extinction at 260 mµ of acid-soluble products.	Percent inhibition.
6,000	0.522	94.6
3,000	0.187	66.2

Purified inhibitor preparation.

µg. protein/ assay	Extinction at 260 mµ of acid-soluble products.	Percent inhibition.
140	0.479	51
70	0.277	29

of pancreatic DNase were required to produce a significant inhibition of the reaction. On the other hand, the system proved to be very sensitive to concentrations of splenic DNase II which produced only a small amount of acid-soluble material.

As will be seen in the following section, the nuclease of the purified inhibitor preparation attacks heat-denatured DNA more rapidly than native DNA and, therefore, the effect of the inhibitor on a DNA nucleotidyltransferase system primed with native DNA was tested. Table 8 shows a comparison of the effects of the inhibitor on the DNA nucleotidyltransferase system with native and denatured DNAs as primers. The incorporation of ^{32}P-TMP into native DNA was only 11% of that obtained with heat-denatured DNA, but the activity of the transferase primed with denatured DNA was substantially inhibited by the inhibitor, whereas that of the transferase primed with native DNA was greatly enhanced. Fig. 20 illustrates the effect of inhibitor concentration on the activity of the DNA nucleotidyltransferase when the system is primed with native DNA. There is a maximum stimulation at a level of 64 µg. inhibitory protein/50 µg. DNA; at higher concentrations of inhibitor the stimulatory effect declines, but at no point within the range of protein concentrations tested

Table 8.

The effect of the purified inhibitor preparation on the incorporation of ^{32}P-TMP into DNA, using native and denatured DNA as primers.

Primer	Counts/min. incorporated/ mg. protein	Effect
Native DNA	5,044	
Native DNA (+ inhibitor)	10,335	105% stimulation in presence of inhibitor
Denatured DNA	45,967	
Denatured DNA (+ inhibitor)	29,659	35.5% inhibition in presence of inhibitor.

The assay medium contained 10 µmoles tris, pH 7.5, 6 µmoles $MgCl_2$, 1 µmole 2-mercaptoethanol, 0.05 µmole EDTA, 0.75 mg. enzyme protein, 50 µg DNA and 125 mµmoles each of dATP, dGTP, dCTP and ^{32}P-TTP. The total volume was 0.3 ml. and incubation was for 1 hr. at 37°.

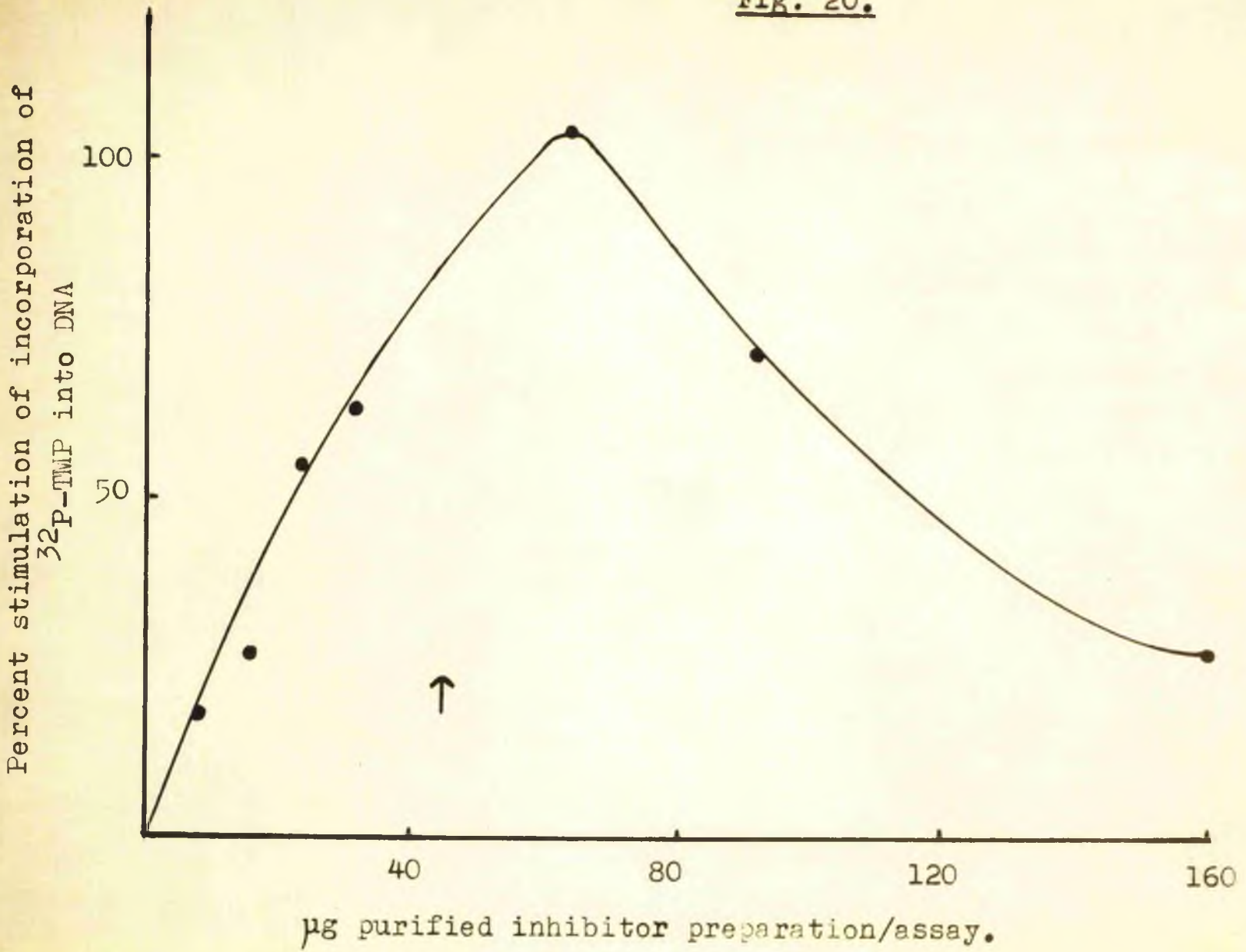
Fig. 20.

The effect of concentration of the purified inhibitor preparation on the activation of the DNA nucleotidyltransferase, when undenatured DNA is used as primer.

The assay medium contained 10 μmoles tris, pH 7.5, 1 μmole 2-mercaptoethanol, 6 μmoles $MgCl_2$, 0.05 μmole EDTA, 50 μg. denatured DNA and 125 mμmoles each of dATP, dGTP, dCTP and ^{32}P-TTP. The total volume was 0.28 ml. and incubation was for 1 hr. at 37°.

↑ Concentration of purified inhibitor preparation which inhibits the incorporation by 30% when denatured DNA is used as primer.

Fig. 20.


Percent stimulation of incorporation of
32P-TMP into DNA
100
50
40
80
120
160
µg purified inhibitor preparation/assay.

was any inhibition of the DNA nucleotidyltransferase observed. The point marked with an arrow on the figure represents the concentration of inhibitor/assay which was found to give an inhibition of 30% when added to a normal DNA nucleotidyltransferase assay, primed with denatured DNA.

3. 6 Characteristics of the nuclease activity in the purified inhibitor preparation.

The properties of the nuclease activity in the purified inhibitor preparation were examined to determine whether this enzyme resembled any of the nucleases whose characteristics have been investigated and defined.

The effect of variations in the $MgCl_2$ concentration on the DNase activity is shown in Fig. 21. The optimal concentration is 1 μmole/100 μg denatured DNA, which corresponds to a molar ratio of $MgCl_2$ to phosphate in the substrate of 3 to 1. The variation of nuclease activity with pH at the optimal Mg^{++} concentration is shown in Fig. 22. There is a broad pH optimum, extending from 6.5 to 8.5.

$MnCl_2$ also stimulates the nuclease activity of the inhibitor preparation, as shown in Fig. 23, and can replace $MgCl_2$ at the optimal concentration, which is about one tenth of that found for $MgCl_2$. $CaCl_2$ does not

Fig. 21.

The effect of $MgCl_2$ concentration on the nuclease activity of the purified inhibitor preparation.

The DNase assay medium contained 20.0 µmoles tris, pH 7.5, 100 µg. denatured DNA and 75 µg. protein from the purified inhibitor preparation in a total volume of 0.275 ml. Incubation was for 1 hr. at 37°.

Fig. 21.

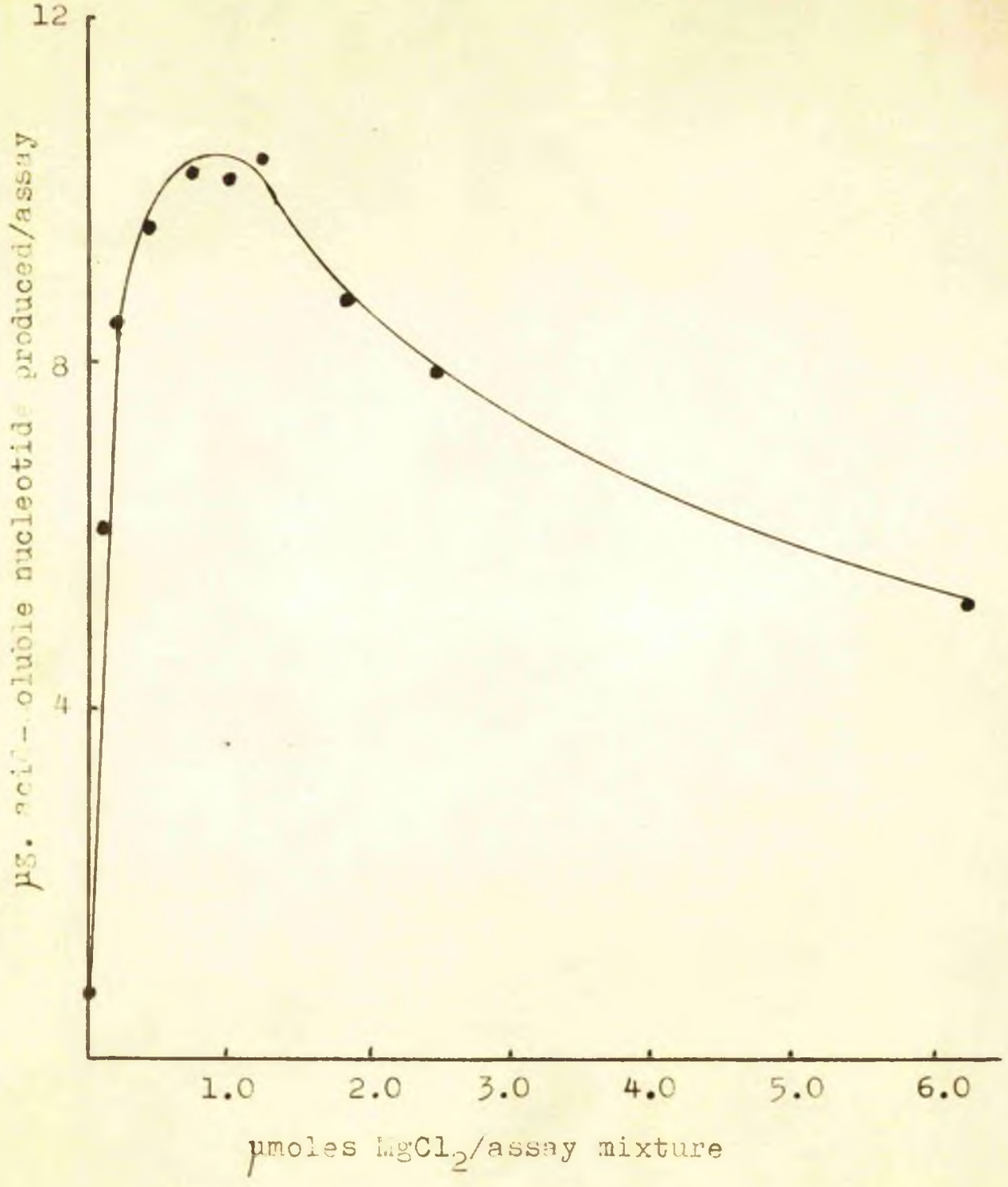

12
8
4
µg. acid-soluble nucleotide produced/assay
1.0
2.0
3.0
4.0
5.0
6.0
µmoles $MgCl_2$/assay mixture

Fig. 22.

The effect of variations in pH on the nuclease activity of the purified inhibitor preparation.

The assay medium contained 1 μmole $MgCl_2$, 100 μg. denatured DNA and 75 μg. inhibitor preparation. 6.6 μmoles phosphate buffer of the appropriate pH were added to assays in the pH range 5.5 to 7.5, while 10 μmoles glycine buffer of the appropriate pH were added to assays in the pH range 7.5 to 10.0. The total volume was 0.375 ml., and incubation was for 1 hr. at 37^{o}.

Fig. 22.

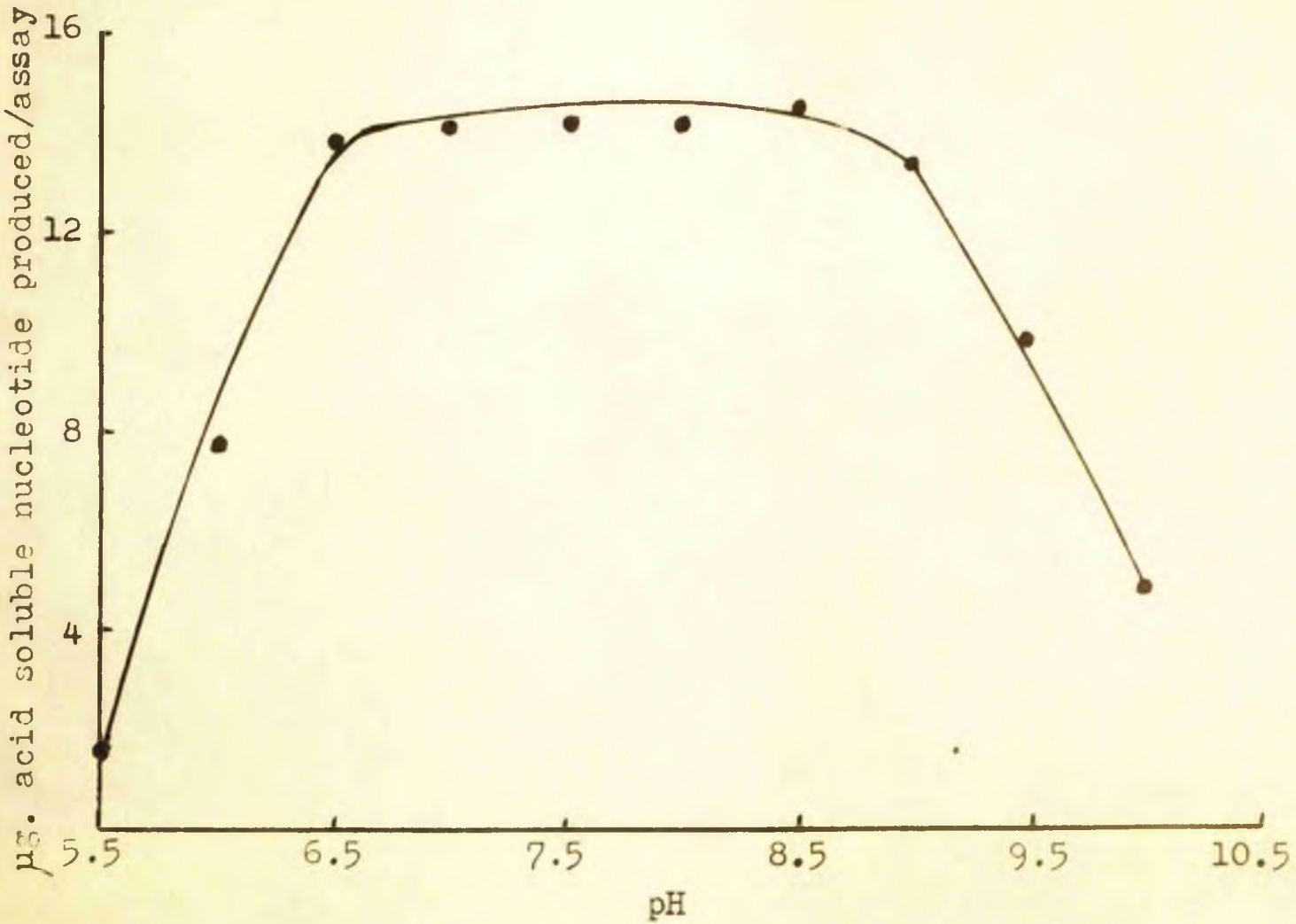
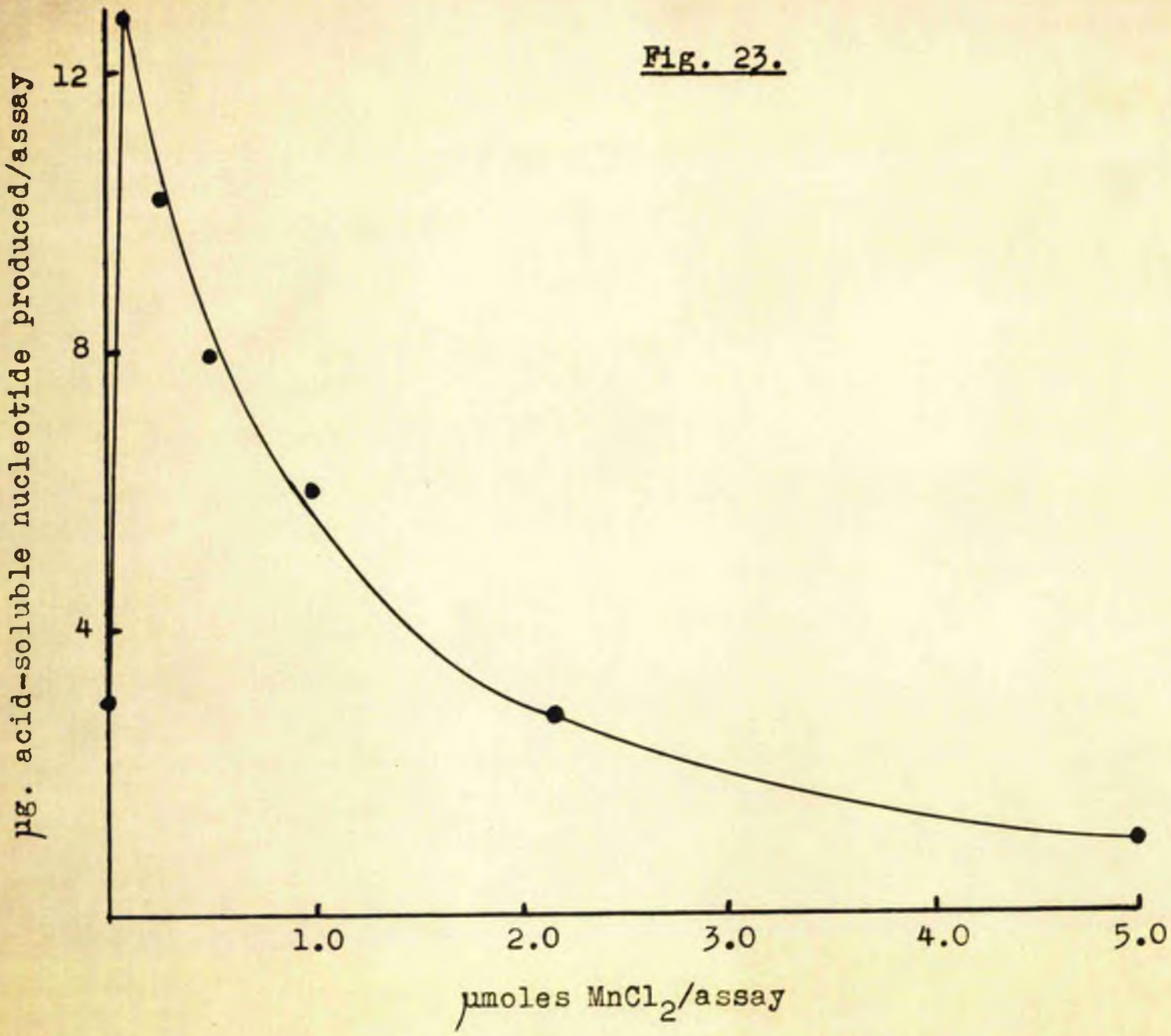

µg. acid soluble nucleotide produced/assay
16
12
8
4
5.5
6.5
7.5
8.5
9.5
10.5
pH

Fig. 23.

The effect of $MnCl_2$ concentration on the nuclease activity of the purified inhibitor preparation.

The assay medium consisted of 10 μmoles tris, pH 7.5, 100 μg. denatured DNA and 75 μg. protein from the purified inhibitor preparation. The total volume was 0.325 ml. and incubation was for 1 hr. at 37°.

Fig. 23.


µg. acid-soluble nucleotide produced/assay
12
8
4
1.0
2.0
3.0
4.0
5.0
µmoles $MnCl_2$/assay

enhance the nuclease activity (Fig. 24), and, in fact, in the presence of an optimal concentration of $MgCl_2$ it strongly inhibits the reaction (Fig. 25). In this system, therefore, Ca^{++} ions have no synergistic action in the presence of $MgCl_2$, as happens with certain other DNases. The inhibition of the nuclease in the presence of increasing NaCl concentration is shown in Fig. 26.

Up until this point, the nuclease had been assayed using thermally denatured DNA as substrate; in further experiments its activity towards undenatured DNA was tested. It was found that heat denatured DNA was a better substrate for the enzyme than native DNA. Fig. 27 shows the results of an experiment in which DNA was heated to various temperatures, and then tested as a substrate for the nuclease activity. Denaturation of the DNA was followed by measuring the increase in extinction at 260 mμ (i.e. the hyperchromic effect). It is clear that the susceptibility of the DNA to attack by the nuclease increases with heating and that this curve exactly parallels the curve for the hyperchromic effect due to the denaturation of the DNA.

The RNase activity of the purified inhibitor preparation was also measured simultaneously with the DNase activity in some experiments. Both RNase and DNase

Fig. 24.

The effect of $CaCl_2$ concentration on the nuclease activity of the purified inhibitor preparation.

The assay medium contained 10 μmoles tris, pH 7.5, 100 μg. denatured DNA and 75 μg. inhibitory protein in a total volume of 0.385 ml. Incubation was for 1 hr. at 37°.

Fig. 24.

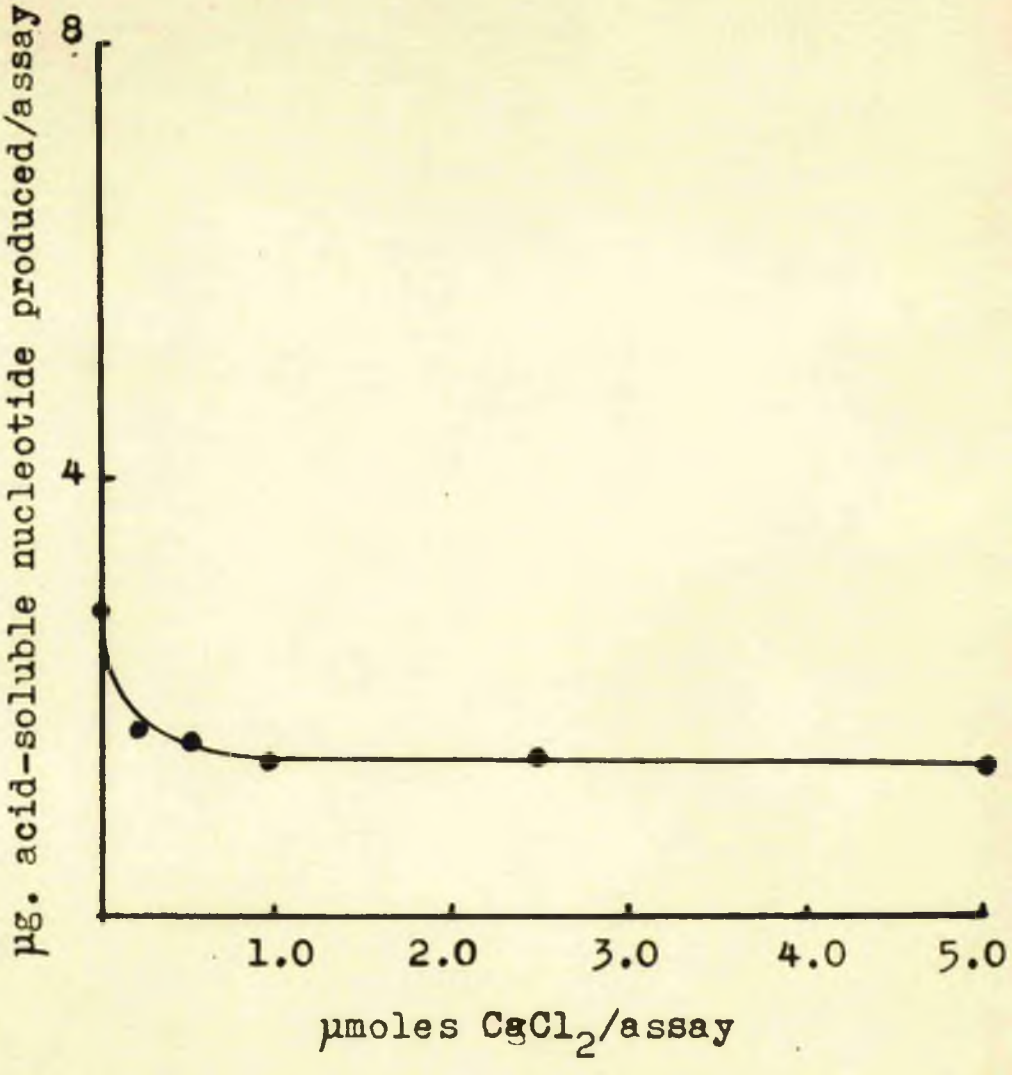

µg. acid-soluble nucleotide produced/assay
8
4
1.0
2.0
3.0
4.0
5.0
µmoles $CaCl_2$/assay

Fig. 25.

The effect of $CaCl_2$ concentration on the nuclease activity of the purified inhibitor preparation in the presence of the optimal concentration of $MgCl_2$.

The assay medium contained 1 µmole $MgCl_2$, 10 µmoles tris, pH 7.5, 100 µg. denatured DNA and 75 mg. protein from the purified inhibitor preparation in a total volume of 0.325 ml. Incubation was for 1 hr. at 37°.

Fig. 25.

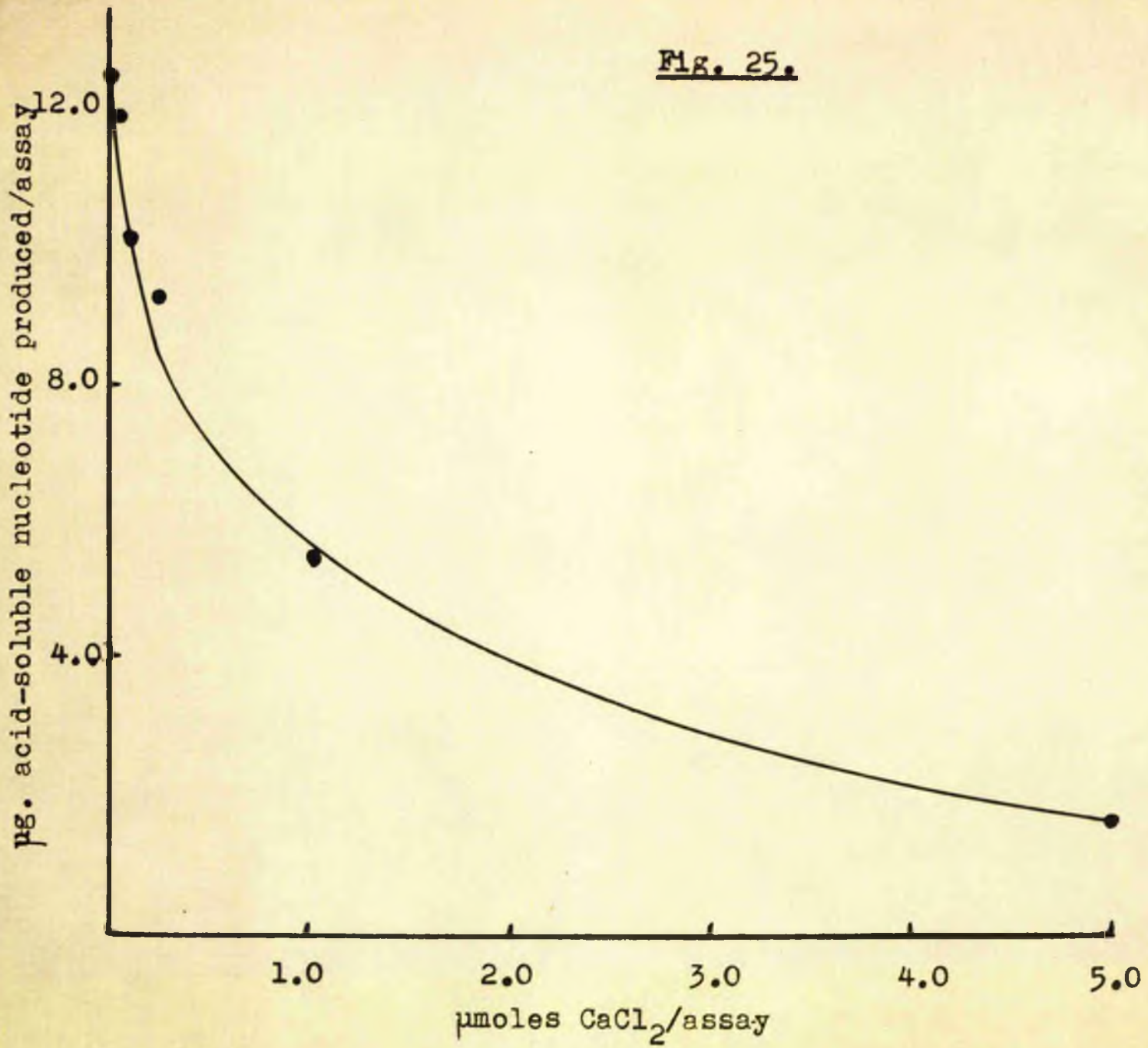

µg. acid-soluble nucleotide produced/assay
12.0
8.0
4.0
1.0
2.0
3.0
4.0
5.0
µmoles $CaCl_2$/assay

Fig. 26.

The effect of NaCl concentration on the nuclease activity of the purified inhibitor preparation.

The assay medium contained 1.0 μmole $MgCl_2$, 10 μmoles tris, pH 7.5, 75 μg. protein from the purified inhibitor preparation and 100 μg. denatured DNA. Incubation was for 1 hr. at 37°.

Fig. 26.

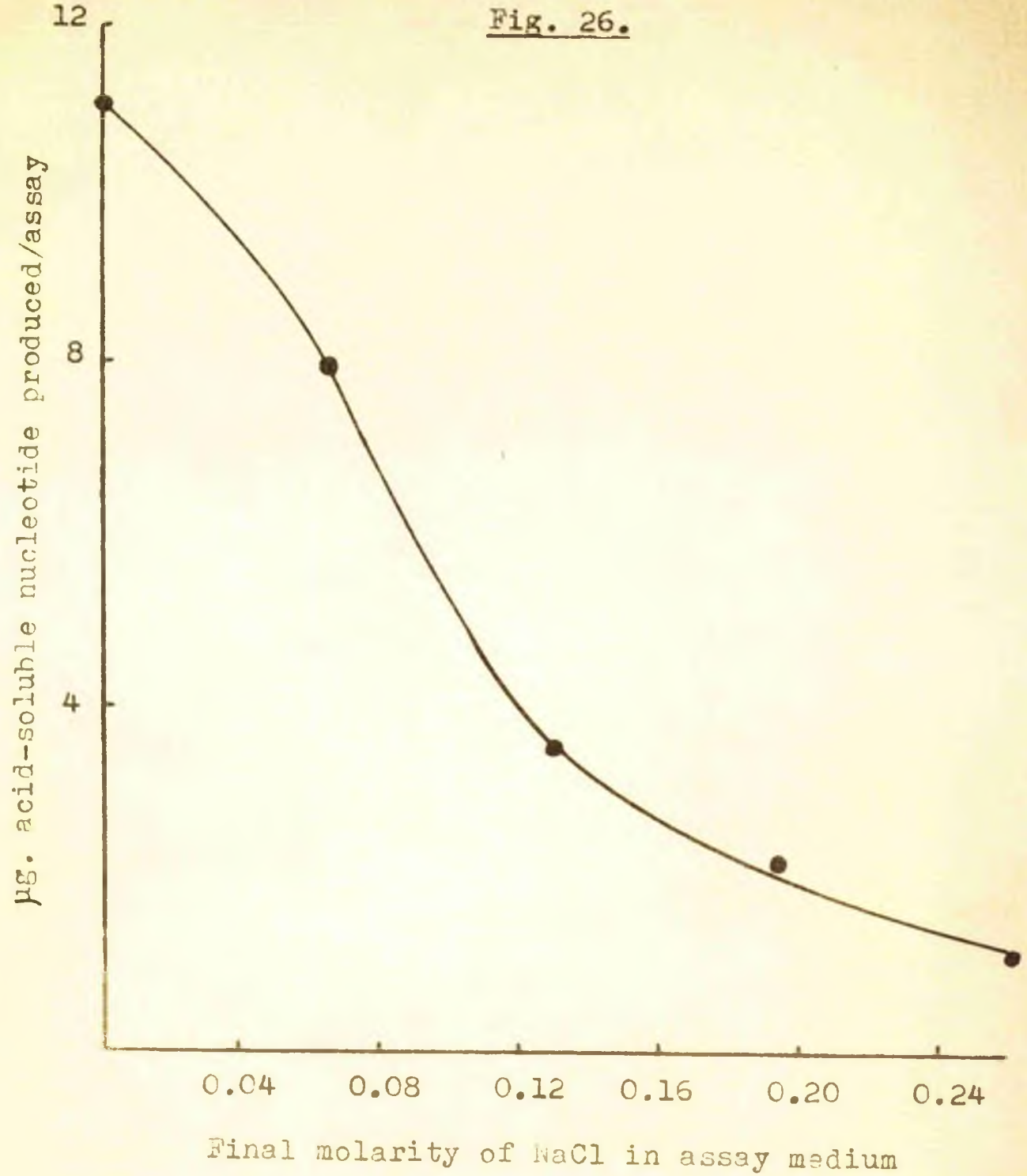

12
8
4
µg. acid-soluble nucleotide produced/assay
0.04
0.08
0.12
0.16
0.20
0.24
Final molarity of NaCl in assay medium

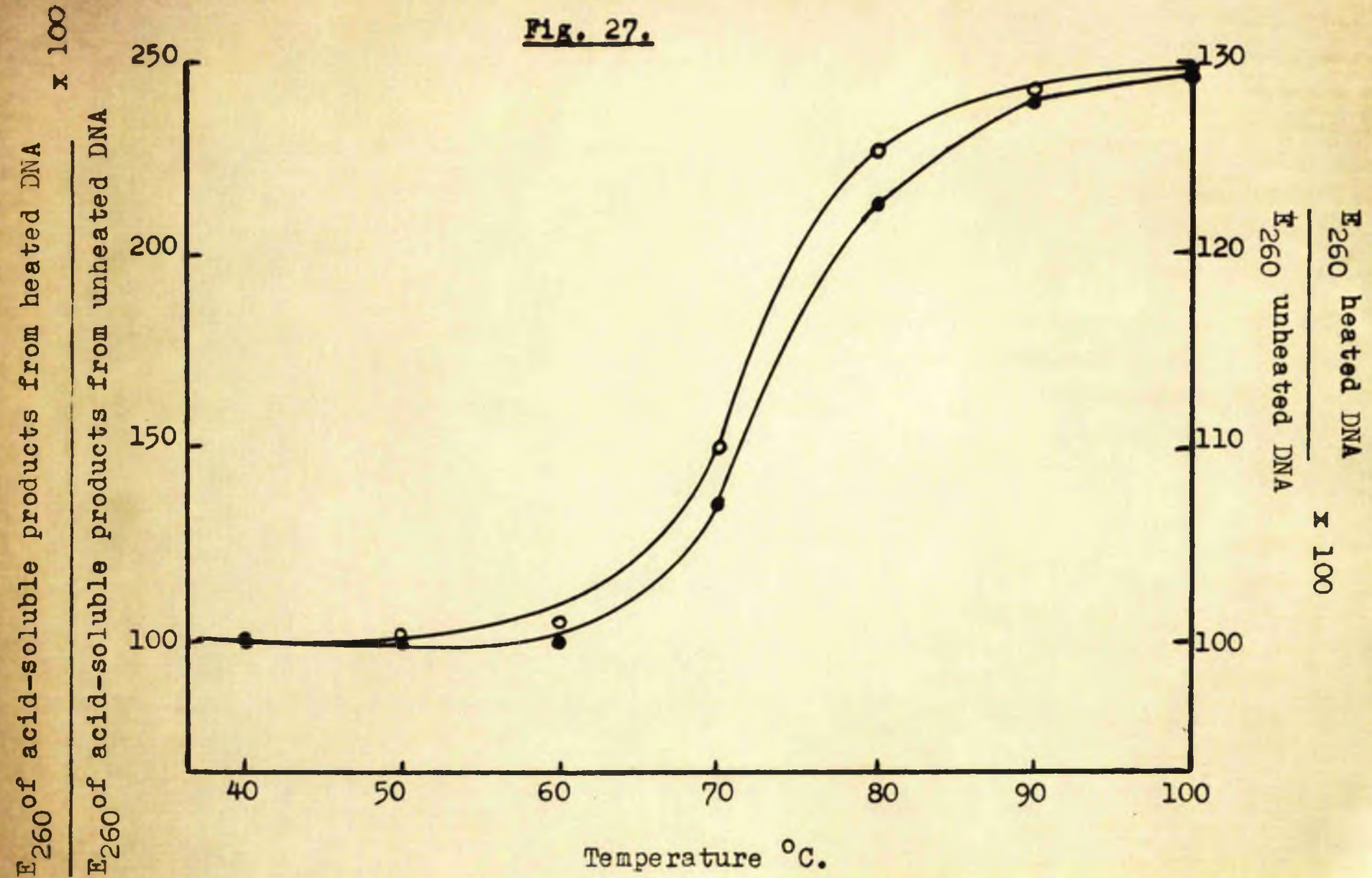
Fig. 27.

The effect of the heat denaturation of DNA on its susceptibility as a substrate for the nuclease of the purified inhibitor preparation.

DNA (0.8 mg./ml) was heated to the temperature shown for 5 min. in a water bath, and the extinction at 260 mμ measured. o——o

DNase activity was measured in the following medium:- 120 μg. DNA, heated to the appropriate temperature, 1 μmole $MgCl_2$, 5 μmoles tris, pH 7.5 and 0.15 mg. protein from the purified inhibitor preparation in a total volume of 0.285 ml. Incubation was for 1 hr. at 37°. ●——●

Fig. 27.


E_{260} of acid-soluble products from heated DNA / E_{260} of acid-soluble products from unheated DNA x 100
250
200
150
100
130
120
110
100
40
50
60
70
80
90
100
Temperature °C.
E_{260} heated DNA / E_{260} unheated DNA x 100

activities were determined at several substrate concentrations. The results of such an experiment are shown in Table 9, and indicate the presence of RNase activity to an extent of about 14% of the DNase activity. These figures do not indicate whether the RNase activity is due to the presence of a separate RNase in the inhibitory fraction, or whether it is an innate property of the nuclease itself.

Feinstein and Hagen (1962) showed that incubation of extracts of mouse kidney and intestine with ovalbumin caused DNase I activity to rise from zero to appreciable levels. Moreover, crystallised commercial DNase lost activity on incubation alone, but not in the presence of albumin or peptone. These authors suggested that the explanation of this phenomenon, in the case of the crude extracts, lay in the protection of the DNase against proteolysis, while coexistent inhibitor was destroyed. Experiments were therefore performed in which crude liver extracts were incubated with various amounts of bovine serum albumin to determined whether this protected or enhanced the nuclease activity. The results of such an experiment are shown in Table 10. Preincubation of the liver extract with bovine serum albumin seemed to have little effect on the DNase activity, which declined slightly with increasing time of

Table 9.

Comparison of the RNase and DNase activities of the purified inhibitor preparation.

µg. substrate	µg. acid soluble nucleotides from RNA	µg. acid soluble nucleotides from DNA	$\frac{\text{RNase}}{\text{DNase}} \times 100$
40	2.32	15.6	14.8
80	3.80	31.0	13.6
160	6.96	49.6	14.2

The assay medium contained 180 µg. purified inhibitor preparation, 1 µmole $MgCl_2$ and 10 µmoles tris, pH 7.5 in a total volume of 0.215 ml. Incubation was for 1 hr. at 37°.

Table 10.

The effect on the DNase activity of liver extracts after preincubation with various concentrations of bovine serum albumin.

Min. of pre-incubation.	Activity (µg. acid-soluble nucleotides produced) in the presence of various amounts of bovine serum albumin (BSA).			
	0.166% BSA	0.083% BSA	0.0166% BSA	0. BSA
0	18.0	17.8	16.7	16.0
25	17.0	15.7	15.4	15.8
45	16.9	16.0	16.6	15.8
75	16.6	16.0	15.8	15.4
120	14.9	14.4	15.0	14.4
175	14.6	14.2	14.8	14.7

5 ml. portions of liver extract, containing 5 mg. protein/ml., were incubated with 10, 5, 1 and 0 mg. bovine serum albumin and 0.5 ml. samples were removed at the times shown in the table.

0.1 ml. portions of these samples were then incubated with 100 µg. denatured DNA, 1.0 µmoles $MgCl_2$ and 8 µmoles tris, pH 7.5, in a total volume of 0.22 ml. Incubation was for 1 hr. at 37°.

incubation. Thus, no evidence for the presence of an inhibitor coexisting with the DNase could be obtained from experiments of this type.

3. 7 Intracellular distribution of enzymes hydrolysing DNA.

The intracellular location of acid and alkaline DNases has been studied by de Duve and his colleagues (Appelmans, Wattiaux and de Duve, 1955; de Duve, Pressman, Gianetto, Wattiaux and Appelmans, 1955; Beaufay, Bendall, Baudhuin and de Duve, 1959). In view of these investigations, it was decided to determine the intracellular location of enzymes hydrolysing denatured DNA at an alkaline pH. Two preparations of particles were made, one of which was disrupted osmotically, and the other sonically.

Table 11 shows the distribution of protein, alkaline DNase (with denatured and undenatured DNAs as substrate) and acid DNase. Figures obtained from two publications by de Duve and his colleagues are also included for purposes of comparison. In both experiments, the recovery of the DNase which preferentially hydrolysed denatured DNA was low (65.2 and 69.1%), while the recovery of the DNase with activity towards native DNA was higher (104.1 and 87.1%). Figs. 28 and 29 illustrate the relative specific activities

Table 11.

Intracellular distribution of protein and enzymes hydrolysing DNA.

Incubation media :-

Alkaline DNase - 150 µg. DNA, native or denatured, 2 µmoles tris, pH 7.5 and 1.3 µmoles $MgCl_2$ in a total volume of 0.36 ml.

Acid DNase - 150 µg. native DNA, 15 µmoles sodium acetate buffer, pH 4.5, and 60 µmoles KCl in a total volume of 0.31 ml.

All fractions were estimated at 3 protein concentrations and a final value arrived at by plotting the 3 values obtained and choosing a mean value.

Figures are expressed as percentages of nuclear + cytoplasmic extract.

N = nuclei, M = mitochondria, L = lysosomes, Mic = microsomes, S = supernatant solution.

MGB.I - particles disrupted osmotically

MGB.II - particles disrupted sonically

CdeD.I - data taken from de Duve, Pressman, Gianetto, Wattiaux and Appelmans (1955)

CdeD.II - data taken from Beaufay, Bendall, Baudhuin and de Duve (1959)

Table 11.

Protein

	N	M	L	Mic.	S	Recovery %
MGB. I	22.2	19.7	1.7	10.2	38.0	91.8
MGB. II	27.5	21.5	1.9	15.9	39.9	106.7
CdeD.I	13.3	16.3	7.4	24.1	37.5	98.9
CdeD.II	22.2	16.3	5.4	22.3	34.1	100.4

Alkaline DNase (native DNA)

MGB. I	24.8	19.2	3.0	10.6	46.5	104.1
MGB. II	17.9	36.5	5.7	8.0	19.1	87.1
CdeD.II	20.6	52.8	12.7	15.7	3.6	105.0

Alkaline DNase (denatured DNA)

MGB. I	6.5	9.0	1.1	10.1	38.5	65.2
MGB. II	17.9	33.6	1.3	2.6	13.8	69.1

Acid DNase

MGB. I	20.3	46.5	8.0	10.6	18.3	103.7
MGB. II	16.7	14.4	14.1	22.7	41.2	109.1
CdeD.I	5.3	33.6	31.2	6.3	18.9	95.3
CdeD.II	17.9	25.4	32.0	15.1	6.9	97.3

Fig. 28.

The intracellular location of enzymes degrading DNA (osmotically disrupted particles).

For details of incubation media etc. see Table 11.

Abscissa - %age of total nitrogen in each fraction. From left to right these are nuclei, mitochondria, lysosomes, microsomes and the supernatant solution after removal of the particles.

Ordinate - Relative specific activity, i.e. %age of total activity/%age of total protein.

Fig. 28.

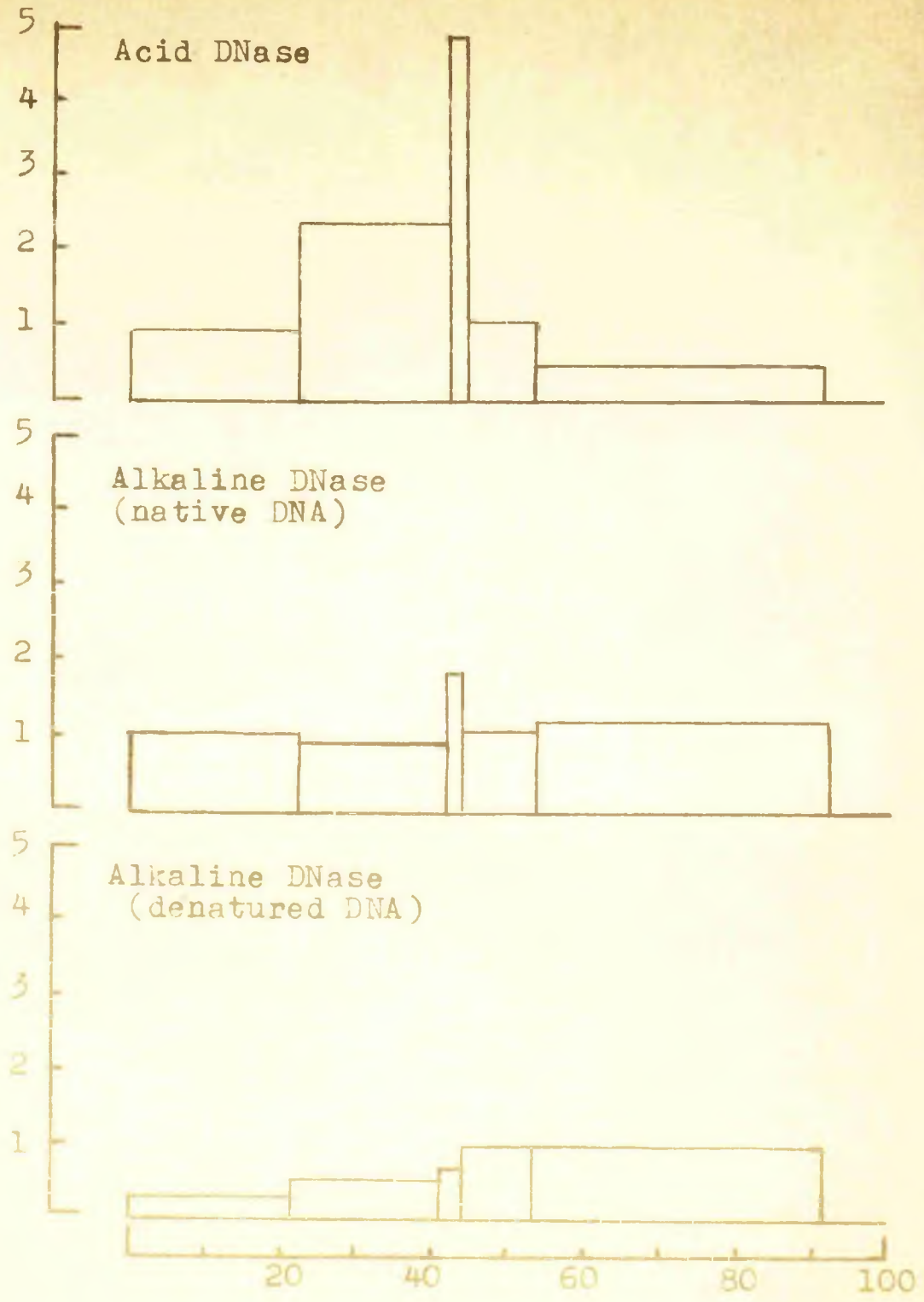

Acid DNase
Alkaline DNase
(native DNA)
Alkaline DNase
(denatured DNA)
5
4
3
2
1
20
40
60
80
100

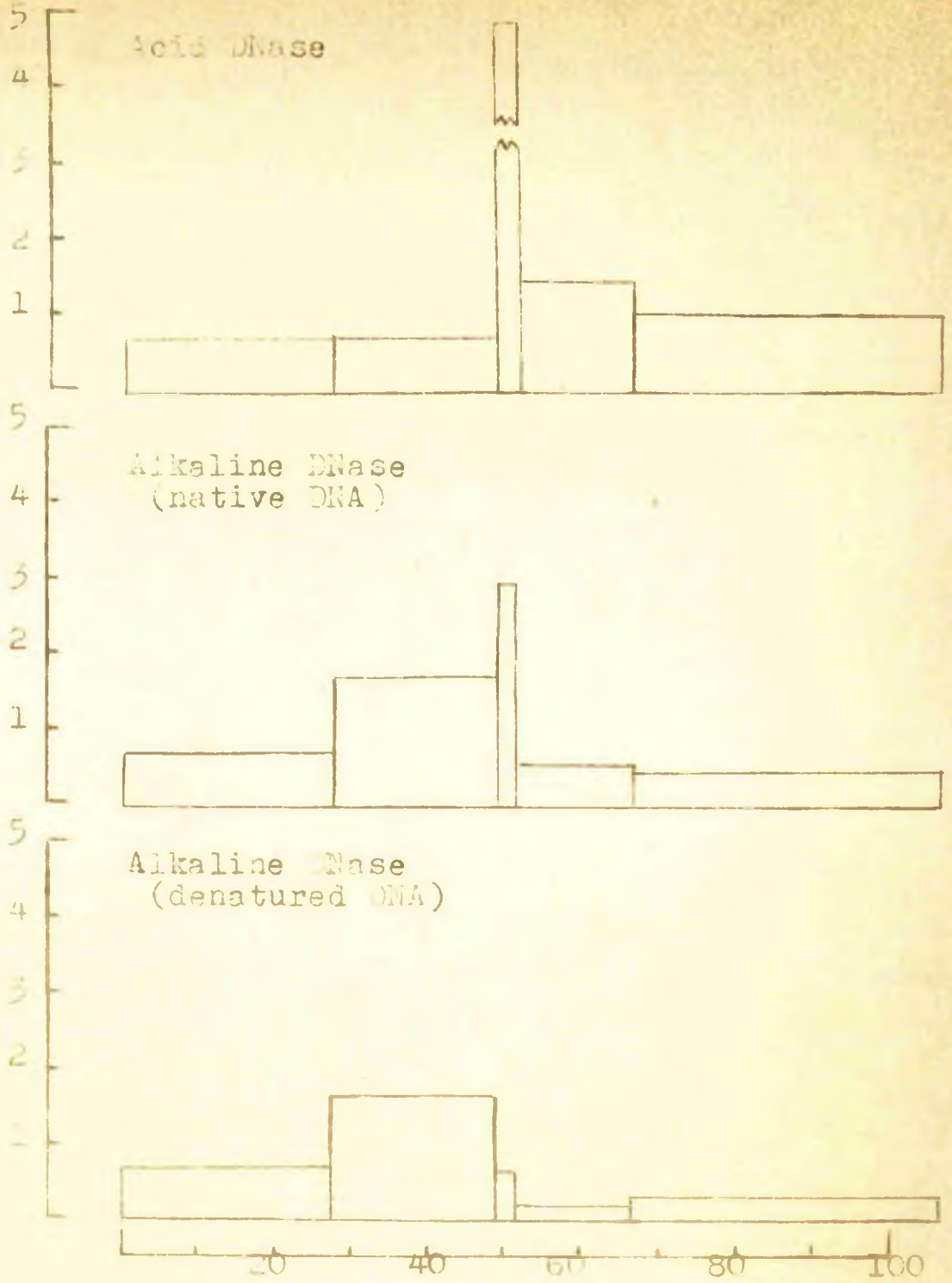
Fig. 29.

The intracellular location of enzymes degrading DNA (sonically disrupted particles).

For details of incubation media etc. see Table 11.

Abscissa and ordinate are as for Fig. 28.

Fig. 29.


Acid DNase
Alkaline DNase
(native DNA)
Alkaline DNase
(denatured DNA)
20
40
60
80
100

of the various fractions using osmotically and sonically disrupted particles respectively. In both cases the lysosomal fraction contained acid DNase of a high specific activity, and this fraction also had the highest specific activity with respect to alkaline DNase (native DNA), although this was not nearly so high as with the acid enzyme. Alkaline DNase hydrolysing denatured DNA seems to be scattered throughout the fractions, with no single one having a particularly high specific activity. These results did not give a clear indication of whether there are two enzymes present in liver, one of which preferentially hydrolyses native DNA while the other preferentially degrades denatured DNA, or whether there is one enzyme present which can hydrolyse both substrates.

3. 8 The products of the action of the purified inhibitory factor on DNA.

Since it appeared that the products of digestion of DNA by the purified inhibitor preparation might contribute to the inhibitory effect on the incorporation of ^{32}P-TMP into DNA, it was decided to separate the digestion products by fractionation on DEAE-cellulose columns and to test their effects on the DNA nucleotidyltransferase system. Fig. 30 shows the elution pattern obtained from DNA

Fig. 30.

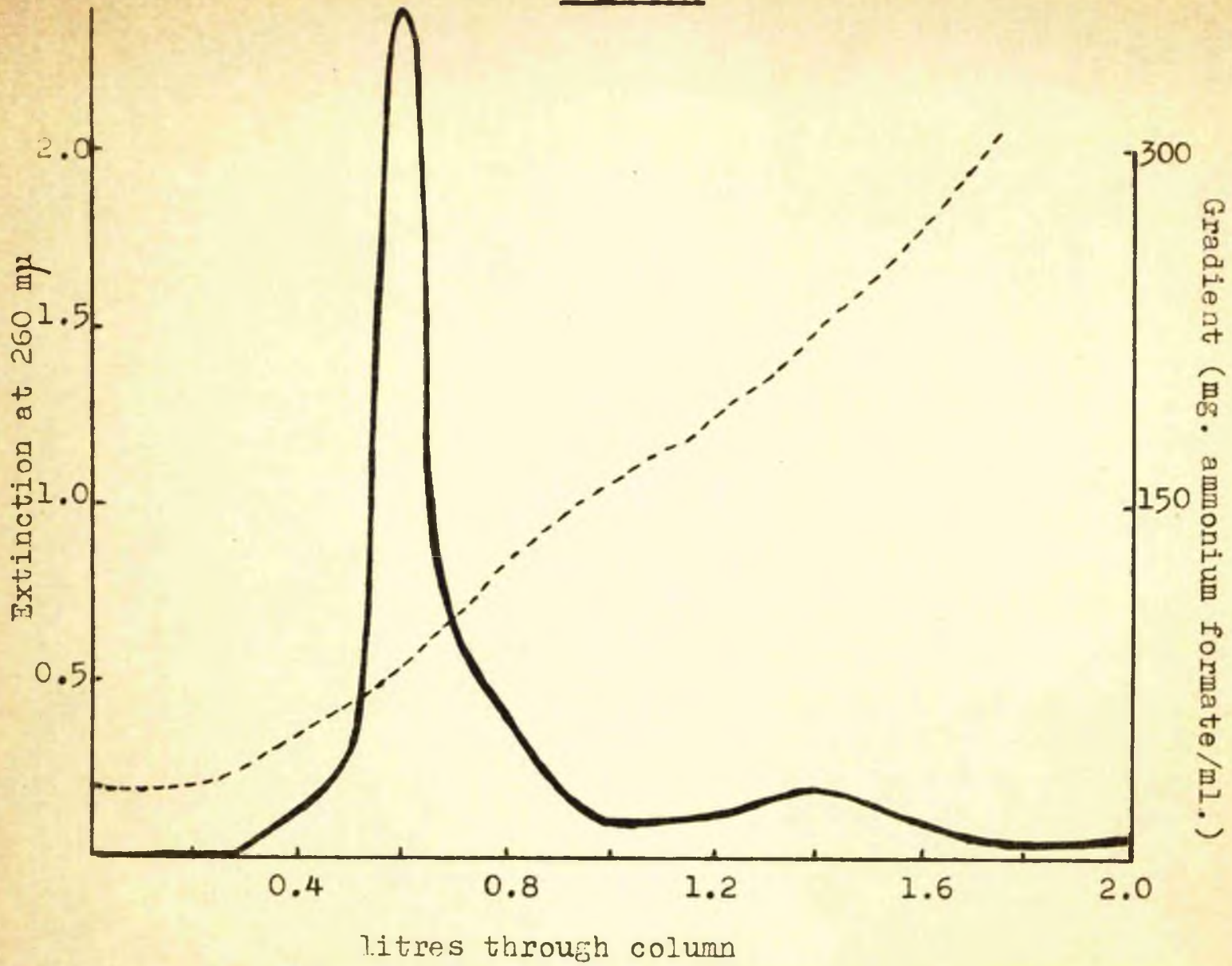
The separation of the products of a 6 hr. digestion of DNA by purified inhibitor preparation on DEAE-cellulose.

The digest contained 40 mg. denatured calf thymus DNA, 1 mmole $MgCl_2$, 800 μmoles tris, pH 7.5, and 10 mg. protein from the purified inhibitor preparation in a total volume of 32 ml. Incubation was for 6 hr. at 37°. The digest was applied to the top of a DEAE-cellulose column and a gradient consisting of 1 l. 0.2 M ammonium formate and 2 l. 3.5 M ammonium formate, pH 5.5 applied. The flow rate of the column was 2 ml./3 min.

—— Extinction at 260 mu.

----- Gradient.

Fig. 30.


2.0
1.5
1.0
0.5
Extinction at 260 mμ
300
150
Gradient (mg. ammonium formate/ml.)
0.4
0.8
1.2
1.6
2.0
litres through column

incubated for 6 hr. with the inhibitor preparation, absorbed on to a column of DEAE-cellulose and eluted with a gradient of 0.2M ammonium formate, pH 7.2, to 3.5M ammonium formate, pH 5.5. Only one large peak emerged, and, after the oligonucleotides in this had been concentrated and freed from salt (as described in Methods section), their effect on the DNA nucleotidyltransferase system was tested. Figs. 31 and 32 illustrate the results of two such experiments. Ascites tumour cell DNA was used as primer in one and calf thymus DNA in the other. The two types of DNA were used, since it was possible that the oligonucleotides might form hydrogen bonds with complementary regions of homologous DNA, but this might not happen with heterologous DNA. In both cases, considerable inhibition was observed with the oligonucleotides. At the highest oligonucleotide concentration (160 µg/assay), the inhibition with ascites tumour cell DNA was slightly greater than with calf thymus DNA. Although 50 µg. oligonucleotide (equivalent to the amount of DNA normally added as primer to the DNA nucleotidyltransferase assays) would not have a profound inhibitory effect, as judged from these figures, it must be remembered that these oligonucleotides are added to a system along with primer DNA, while under normal circumstances it is the primer itself

Fig. 31.

The effect on the incorporation of ^{32}P-TMP into DNA of oligonucleotides isolated from a digest of calf thymus DNA by the purified inhibitor preparation.

The assay medium contained 10 µmoles tris, pH 7.5, 1 µmole 2-mercaptoethanol, 6 µmoles $MgCl_2$, 0.05 µmole EDTA, 50 µg. denatured ascites tumour cell DNA, 0.75 mg. enzyme protein and 125mµmoles each of dATP, dGTP, dCTP and ^{32}P-TTP in a total volume of 0.305 ml. Incubation was for 1 hr. at 37°.

▲——▲ no added oligonucleotide.

■——■ 16 µg. oligonucleotide/assay.

●——● 80 µg. oligonucleotide/assay.

o——o 160 µg. oligonucletide/assay.

Fig. 31.

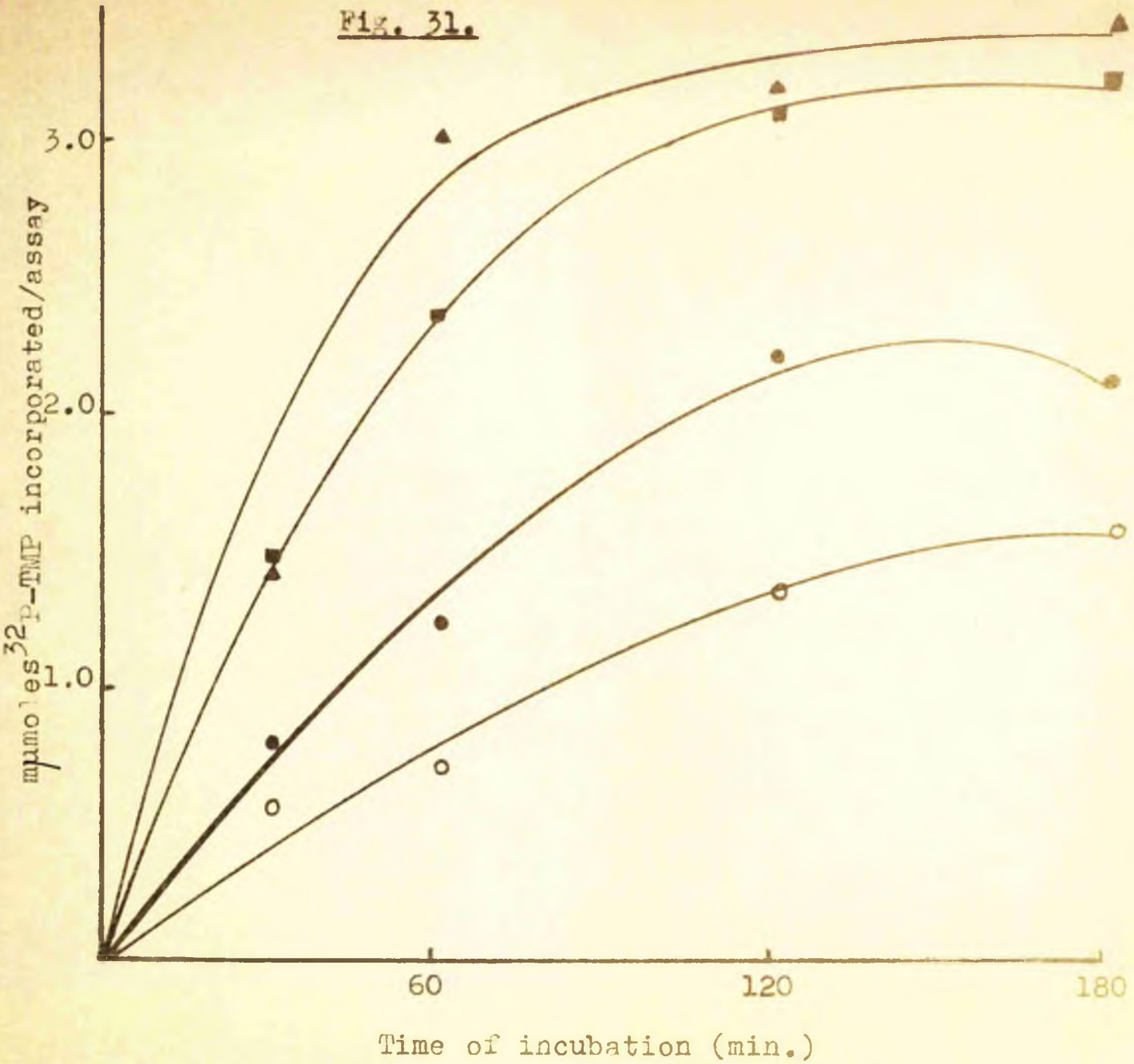

3.0
2.0
1.0
mμmoles ^{32}P-TMP incorporated/assay
60
120
180
Time of incubation (min.)

Fig. 32.

The effect on the incorporation of ^{32}P-TMP into DNA of oligonucleotides isolated from a digest of calf thymus DNA by the purified inhibitor preparation.

The incubation medium was as for Fig. 31, except that calf thymus DNA was used as primer instead of ascites tumour cell DNA.

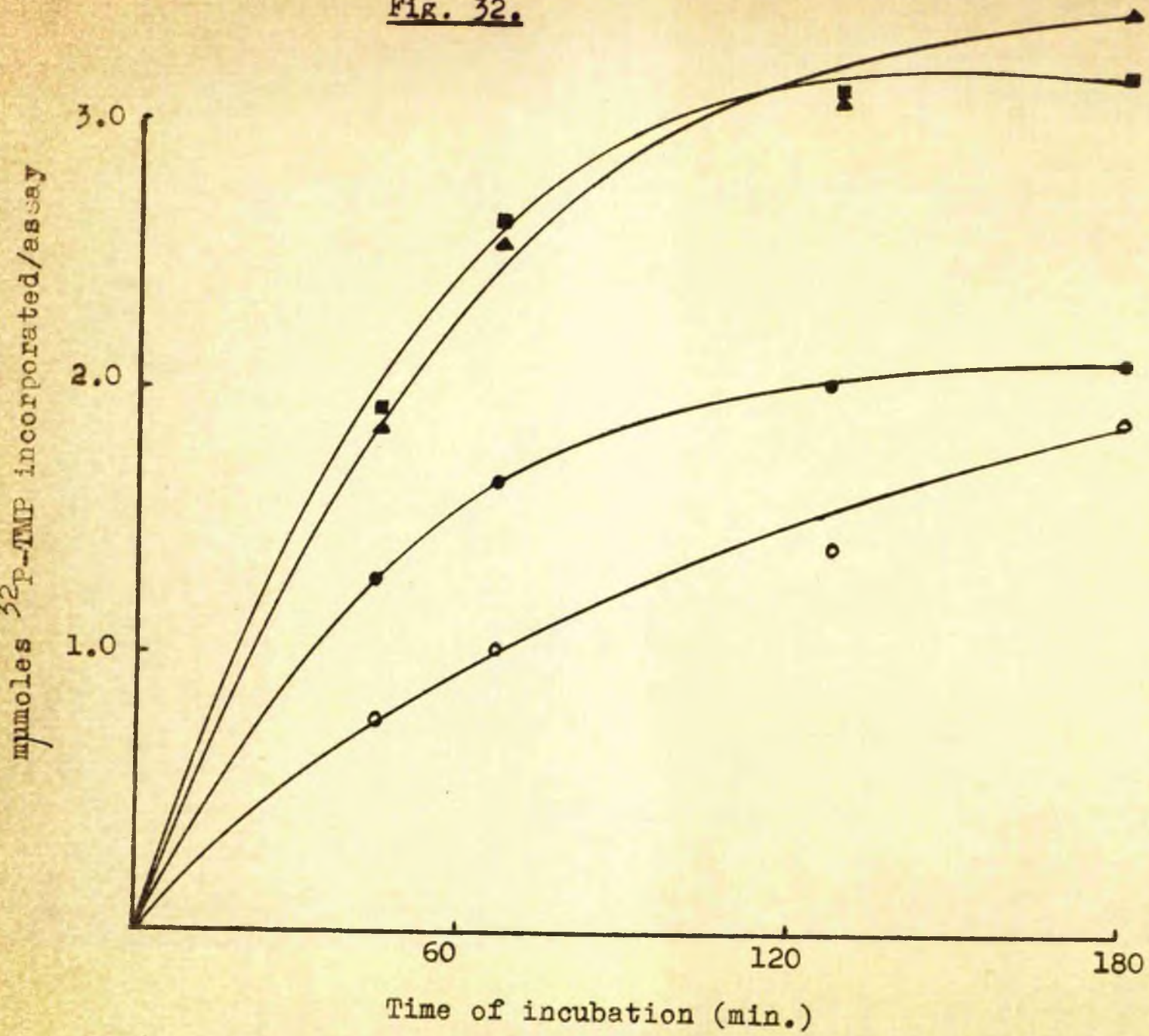
▲——▲ no added oligonucleotide.

■——■ 16 µg. oligonucleotide/assay.

●——● 80 µg. oligonucleotide/assay.

o——o 160 µg. oligonucleotide/assay.

Fig. 32.


3.0
2.0
1.0
mμmoles ^{32}P-TMP incorporated/assay
60
120
180
Time of incubation (min.)

that is being converted to the inhibitory oligonucleotides.

Fig. 33 shows the elution pattern obtained after 1 and 22 hr. digestion of DNA by the purified inhibitor preparation. The two experiments were run under exactly analagous conditions, i.e. length of column, gradient, etc. The pattern obtained after the 1 hr. digestion would represent the condition of the priming DNA at the end of the incubation of the DNA nucleotidyltransferase assay. At this stage, only very small amounts of the smaller fragments seen in the 22 hr. digestion were seen. This would seem to preclude a completely exonucleolytic mechanism of hydrolysis by the nuclease, since, if this were the case, relatively large amounts of mononucleotides would be expected relatively early in the digestion. When concentrated and freed from salt, the components of the peaks of the 22 hr. digest were found not to inhibit, and, in fact, in some cases, to stimulate the incorporation. However, the oligonucleotides isolated from the 1 hr. digestion were found to inhibit in exactly the same way as those isolated from the digest depicted in Fig. 29.

Since it had been found (Kair, 1962) that oligonucleotides bearing a 3'-phosphoryl terminal group inhibited the DNA nucleotidyltransferase, the effect of incubating the

Fig. 33.

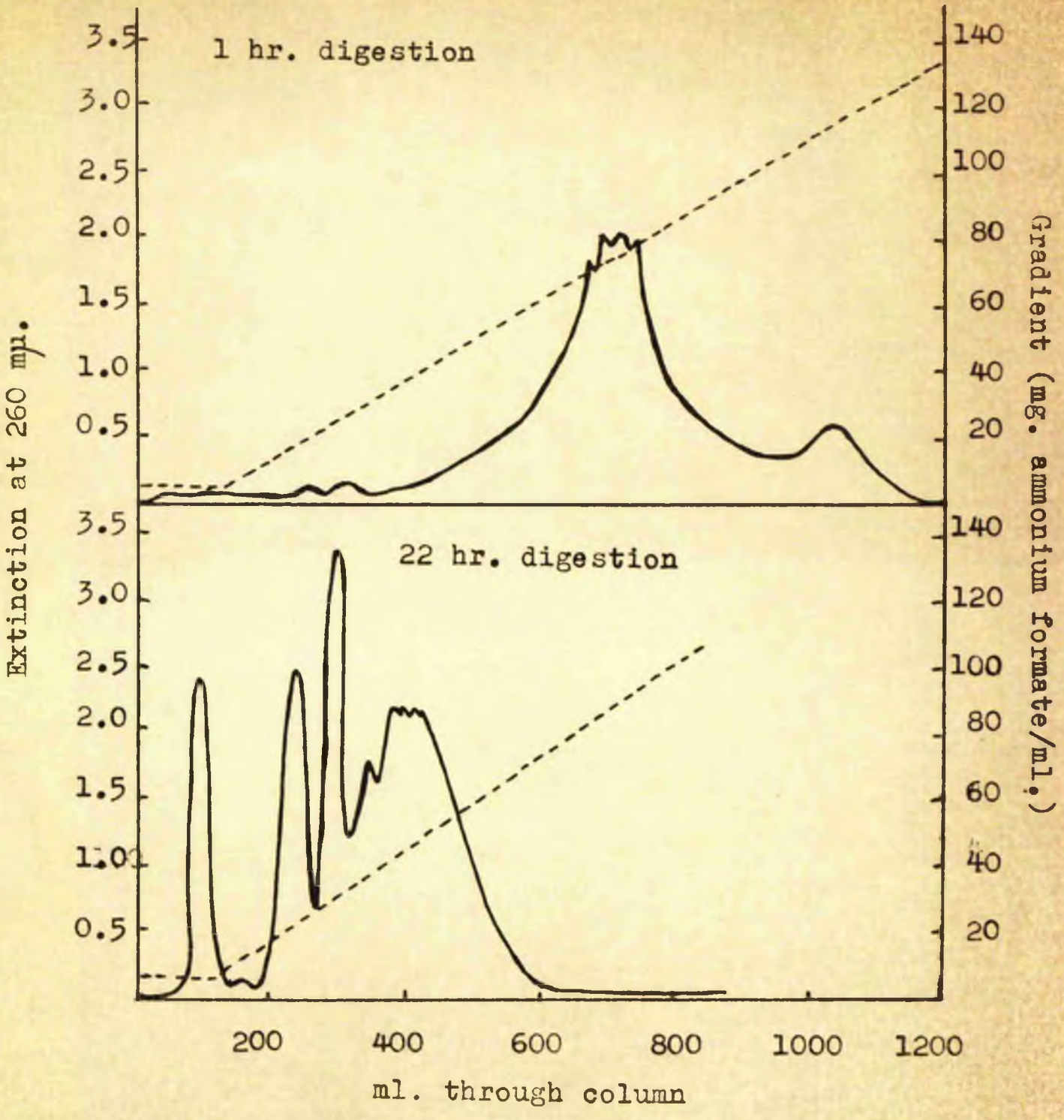
Comparison between the products of 1 and 22 hr. digestion of the purified inhibitor preparation on DNA, after fractionation on a DEAE-cellulose column.

The digest contained 80 mg. denatured calf thymus DNA, 2 mmoles $MgCl_2$, 1.6 mmoles tris, pH 7.5, and 30 mg. protein from the purified inhibitor preparation in a total volume of 56.4 ml. After incubation had proceeded for 1 hr., half of the digest was removed and the remainder incubated for a further 21 hr. at 37°. Both digests were put on to exactly similar columns of DEAE-cellulose and gradients consisting of 1.5 l. 0.05M and 2 l. 3.5M ammonium formate, pHs 7.5 and 5.5 respectively, applied. The flow rate was 2 ml./3 min.

—— Extinction at 260 mμ.

----- gradient.

Fig. 33.


1 hr. digestion
22 hr. digestion
Extinction at 260 mμ.
Gradient (mg. ammonium formate/ml.)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
140
120
100
80
60
40
20
200
400
600
800
1000
1200
ml. through column

inhibitory oligonucleotides with a bacterial alkaline phosphatase preparation, which can hydrolyse phosphate groups from oligonucleotides having either a 3' or 5'-phosphate terminal group, was tested. The results of this experiment are shown in Fig. 34. It was important that the phosphatase was completely inactivated, since it might have been inhibitory to the DNA nucleotidyltransferase by removing phosphate groups from the primer or the substrate. The method of denaturation seemed to be sufficiently effective, since the addition of the denatured phosphatase to the DNA nucleotidyltransferase assays did not inhibit incorporation. However, incubation of the isolated oligonucleotides with the phosphatase did not diminish their ability to inhibit the DNA nucleotidyltransferase, suggesting either that the phosphate group is not involved in the inhibition, or that the phosphatase had not been effective in removing the terminal phosphate group.

A "limit" digest of DNA was prepared by incubating DNA with the purified inhibitor preparation for a total of 68 hr., with further additions of the inhibitor from time to time. This digest was then fractionated on a column with a gradient consisting of 2 l. 0.01M ammonium bicarbonate, pH 8.6, and 2 l. 0.4M ammonium bicarbonate, pH 8.6 (Fig. 35). The main peak obtained was concentrated,

Fig. 34.

The effect of bacterial alkaline phosphatase on the inhibitory oligonucleotides produced by the action of the purified inhibitor preparation on calf thymus DNA.

The oligonucleotides used were isolated from the 1 hr. digestion peak illustrated in Fig.32. 32 extinction (at 260 mμ) units of oligonucleotide and 10 μg. bacterial alkaline phosphatase were incubated alone and together in a total volume of 0.52 ml. for 1 hr. and then the enzyme was denatured (see Methods section). In addition, 10 μg. phosphatase were incubated alone, and after denaturation of the enzyme 32 extinction units of oligonucleotide were added.

The constituents of the DNA nucleotidyltransferase assay medium were 10 μmoles tris, pH 7.5, 6 μmoles $MgCl_2$, 1 μmole 2-mercaptoethanol, 0.05 μmole EDTA, 0.75 mg. enzyme protein, 50 μg. denatured DNA, 125 mμmoles each of dATP, dGTP, dCTP and ^{32}P-TTP and 0.3 ml. of the appropriate preincubated preparation. The total volume was 0.42 ml., and incubation was for 1 hr. at 37°.

△——△ Control - no oligonucleotide, no phosphatase.

■——■ Phosphatase alone.

●——● Phosphatase and oligonucleotides.

○——○ Oligonucleotides alone.

□——□ Phosphatase - oligonucleotides added after denaturation.

Fig. 34.

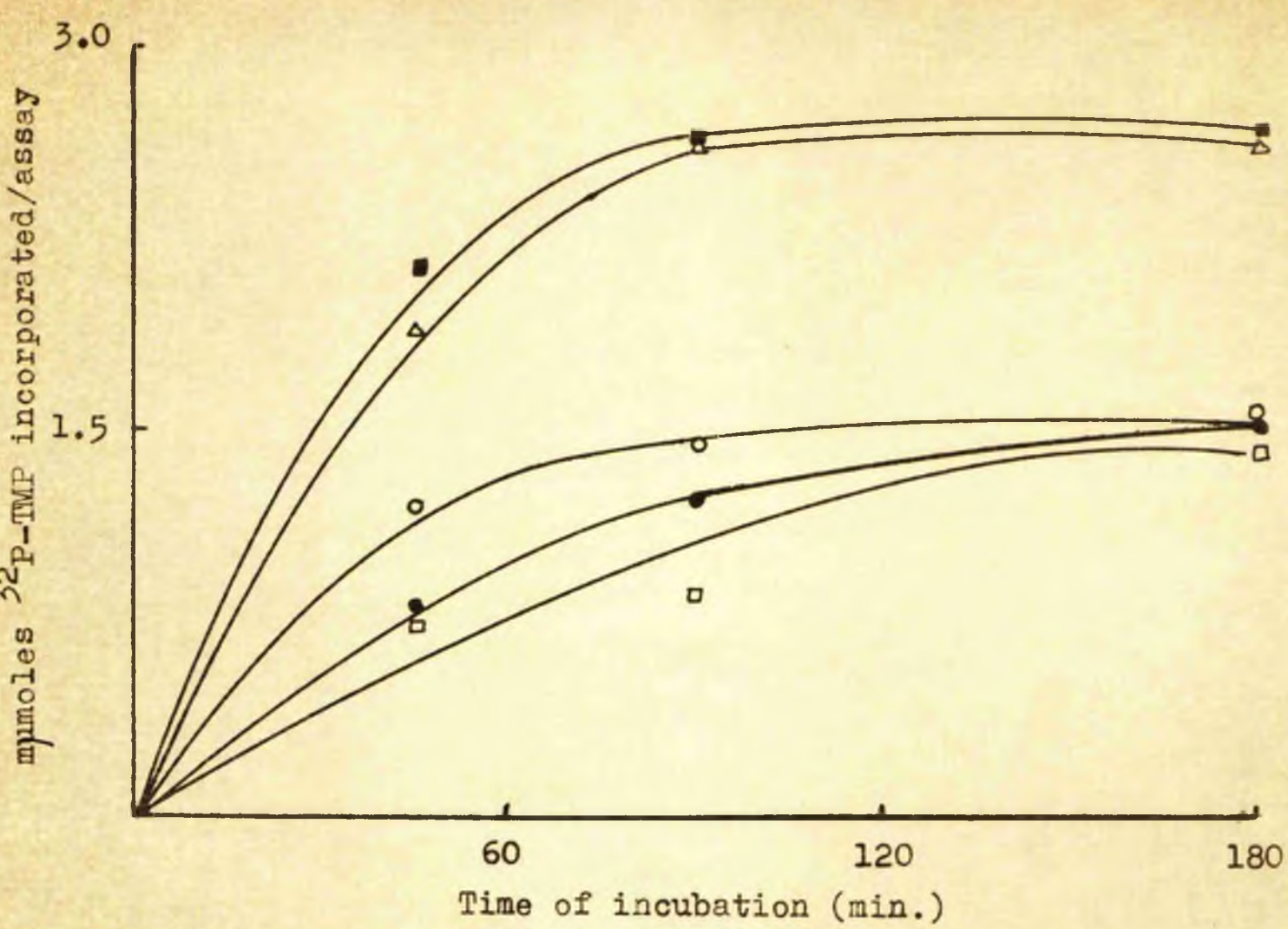
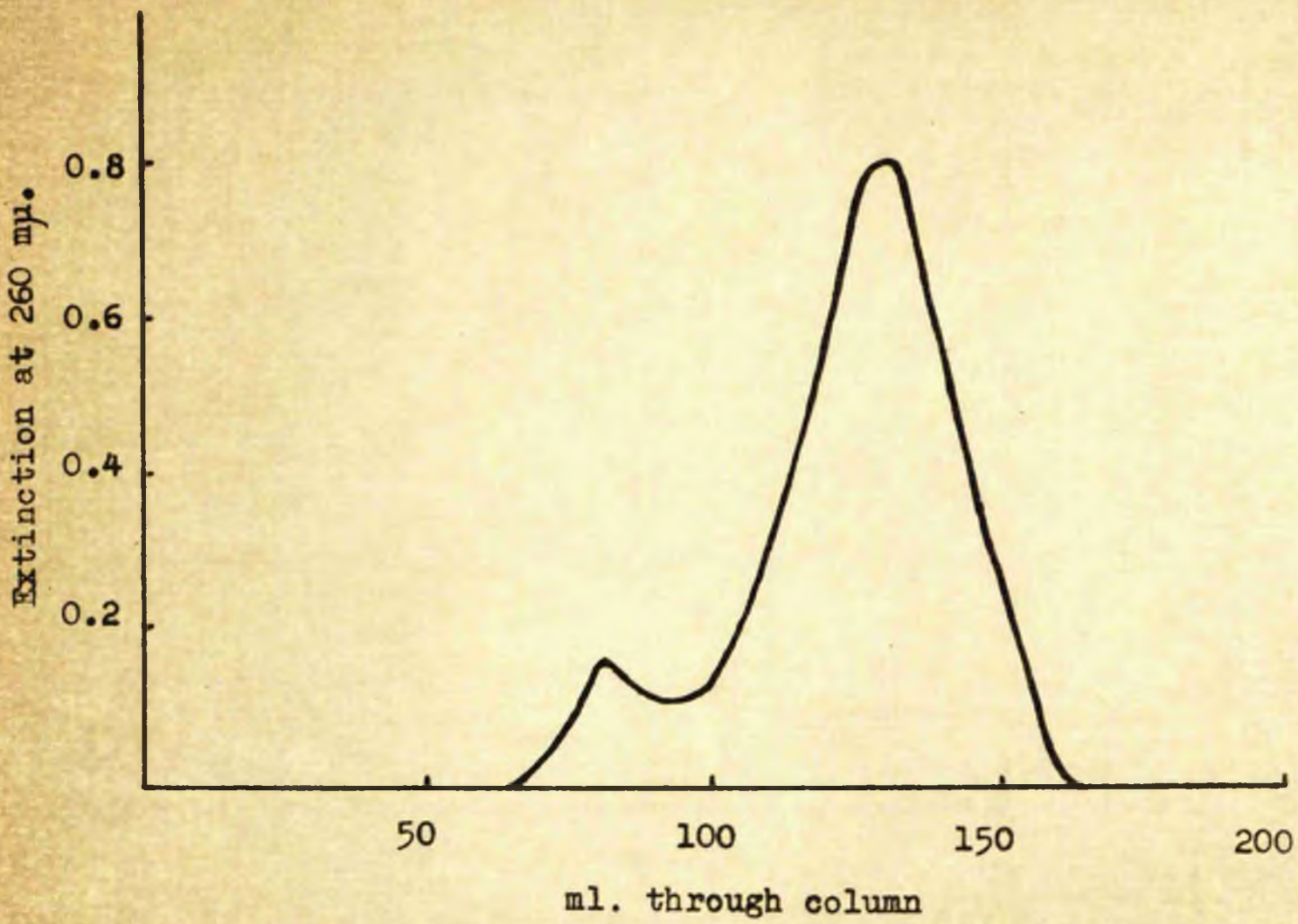

3.0
1.5
mμmoles ^{32}P-TMP incorporated/assay
60
120
180
Time of incubation (min.)

Fig. 35.

Fractionation of the products of a "limit" digest of calf thymus DNA, produced by the action of the purified inhibitor preparation.

The incubation medium contained 40 mg. denatured calf thymus DNA, 2 mmoles $MgCl_2$, 800 μmoles tris, pH 7.5 and 12 mg. of protein from the purified inhibitor preparation together with a small amount of toluene, to prevent bacterial growth, in a total volume of 27.4 ml. After 7 hr. incubation at 37°, 5 mg. purified inhibitor preparation were added, and a further 2.5 mg. after 60 hr. Incubation was continued for a further 1 hr. after the last addition. The sample was absorbed on to a column and a gradient consisting of 2 l. 0.01 M and 2 l. 0.4 M ammonium bicarbonate, pH 8.6, applied.

Fig. 35.


Extinction at 260 mμ.
0.8
0.6
0.4
0.2
50
100
150
200
ml. through column

the buffer removed and the nature of the products investigated using paper chromatography in different solvents. The results are illustrated in Fig. 36, and it is apparent that after this length of incubation time the main products are deoxyribonucleosides.

In view of the possibility that these might have arisen by microbial action during the long incubation time (even although toluene was added to the incubation medium to prevent this), a 24 hr. digest was prepared and fractionated on a similar DEAE-cellulose column. The column was washed first with 200 ml. 0.01M ammonium bicarbonate, pH 8.6, and then a gradient consisting of 1.8 l. 0.01M ammonium bicarbonate and 2 l. 0.4M ammonium bicarbonate, pH 8.6, applied. The pattern obtained from this fractionation is shown in Fig. 37. The fractions were pooled as indicated at the top of the Figure, concentrated and the salt removed (see Methods section) and their nucleotide content examined by paper chromatography. The U.V. absorption spectra were compared with those of known deoxyribonucleotides, and since available data on the spectra of the deoxyribonucleosides and deoxyribonucleoside monophosphates were rather scanty, the spectra of each of the four deoxyribonucleosides and deoxyribonucleoside monophosphates were determined at

Fig. 36.

The separation by paper chromatography of the products obtained from a "limit" digest of DNA by the purified inhibitor preparation.

Solvents.

I Isobutyric acid/ammonia/EDTA/water.

II Ethanol/ammonium acetate/EDTA.

Fig. 36.

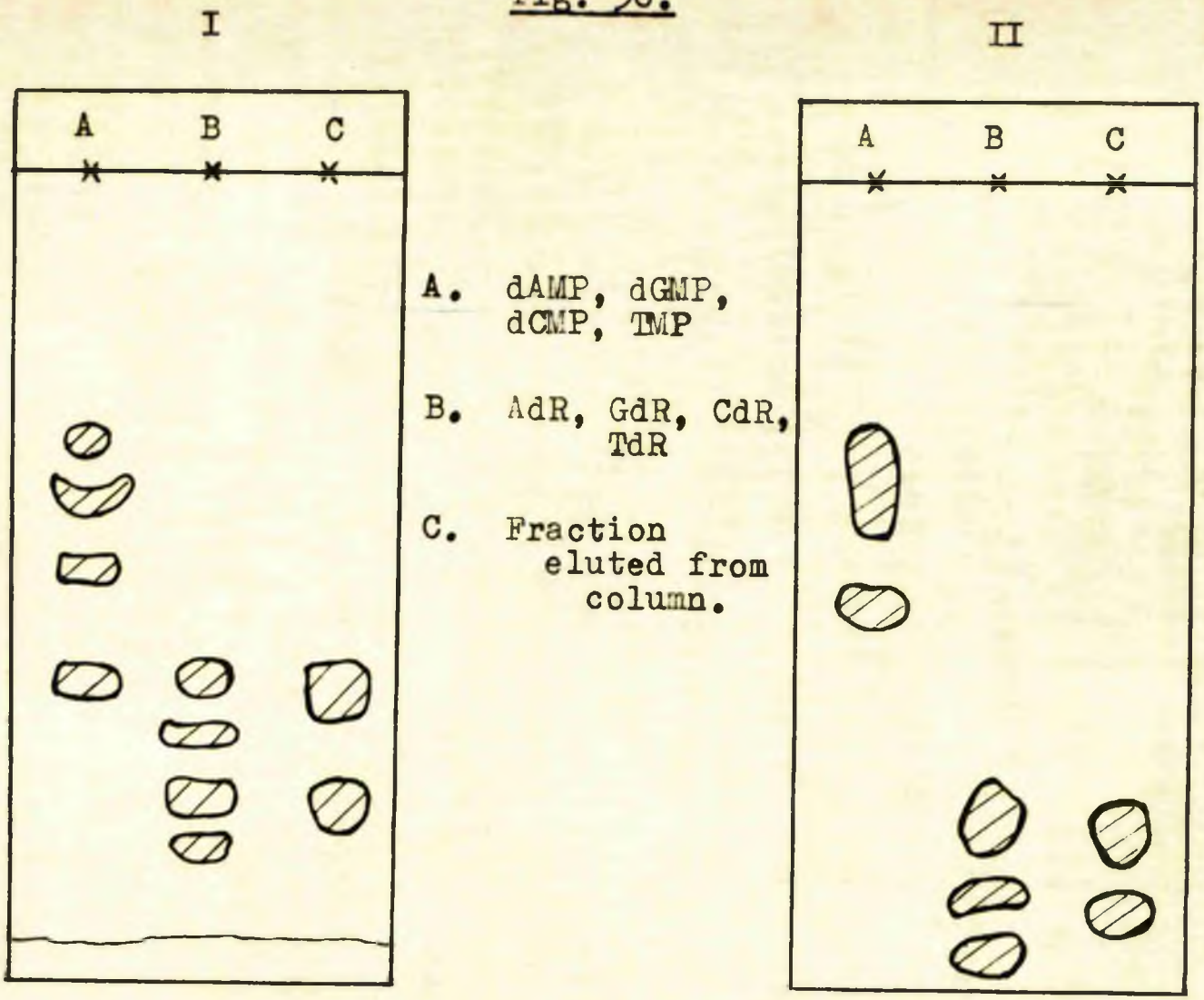
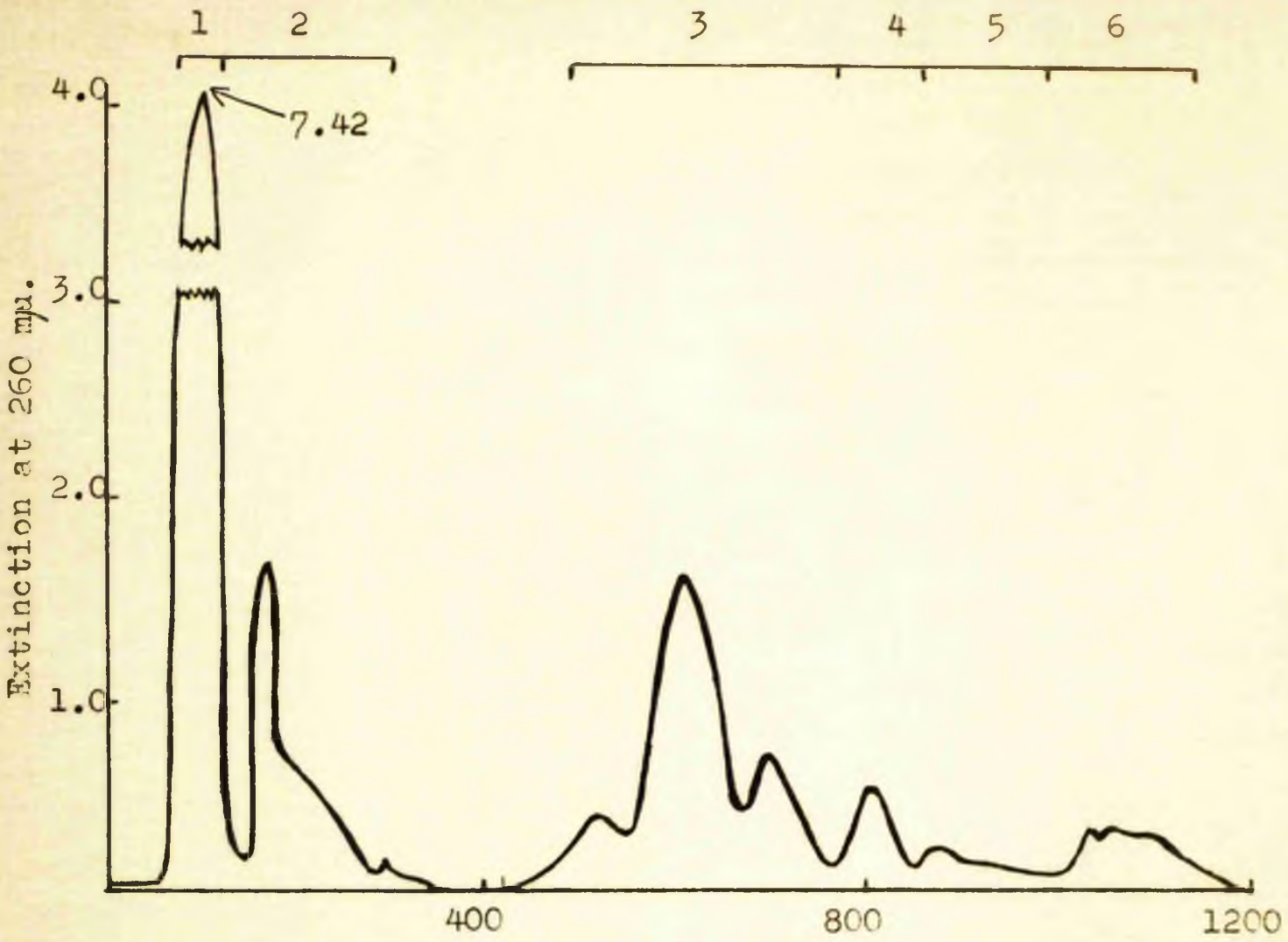

I
II
A
B
C
A
B
C
A. dAMP, dGMP, dCMP, TMP
B. AdR, GdR, CdR, TdR
C. Fraction eluted from column.

Fig. 37.

The fractionation of the products of the action of the purified inhibitor preparation on DNA on DEAE-cellulose (24 hr. digestion).

The digest contained 40 mg. denatured calf thymus DNA, 1 mmole $MgCl_2$, 300 μmoles tris, pH 7.5 and 15 mg. purified inhibitor preparation in a total volume of 27.4 ml. After 6 hr. incubation, a further 15 mg. inhibitor preparation were added and incubation was continued for a total of 24 hr. The digest was applied to the top of a column of DEAE-cellulose, which was eluted with 200 ml. 0.01M ammonium bicarbonate, pH 8.6, and then by a gradient consting of 1.8 l. 0.01M and 2 l. 0.4M ammonium bicarbonate, pH 8.6. The flow rate was 2 ml./3 min.

Fig. 37.


1
2
3
4
5
6
4.0
3.0
2.0
1.0
7.42
Extinction at 260 mμ.
400
800
1200
ml. through column

pHs 2.0, 7.5 and 12.0. The main features of the spectra are indicated in Table 12.

Several different solvent systems were employed in the investigation of the pooled eluates from the DEAE-cellulose columns, and the compounds found in the fractions are shown in Table 13. The solvents used were isopropanol/HCl/water and isobutyric acid/ammonia/water for 1 dimensional chromatography, and isopropanol/HCl/water then butanol/ammonia/water and ethanol/ammonium acetate then ammonium sulphate/water/isopropanol for two dimensional chromatography. This last combination of solvents was found to be particularly useful, since nucleosides, nucleoside monophosphates and dinucleotides can be readily resolved. A chromatogram obtained using these solvents is shown in Fig. 38. The spots found in the various solvents were identified both by their R_f values and by their spectra after elution from the paper. The presence of deoxyinosine deserves special mention; in several solvents a spot, containing a fairly large amount of ultraviolet-absorbing material, was seen whose R_f values did not correlate with any of the known constituents of DNA. On elution it was found that this spot had spectral characteristics which corresponded exactly to deoxyinosine, and that the R_f values, where these were known, also

Table 12.

Spectral data on the deoxyribonucleosides and deoxyribonucleoside monophosphates. The spectra were determined in a Cary recording spectrophotometer, each at a concentration of 0.1 μmole/ml.

	λ_{min}	λ_{max}	$\frac{250}{260}$	$\frac{250}{260}$	ϵ_{min}	ϵ_{max}
pH 2.0						
AdR	228	258	0.84	0.19	2.6	14.8
GdR	225	254	1.14	0.67	1.1	11.0
CdR	239	279	0.53	1.85	2.7	14.5
TdR	232	266	0.63	0.67	1.4	8.2
dAMP	229	258	0.81	0.25	3.3	15.4
dGMP	227	254	1.06	0.68	2.6	11.6
dCMP	240	279	0.48	1.93	1.7	12.9
TMP	235	265	0.68	0.68	2.3	8.6
pH 7.5						
AdR	226	260	0.78	0.15	2.2	15.2
GdR	223	252	1.17	0.68	3.3	14.1
CdR	250	270	0.85	0.95	6.4	9.1
TdR	235	265	0.66	0.71	2.5	8.7
dAMP	228	260	0.85	0.18	4.8	16.4
dGMP	225	252	1.15	0.66	4.4	13.6
dCMP	251	270	0.72	0.93	6.9	9.7
TMP	235	265	0.68	0.70	2.8	8.9
pH 12.0						
AdR	226	259	0.78	0.14	2.1	15.1
GdR	230	261	0.88	0.60	3.9	11.2
CdR	250	270	0.82	0.87	6.5	9.4
TdR	245	265	0.76	0.61	4.4	6.6
dAMP	228	260	0.76	0.16	2.6	15.5
dGMP	230	262	0.88	0.60	4.0	10.8
dCMP	248	271	0.80	0.94	5.7	8.8
TMP	245	265	0.76	0.68	3.2	4.8

Table 13.

Products found in various fractions of the DNA digest depicted in Fig. 37.

Fraction	Compounds found
1	AdR, TdR, CdR (+ 3 very minor components)
2	IdR (+ 1 very minor component)
3	dAMP, TMP, dCMP
4	dGMP, very small amount dAMP
5	dGMP, at least 4 dinucleotides
6	At least 4 dinucleotides

Fig. 38.

Positions of deoxyribonucleosides and deoxyribonucleoside monophosphates on a chromatogram, after running in I ethanol/ammonium acetate for 16 hr. and II isopropanol/water/ammonium sulphate for 12 hr.

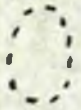
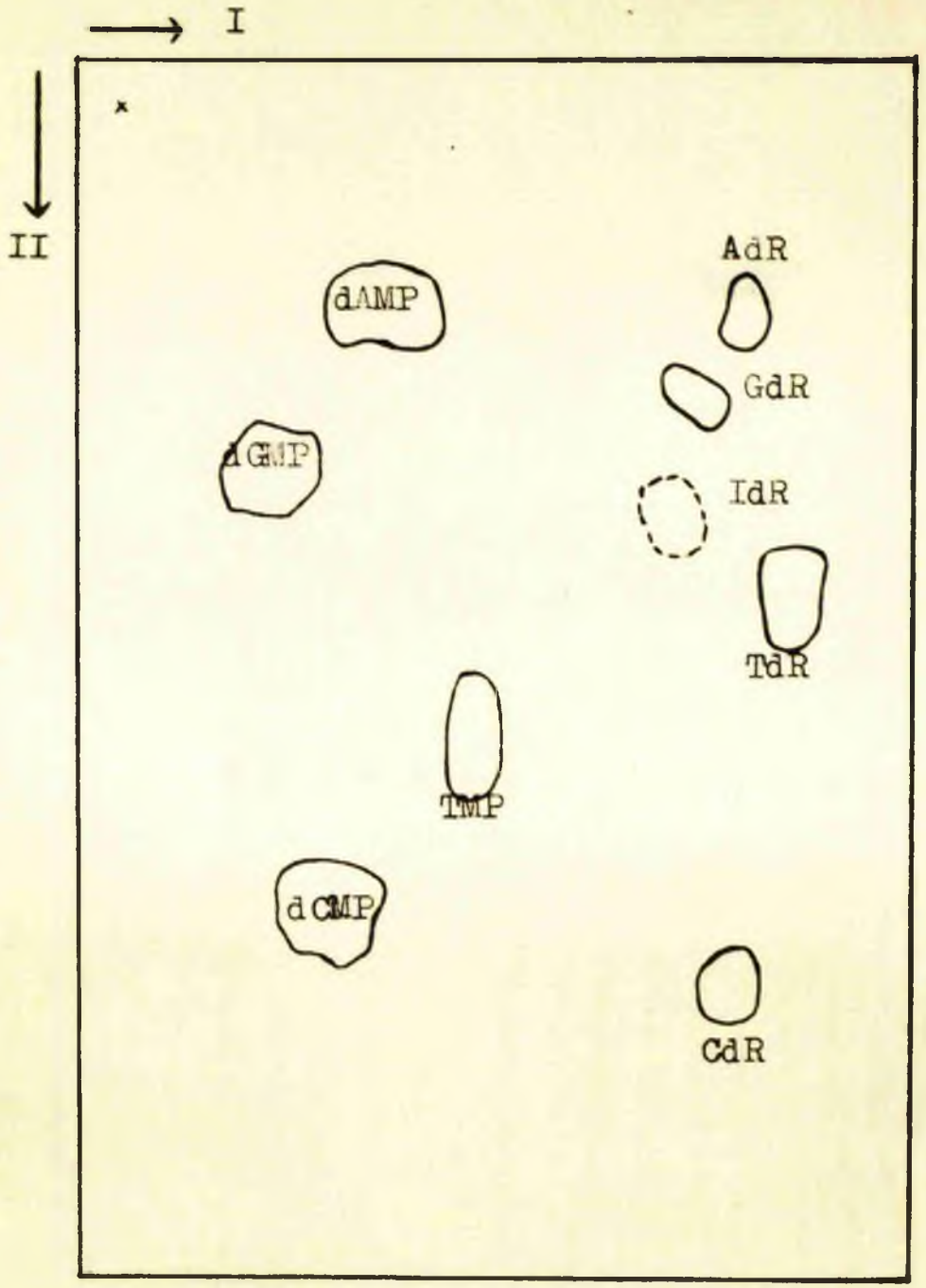
 indicates the position of IdR found on a different chromatogram.

Fig. 38.



corresponded.

In order to investigate whether this deoxyinosine was present in non-terminal positions of the oligonucleotides, a 2 hr. digestion of DNA was carried out, the products chromatographed on a DEAE-cellulose column and split into fractions as shown in Fig. 39. The oligonucleotide fractions were concentrated and the buffer removed. The fractions were treated with bacterial alkaline phosphatase and then with purified snake venom diesterase. The fractions after the action of the enzymes were chromatographed in the ethanol/ammonium acetate; ammonium sulphate/water/isopropanol solvents and the identity of the spots established from their position on the chromatogram and from their spectra. The contents of the fractions, whose identities were established both chromatographically and spectroscopically, are shown in Table 14. Although the data are incomplete, due to the small amounts of the components in the digests, no dIMP could be found in fractions 3 and 4 which contained the largest amounts of ultraviolet absorbing material. This suggests that no dIMP occurs in positions other than the terminal ones in the oligonucleotides.

The possibility that IdR (or dIMP), AdR, GdR, CdR or

Fig. 39.

The fractionation of the products of the action of purified inhibitor preparation on DEAE-cellulose (2 hr. digestion).

The digest, containing 40 mg. denatured calf thymus DNA, 1 mmole $MgCl_2$, 300 µmoles tris, pH 7.5 and 30 mg. protein from the purified inhibitor preparation, was incubated in a total volume of 32.4 ml. for 2 hr. The digest was applied to a DEAE-cellulose column and a gradient consisting of 2 l. 0.01M and 2 l. 0.4M ammonium bicarbonate, pH 8.6, was applied. The flow rate was 2.5 ml./3 min.

Fig. 39.

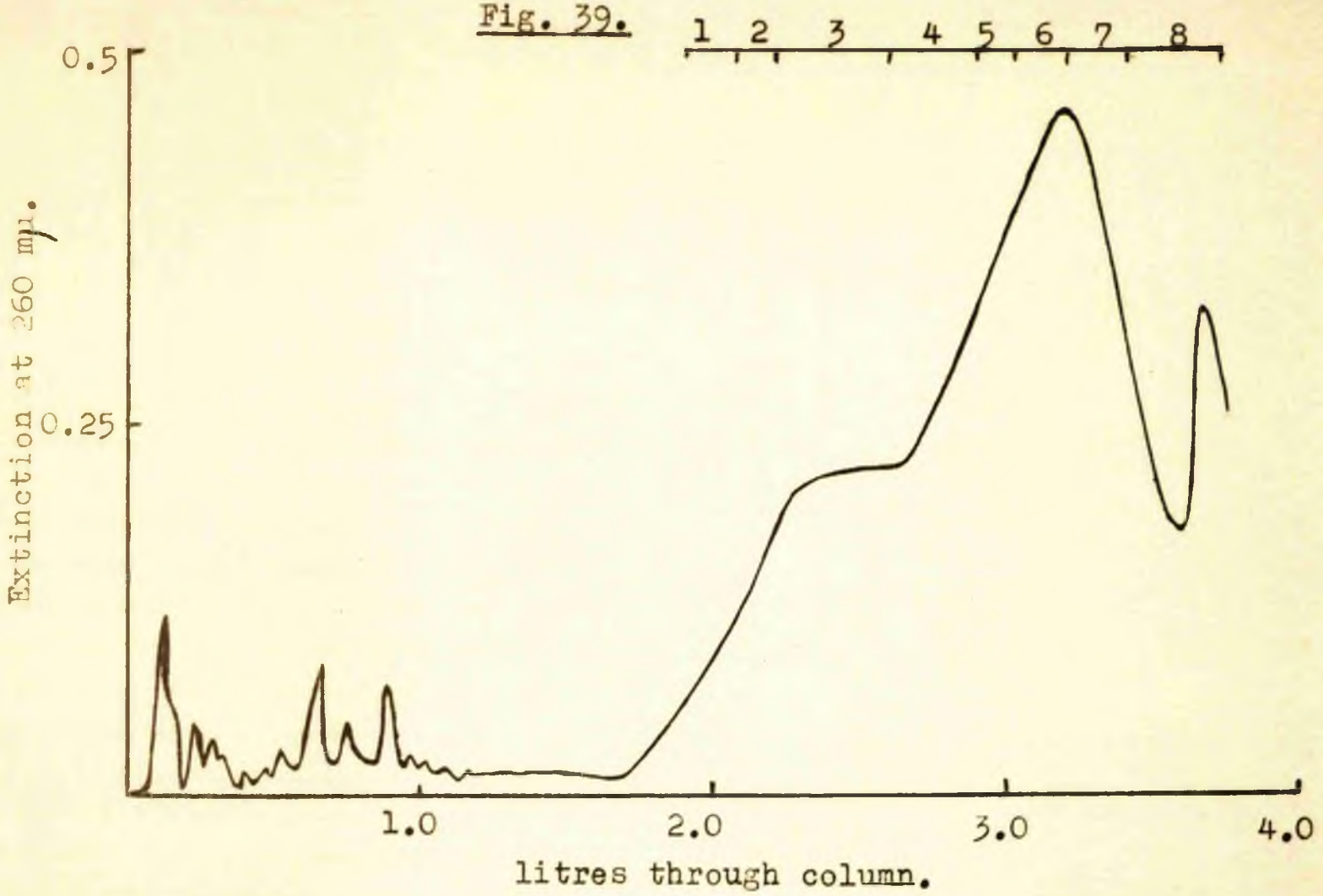

1 2 3 4 5 6 7 8
0.5
0.25
Extinction at 260 mμ.
1.0
2.0
3.0
4.0
litres through column.

Table 14.

Products found in various fractions of the DNA digest depicted in Fig. 39.

Fraction	Compounds found
1	CdR, IdR
2	AdR
3	CdR, TdR, AdR, GdR, TMP, GMP, AMP, CMP
4	TMP, CMP, GMP, AMP
5	TMP, CMP, GMP, AMP
6	AMP, GMP, TdR, IdR
7	GdR
8	-

TdR might be responsible for the inhibition of the DNA nucleotidyltransferase seemed remote, since inhibition was observed with those fractions which were eluted from a DEAE-cellulose column at a salt concentration corresponding to fairly large oligonucleotides (approximately 8 to 12 residues, as determined from the amount of salt required to elute them from the column) and also since these compounds are not produced until the digestion had proceeded for fairly long periods. However, the possibility that these compounds might inhibit was explored by including dIMP and AdR, GdR, CdR and TdR in DNA nucleotidyltransferase assays in amounts which would approximate to their possible production from DNA. The results of these experiments are shown in Tables 15 and 16. Table 15 indicates that none of the nucleosides has an inhibitory effect, and Table 16 demonstrates that dIMP does not inhibit, but in fact stimulates at certain concentrations.

Table 15.

The effect of nucleosides on the incorporation of ^{32}P-TMP into DNA by the DNA nucleotidyltransferase.

Addition	Counts/min. incorporated/assay
None (Control)	5,142
1 µmole AdR/assay	6,984
1 µmole GdR/assay	6,564
1 µmole CdR/assay	5,646
1 µmole TdR/assay	5,190

The incubation medium contained 10 µmoles Tris, pH 7.5, 1 µmole 2-mercaptoethanol, 6 µmoles $MgCl_2$, 0.05 µmole EDTA, 50 µg denatured DNA and 125 mµmoles each of dATP, dGTP, dCTP and ^{32}P-TTP in a total volume of 0.30 ml. Incubation was for 1 hr. at 37°.

Table 16.

The effect of dIMP on the incorporation of ^{32}P-TMP into DNA by calf thymus DNA nucleotidyltransferase.

mμmoles dIMP/assay	Counts/min. incorporated/assay.
0	8,335
2.5	10,540
10.0	12,412
25	12,026
75	9,062
100	8,940

The assay medium was as for Table 15.

SECTION IV

D I S C U S S I O N

Section IV. Discussion.

4. 1 Inhibition of DNA nucleotidyltransferase by calf serum.

Inhibition of the incorporation of ^{32}P-TMP amounting to 68% was caused by the addition of calf serum to DNA nucleotidyltransferase assays. Since the major portion of this inhibitory activity was removed by dialysis, it seemed that the small molecular components or ions present in the serum might be responsible for the inhibition. The amount of Na^+ added to the assays from the serum was found to correspond to a final concentration of 0.05M, not including the additional Na^+ which was present in the substrates, primer DNA and the enzyme preparation. Fig. 10 indicates that 0.05M NaCl inhibits the DNA nucleotidyltransferase reaction by 75%, so that the concentration of Na^+ added from the serum is sufficient to account for the observed inhibition. The small residual inhibitory activity of the serum after dialysis and heating is unexplained, but this could be due to incomplete dialysis, or to the presence of another inhibitory factor, which is heat-stable and non-dialysable.

Walwick and Main have studied the effects of monovalent cations on the incorporation of ^{3}H-TdR into DNA by extracts of rat thymus. In a preliminary report (Walwick and Main,

1959) it was found that Na^+ at a concentration of 50mM inhibited to the extent of about 62% when care was taken to eliminate other sources of Na^+ in the incubation medium. In a more recent paper (Walwick and Main, 1962) it has been demonstrated that when other sources of Na^+ were eliminated from the reaction medium, low concentrations of Na^+ (40mM) stimulated the incorporation about 2.5-fold at pH 7.3. However, when the Na^+ concentration was increased further, it rapidly became inhibitory and only 30% of the enzyme activity remained at an NaCl concentration of 160mM. At pH 7.9, the optimal Na^+ concentration decreased to 20mM, and at a concentration of 60mM 35% inhibition was observed. The explanation for the lower inhibition found with equivalent Na^+ concentrations to those shown in Fig. 10 lies in the precautions taken by Walwick and Main for the elimination of monovalent cations. The medium used in the present studies for the homogenisation of the calf thymus is itself 6mM with respect to KCl and the substrate and primer could contribute a further amount.

Bollum (1960) also reported the inhibition of calf thymus DNA nucleotidyltransferase by NaCl. He found that 0.05M NaCl inhibited the incorporation of ^{32}P-TMP into DNA by 40%. Once again, a lower figure was obtained than that

shown in Fig.10. The explanation probably lies in the fact that Bollum was using a purified enzyme, which doubtless contained a lower concentration of monovalent cations than did the crude extract, and, in addition, the assay conditions used were slightly different.

In view of the fact that the main inhibitory factor of calf serum was both dialysable and heat-stable, it seems likely that this inhibition of the DNA nucleotidyltransferase by the serum is entirely attributable to its content of monovalent cations.

4. 2 The phosphatase activity of extracts of rat liver.

The inhibition of the DNA nucleotidyltransferase by extracts of rat liver and kidney was observed to be different from that produced by calf serum, since the activity was non-dialysable and very heat-labile. It seemed likely, therefore, that the inhibition was due to a protein.

The removal of one or more of the phosphate groups from the substrate deoxyribonucleoside triphosphates would lead to inhibition of the incorporation of ^{32}P-TMP into DNA, since the DNA nucleotidyltransferase can use only the triphosphates, unless an energy source, such as ATP

and the appropriate kinases, are available to convert the deoxyribonucleosides or their monophosphates or diphosphates to the corresponding triphosphates. Enzymes which hydrolyse ATP and other deoxyribonucleoside triphosphates are known to be widely distributed in subcellular fractions of liver and in many other tissues (Kielley, 1961). In view of the possibility that hydrolysis of the substrates might be responsible for the inhibitory effect, extracts of rat liver were fractionated to determine whether phosphatase and inhibitory activities could be correlated.

When an extract of liver was fractionated with acid, a fraction containing high inhibitory and ATPase activity was precipitated when the pH was reduced only slightly (Table 2), and it may be that the inhibitory effect of this fraction is due to its nucleoside triphosphatase activity. However, the ratios of the inhibitory activities to the phosphatase activities in the subsequent fractions were sufficiently different to suggest that not all of the inhibitory activity was due to phosphatase. The two activities were more clearly resolved when the fraction produced by reducing the pH from 7.1 to 4.5 was further fractionated with ammonium sulphate (Fig. 12). The relative activities of the inhibitor and the ATPase were

clearly quite different, since the ATPase was precipitated by relatively low concentrations of ammonium sulphate, while the major portion of the inhibitory factor was not precipitated until higher concentrations were reached.

The possibility remained that the ATPase activity did not reflect the true activity of enzymes hydrolysing all or one of the four deoxyribonucleoside triphosphates. Several workers have reported enzymes which hydrolyse different nucleoside phosphates at different rates. For instance, Cooper and Lehninger (1957) described an ATPase from liver mitochondria that hydrolyses the other ribonucleoside triphosphates slowly, and Plaut (1955) showed the presence, again in liver mitochondria, of an enzyme which hydrolyses IDP, UDP and GDP, but not ADP or CDP. However, it seemed likely that a large proportion of the ATPase activity observed in the liver extract might be of the non-specific type, and that the more specific phosphatases might be quantitatively less important. Table 3 shows the extent of the hydrolysis of the four deoxyribonucleoside 5'-triphosphates by fractions prepared from rat liver, and strongly suggests that the ability of these preparations to hydrolyse the substrates and to inhibit the DNA nucleotidyltransferase are unrelated.

It is interesting to note that TTP appears to be hydrolysed at a slower rate than any of the other triphosphates. This may relate to observations that the enzymes phosphorylating TdR are present in low concentrations in non-proliferating tissues whereas those phosphorylating the other deoxyribonucleosides are present in approximately equal amounts, whether the tissue is growing or not. (Canellakis, Jaffe, Mantsavinos and Krakow, 1959; Bollum and Potter, 1959; Reichard, Canellakis and Canellakis, 1961).

It was not clear whether the inhibitory activity of kidney extracts was due solely to phosphatase action, but since this tissue contains such large amounts of these enzymes, further studies on the inhibition of DNA nucleotidyltransferase were carried out with liver preparations.

4. 3 Purification of the inhibitor from liver extracts.

Table 4 shows the partial purification of the inhibitory factor from rat liver. The three steps described usually produced a purification of 10 to 15-fold. Although the purification was not large, the recovery of inhibitory units was around 20% as compared with about 2% of the phosphatase units. Thus the phosphatase activity was

reduced to a level at which it could not seriously interfere with the assay of the DNA nucleotidyltransferase. Further experiments to explore the mode of action of the inhibitor were carried out with this preparation, since additional purification seemed to be difficult owing to the instability of the inhibitor when absorbed on to gels or substituted celluloses.

4. 4 The mode of action of the purified inhibitor preparation.

Since destruction of the deoxyribonucleoside triphosphates had been eliminated as a mode of action of the inhibitor, there remained the possibility that either the DNA nucleotidyltransferase or the DNA primer was affected by the inhibitor preparation. The results depicted in Figs. 14 and 15 make it clear that the major effect of the inhibitory factor is on the primer DNA. Increasing the concentration of DNA in the DNA nucleotidyltransferase assays in the presence of inhibitor partially reverses, but does not completely abolish the inhibition (Table 5). The purified inhibitor preparation contains appreciable DNase activity (Fig.16) and the close correlation between the inhibitory and the DNase activities during the purification procedure (Fig.17) strongly suggests that the

DNase activity is in some way responsible for the inhibition of the DNA nucleotidyltransferase activity.

Several mechanisms can be postulated for the inhibition that results from the action of nuclease on the primer. If the nuclease behaved as an exonuclease, the deoxyribonucleotide units attached to the primer by DNA nucleotidyltransferase might be removed almost as fast as they were added. If it behaved as an endonuclease, the primer might be degraded almost completely to non-priming fragments, or could have been converted to fragments which were actively inhibitory to the DNA nucleotidyltransferase. In the latter case, the fragments might appear to be inhibitory for one of two reasons. They could actively inhibit the DNA nucleotidyltransferase, possibly in a competitive fashion, or the primer could have been degraded to small acid-soluble fragments which were capable of priming and having mononucleotide units attached to them, but which were too small to be detected by the assay method used.

Bollum (1962) has shown that deoxyribooligonucleotides containing more than two mononucleotide units and a free 3'-hydroxyl group serve as primers for calf thymus DNA nucleotidyltransferase, by acting as a nucleus for chain extension, which takes place at the 3'-hydroxyl group.

It is known that these oligonucleotides have units attached by internucleotide bonds rather than by the formation of hydrogen bonds with the original oligonucleotide, since in the case of the trinucleotide pTpTpT, all four deoxyribonucleoside triphosphates were used as substrates, whereas, if the latter mechanism were operative, only dAMP would have been added. If the 3'-hydroxyl group of pTpTpT is acetylated, the oligonucleotide loses its priming ability, and the oligonucleotides prepared from a DNase II digest of DNA, which have a 3'-phosphoryl terminal group, cannot act as primers. Thus, it seems that the only requirements for a "chain extension primer" are at least 2 nucleotide units and a free 3'-hydroxyl group. Keir (1962) has shown that DNA nucleotidyltransferase from Landschutz ascites tumour cells is stimulated by the addition of 5'-phosphoryl terminal oligonucleotides. Both the stimulation and inhibition effects of these oligonucleotides can be reduced by removing the terminal phosphate groups from the oligonucleotides with bacterial alkaline phosphatase.

It seems feasible, therefore, that at least part of the inhibitory effect of the nuclease on the DNA nucleotidyltransferase system could be due to the products of the action of the nuclease on DNA. The oligonucleotides might act as "chain extension primers", but the products

of this reaction might be too small to be detected by the assay method used. Alternatively, the products of the nuclease action might inhibit by virtue of having a 3'-phosphoryl terminal group or some similar characteristic which would make them competitive inhibitors in the DNA nucleotidyltransferase system.

When the inhibitory nuclease was added to a system which had already been allowed to synthesise DNA, and the whole incubated for a further hour, the total amount of ^{32}P-TMP incorporated into DNA decreased as compared with the level of incorporation found after one hour (Table 6). Thus, the inhibitor preparation must have some other mode of action besides the production of inhibitory oligonucleotides.

Direct proof that the products of the action of the nuclease on DNA were inhibitory was obtained by pre-incubating DNA with the nuclease, heating to inactivate the nuclease and adding the products of the incubation to a normal assay medium, containing increasing amounts of primer DNA. Fig.19 shows that even with large amounts of primer (250 µg./assay), the incorporation never reaches the control level. This suggests that the products of the action of the nuclease on DNA are inhibitory, even

when a large excess of DNA is added to the transferase assay.

The effects of two purified nucleolytic enzymes, pancreatic DNase I and splenic DNase II on the incorporation of ^{32}P-TMP into DNA are illustrated in Table 7. Whereas relatively large amounts of pancreatic DNase I are required to inhibit the transferase reaction, DNase II proved to be very inhibitory, even although the conditions of the transferase assay are far from optimal for this enzyme. Mantsavinos and Canellakis (1959) demonstrated that the inclusion of 1 µg. DNase/12 µg. DNA in a DNA nucleotidyl-transferase assay stimulated the reaction. Their assay contained the four deoxyribonucleoside monophosphates (dCMP labelled with ^{14}C) and was therefore dependent on the nucleoside mono- and diphosphate kinases for the conversion of the monophosphates to the triphosphates, as well as on the DNA nucleotidyltransferase. In these experiments native DNA was used as primer, and it is possible that the DNase functioned by rendering portions of the DNA double helix more suitable as a template, due to the production of a molecule which had some structural feature in common with thermally denatured DNA. Alternatively, the DNase I could have stimulated the reaction by giving rise to additional 3'-hydroxyl terminal oligo-

nucleotides, which were suitable as sites for chain extension.

It has also been shown that the priming capacity of DNA can be increased by pre-treating the primer with minute amounts of DNase I (Lehman, 1959; Keir, Binnie and Smellie, 1962). Lehman (1959) stated that when the ratio of DNA to DNase was 2×10^5, a 2 to 3-fold stimulation in reaction rate was observed with the E. coli DNA nucleotidyl-transferase. Keir, Binnie and Smellie (1962) showed that treatment of native DNA with 1 mμg DNase/100 μg. DNA, followed by thermal denaturation, increased the priming capacity 11-fold in the ascites tumour cell system. Sarkar (1961) treated DNA with DNase I, and then re-isolated the product by a modification of the method of Kay, Simmons and Dounce (1952). "Slightly degraded" and "extensively degraded" preparations of DNA were produced by incubating 1 mg. DNase I with 100 mg. DNA for 2 and 10 minutes respectively. In both cases the product (both before and after heating) was superior to unheated DNA as a primer for Bollum's calf thymus DNA nucleotidyltransferase system.

The experiments in the present study differ, however, from those discussed above. Mantsavinos and Canellakis (1959) used native DNA as primer, and all the others pre-treated the DNA with DNase, rather than adding the enzyme

directly to the incubation medium. Heat denatured DNA is known to be less effective as a substrate for pancreatic DNase I than native DNA, and the explanation of the finding that DNase I does not inhibit the DNA nucleotidyltransferase at the level of 1 µg./µg. DNA may be the low activity of DNase I towards thermally denatured DNA. Higher concentrations of DNase I do inhibit the transferase, and when the concentration is increased to a level of 10 µg./µg. DNA, the inhibition is very substantial (Table 7). However, this is a very large concentration of enzyme, and an amount of DNase I which produces acid-soluble products to an extent equivalent to that of the purified inhibitor preparation reduces the incorporation of ^{32}P-TMP into DNA by only 6.5%, as compared with the 51% reduction produced by the inhibitor. These results suggest that the inhibition of the DNA nucleotidyltransferase is not directly proportional to the amount of acid-soluble fragments produced.

The effect of splenic DNase II on the incorporation of ^{32}P-TMP into DNA is more dramatic. A large amount of the enzyme is required to produce an appreciable release of acid-soluble nucleotides, since DNA nucleotidyltransferase assays are carried out at an alkaline pH, low ionic strength

and in the presence of Mg^{++}, all of which conflict with the optimal conditions for DNase II activity. However, production of even a small amount of acid-soluble material is sufficient to give rise to a considerable inhibition of incorporation of ^{32}P-TMP into DNA. Presumably this is due to the production of oligonucleotides having 3'-phosphoryl terminal groups, which are potent inhibitors of the DNA nucleotidyltransferase.

Since all these experiments were carried out using thermally denatured DNA, the effect of the purified inhibitor on the incorporation of ^{32}P-TMP into DNA was tested using native DNA as primer. An appreciable incorporation of ^{32}P-TMP was found in a system primed with native DNA (Table 8), and this is in contrast to the results of Bollum (1960) who stated that there was no incorporation of ^{32}P-CMP into native DNA. The possibility exists that there is an activating nuclease or some other enzyme capable of breaking the hydrogen bonds in double stranded DNA present in the crude thymus extract, but absent from Bollum's purified fraction. The addition of a purified inhibitor preparation to an assay containing native DNA caused a 105% stimulation of the incorporation of ^{32}P-TMP into the DNA and Fig. 20 shows that this stimulation was dependent on

the concentration of inhibitor added. Within the range of protein concentrations tested, the stimulatory effect exhibited a fairly sharp maximum, but even at the highest protein concentration, the inhibitor preparation did not inhibit incorporation into native DNA. It is possible that the stimulation was due to the conversion of sections of double stranded DNA to single stranded priming regions. There may therefore be two enzymes present, one of which acts on native DNA, making it a more efficient primer, and the second, the true inhibitory factor, which acts only on heat denatured DNA. If only one nuclease is present, it must, initially at least, have a different mode of action on native and thermally denatured DNA.

These studies suggest that some nucleolytic enzymes may play an important part in the in vivo mechanism of DNA replication. All mammalian systems so far investigated have shown a marked preference for thermally denatured, rather than for native DNA as primer, and it may be that when cell division is about to take place, the action of a nuclease is required to convert the native DNA to a template for replication. However, this nuclease action would have to be very closely controlled, since it is obviously important that the breakage of internucleotide

linkages should not take place if the integrity of the DNA and the information that it contains are to be maintained. The role of nucleolytic enzymes in normal rat liver is therefore obscure, since the synthesis of DNA in this tissue is extremely slow, and it is difficult to see what useful function the nucleases present fulfil, unless they are present, but can exert their action only if some special circumstance arises. The inhibitors of nucleases mentioned above, which occur in normal tissues, may have some part to play in maintaining the nucleases in an inactive state when DNA synthesis is not taking place. Lehman, Roussos and Pratt (1962a) have shown that there is an endonuclease in E. coli which is found bound to inhibitory RNA in crude extracts, and it may be that the activity of this nuclease is controlled by the inhibitory RNA in the intact organism.

4. 5 Comparison between the properties of the purified inhibitor and other known nucleases.

The characteristics of the DNase activity of the purified inhibitor preparation are presented in Section 3.6. In summary, the activity has a broad pH optimum, extending from pH 6.5 to 8.5 and a Mg^{++} requirement which is replaceable by Mn^{++} but not by Ca^{++}. It is not activated by Ca^{++} synergistically in the presence of Mg^{++}, and is inhibited by

increasing the ionic strength of the medium. The preparation has a small amount of RNase activity and hydrolyses thermally denatured DNA more rapidly than native DNA.

The nuclease clearly does not belong to the DNase II class, typified by the enzyme from spleen (Koerner and Sinsheimer, 1957) for several reasons. Its alkaline pH optimum, Mg^{++} requirement and the inhibition noted on increasing the ionic strength of the medium are all in contrast to the known properties of the DNase II type of enzyme. Its alkaline pH optimum and Mg^{++} requirement both suggest that it belongs to the DNase I group of enzymes. However, it has several properties which differ from the classical enzyme of this type, crystalline pancreatic DNase I. The two activities are similar in pH optimum, and pancreatic DNase I is known to have a broad pH optimum curve, which varies with the nature of the ions present in the medium (Miyaji and Greenstein, 1951; Erkama and Suutarinen, 1959). It seems probable that different methods of estimating DNase activity give different values for the pH optimum. Shaok (1957) showed that the DNase activity of crude extracts of rat liver had a broad pH optimum, ranging from 6.8 to 8.2, when changes in the viscosity of DNA were used as a measure of the enzyme activity. The

optimal Mg^{++} concentrations for pancreatic DNase I and the nuclease of the purified inhibitor preparation are very similar, both when calculated in terms of molarity and of the molar ratio of Mg^{++} to the total phosphorus of the substrate. Miyaji and Greenstein (1951) found this latter ratio to be 3/1 for pancreatic DNase, which is exactly the value found for the nuclease of the purified inhibitor fraction. The two enzymes differ, however, in their activation by Mn^{++} ions. Wiberg (1958), who measured the liberation of acid during the hydrolysis by DNase I of the phosphodiester bonds of DNA, by observing the decrease in extinction of a p-nitrophenol buffer, found that the activation by Mn^{++} is at least 3.5 times as great as the activation produced by any equivalent concentration of Mg^{++}. The nuclease in the purified inhibitor preparation, however, was found to be activated to about the same extent by both Mn^{++} and Mg^{++}, although the optimal Mg^{++} concentration was about 10 times that found for the optimal Mn^{++} concentration. This might be accounted for on the basis of the different methods of estimation, but this seems unlikely, since the differences involved are substantial.

Wiberg also demonstrated that Ca^{++} ions could activate pancreatic DNase (to about 6% of the extent obtained with equivalent concentrations of Mg^{++}) and that Ca^{++} in the

presence of Mg^{++} (Mg^{++}/Ca^{++} varying between 1 and 100) caused an activation of up to 3-fold. Neither of these observations was true for the nuclease of the purified inhibitor preparation (Figs. 24 and 25). Ca^{++} did not activate, but slightly inhibited the small activity seen in the absence of Mg^{++}, and no synergistic effect of Ca^{++} in the presence of Mg^{++} could be shown. Ratios of Mg^{++}/Ca^{++} varying between 0.1 and 10 were used, but only a decrease in activity was observed when Ca^{++} was added to Mg^{++} activated assays.

Increasing the NaCl concentration above 0.05M is known to inhibit pancreatic DNase I (Greenstein, Carter and Chalkley, 1946), and this was also observed in the case of the purified inhibitor preparation (Fig. 26). This inhibition was also noted by Shack (1957) using crude extracts of rat liver.

The effect of the thermal denaturation of DNA on its susceptibility to the liver nuclease is shown in Fig. 27. This indicates that denatured DNA is 2.5 times more susceptible to attack than native DNA. Kurnick (1954) has demonstrated that heat denaturation of DNA (for periods up to 7 hr. at 100°) greatly reduced the affinity for pancreatic DNase, and similar results have been obtained by Dirksen and Dekker, (1958), using the release of protons during hydrolysis as a measure of DNase activity. This then further

distinguishes the nuclease activity of the inhibitor preparation from that of pancreatic DNase I.

Only two enzymes have been characterised which hydrolyse thermally denatured DNA more rapidly than native DNA. These are the phosphodiesterase from E. coli described by Lehman (1960), and micrococcal nuclease from Staph. aureus (M. pyogenes var. aureus)(Cunningham, Catlin and Privat de Garilhe, 1956; Cunningham, 1956). Lehman found that the purified E. coli phosphodiesterase hydrolysed heat denatured DNA at a rate 300 times greater than native DNA, and that the enzyme preferentially degraded denatured DNA in a mixture of heated and unheated DNA. This enzyme, which has an optimum pH of 9.2 to 9.7, is activated by Mg^{++} and inhibited by Mn^{++} or Ca^{++}. The products of its action are the deoxyribonucleoside 5'-monophosphates. Micrococcal nuclease has been shown by Dirksen and Dekker (1958, 1960) to hydrolyse thermally denatured DNA four times as rapidly as native DNA, but under conditions of high ionic strength, differences of 20 to 100-fold could be obtained. This effect was attributed to the known stabilising effect of high ionic strength on the double stranded structure of native DNA. This enzyme has an optimal pH of 8.6 and requires the presence of 0.01M Ca^{++} for maximal activity. The products of its action are the

deoxyribonucleoside 3'-monophosphates and dinucleotides and higher oligonucleotides. No enzyme from animal cells having the property of hydrolysing heat-denatured DNA more rapidly than native DNA has previously been reported.

The liver nuclease has a certain amount of activity towards RNA, which could be attributed to the enzyme itself or to a contaminating RNase activity. Since the enzyme is not highly purified, the second explanation seems quite possible. Crystalline pancreatic DNase I has no detectable activity towards RNA, and while most preparations of splenic DNase II attack RNA, a method has been described by Maver, Petersen, Sobers and Greco (1959), which gives rise to a preparation free from RNase activity. Micrococcal nuclease hydrolyses RNA in addition to DNA (see Section 1.3b) and E. coli phosphodiesterase hydrolyses RNA, but at a much reduced rate, and it is not clear whether this is an intrinsic property of the enzyme, or whether it is due to a contaminating activity. In fact, few enzymes, apart from pancreatic DNase I, have been purified sufficiently for it to be stated categorically that the enzymes have or do not have RNase activity.

It is well established that there is a specific inhibitor for the DNase I type of enzyme activity in several

tissues (Feinstein, 1960). Feinstein and Hagen (1962) have demonstrated that extracts of mouse kidney and intestine, which normally show no DNase I activity, exhibit appreciable activity if they are first incubated with ovalbumin. Similar DNase I inhibitors have been found in other tissues. The first to be discovered was that in the crop of pigeons (Dabrowska, Cooper and Laskowski, 1949). This was found to be protein in nature, and to increase when the glandular mucosa of the crop became hypertrophic. The presence of an inhibitor has also been reported in several other tissues (Cooper, Trautmann and Laskowski, 1950; Henstell and Freedman, 1952; Kurnick, Schwartz, Pariser and Lee, 1953). The effect of incubating crude liver extract with serum albumin was therefore tested (Table 10). It seemed possible that the nuclease and its inhibitor might coexist in the crude liver extract, since, on fractionation with acid, more than 100% of the nuclease activity was frequently obtained. However, the results failed to show any release of inhibitor on incubation of the enzyme with albumin, and therefore provided no evidence for the existence of an inhibitor of the nuclease in crude liver extracts.

The nuclease purified from rat liver has properties which distinguish it from any of the hitherto known and well

defined nucleases. It would appear from its pH optimum and ionic requirements to belong to the DNase I group of enzymes. However, it differs from pancreatic DNase I in its ability to hydrolyse thermally denatured DNA more rapidly than native DNA, in its response to Ca^{++} in the presence of Mg^{++} and in behaving differently in the presence of Mn^{++}. It also differs in being more inhibitory than crystalline pancreatic DNase I when included in the DNA nucleotidyltransferase assay.

Razzell (1961a) has shown that a phosphodiesterase (identified as such by its ability to hydrolyse thymidine 5'-p-nitrophenyl phosphate) with an alkaline pH optimum is present in many tissues, including rat liver. The enzyme is activated only slightly by Mg^{++}, but is sensitive to EDTA and has been purified from hog kidney (Razzell, 1961b). The possibility existed that the rat liver nuclease might be identical with this phosphodiesterase, but the purification steps which Razzell used included trypsin and heat treatment, in which the protein was incubated with trypsin at 60° for 24 hr. It is obvious from the data obtained on the heat inactivation of the nuclease (Fig. 18) that the majority of its activity would be destroyed by such a treatment. It appears, therefore, that the liver nuclease cannot be identical

with the phosphodiesterase obtained from hog kidney. There remain two possibilities as to the identity of the nuclease. Either it is the enzyme causing the DNase I activity of liver observed by several workers or alternatively it may be a minor nuclease whose activity was only detected because denatured DNA was used as primer in the DNA nucleotidyltransferase assay. It is possible that there is more than one nuclease in the purified inhibitor preparation, but that the true inhibitory activity is due to an enzyme which is specific for denatured DNA, while the activity observed with native DNA is due to a contaminating enzyme.

4. 6 The presence of enzymes hydrolysing DNA in subcellular fractions of rat liver.

When rat liver was fractionated according to the methods of de Duve and his colleagues (see Section 2.13), the distribution of protein in the fractions corresponded closely to those found by de Duve. The microsomal and lysosomal fractions were the only ones which seemed to vary from one or other of the published values quoted in Table 11. The microsomal fraction appeared to contain only about half the protein which de Duve obtained, but the explanation for this probably lies in the fact that de Duve measured nitrogen

content, whereas in the experiments described above protein was measured by the method of Lowry, Rosebrough, Farr and Randall (1951). Thus, the RNA and phospholipid nitrogen of the microsomal fraction would be included in measurements by the micro-Kjeldahl, but not by the Lowry protein estimation. The amount of protein in the lysosomal fraction was also low, when compared with de Duve's figures. Lysosomes were not easy to isolate, since a considerable amount of experience and judgement is necessary in gauging their separation from other particles. The particles obtained, however, did tally closely with de Duve's description, and it was assumed that they were lysosomes, even although the yield was poor.

The choice of method for the disruption of the particles was a matter of some difficulty. De Duve added the detergent Triton X-100 to his incubation medium for this purpose, but it was felt that this treatment might inhibit some enzymes and not others. Since the relative activities of the enzyme (or enzymes) hydrolysing native and denatured DNA were to be examined, it seemed inadvisable to employ the detergent without examining fully the effects on the different activities. De Duve compared only the relative activity of enzymes in different fractions, so that any

inhibition by the detergent was less important. Two other methods of cellular disruption were therefore employed: osmotic disruption in the first experiment and sonic disruption in the second.

The pattern of distribution of alkaline DNase hydrolysing native DNA differed from that found by de Duve. The chief differences lay in the relatively low activity in the microsomes and the relatively high activity in the supernatant fraction. The specific activities of the fractions also differed; Beaufay, Bendall, Baudhuin and de Duve (1959) found that the mitochondrial fraction had the highest specific activity, while from the experiments illustrated in Figs. 28 and 29 the lysosomal fraction was found to have the highest specific activity. The only major difference in the assay used to obtain these results and the method employed by de Duve lay in the much larger amount of DNA added by de Duve to the assays. In the case of the acid DNase activity, the lysosomal fraction contained a smaller amount of the total activity, as expected from the protein distribution. However, the relative specific activities of the fractions were quite similar to those found by de Duve. The lysosomal fraction has by far the highest specific activity of all the fractions. The

distribution of enzymes hydrolysing thermally denatured DNA did not vary very significantly from those hydrolysing native DNA. However, the small overall recovery of the former (at least 20% lower than for the latter) may indicate that the two activities are different, although they may have a similar subcellular distribution.

The phosphodiesterase described by Razzell (1961a) must contribute to the level of alkaline DNase activity observed in the various subcellular fractions. Razzell demonstrated that the phosphodiesterase activity was widely distributed in the subcellular fractions, but that the highest specific activity occurred in the microsomal fraction, which in his experiments would have included the lysosomes.

4. 7 The nature of the products of the action of the liver nuclease on DNA, and their effect on the DNA nucleotidyltransferase.

The chromatography of the products of RNase digestion of RNA on DEAE-cellulose columns has been studied by Staehelin (1961). The mononucleotides and small oligonucleotides were separated using chiefly gradients of ammonium bicarbonate, at pH 8.6, but it was noted that at pH 5.5, the oligonucleotides, apart from those containing guanine, were separated according to the total charges carried. Initially, therefore, it was decided to use this low pH gradient to

fractionate the digest. The separation of the products obtained by incubating the purified inhibitor preparation with thermally denatured DNA is shown in Fig. 30. The one peak which emerged must be very heterogeneous, but the average size of the oligonucleotides must be quite large, of the order of 6 to 10 nucleotide units, in view of the volume and concentration of buffer required to elute them from the column.

After the oligonucleotides had been isolated from the column they were found to inhibit the incorporation of ^{32}P-TMP into DNA (Figs. 31 and 32). It was possible that the oligonucleotides from calf thymus DNA might be capable of hydrogen bonding with a complementary series of nucleotides on the calf thymus primer, thus assisting the polymerisation reaction by providing extra sites for the addition of nucleotide units. Hence, the oligonucleotides were added to DNA nucleotidyltransferase assays primed with DNA from both ascites tumour cells and calf thymus glands to examine this possibility. The oligonucleotides were very slightly more inhibitory when ascites tumour cell DNA was used as primer, and, therefore, the above mechanism may be operating, but it does not seem to be of great quantitative significance.

When the products of the action of the purified inhibitor preparation on DNA for 1 hr. and 22 hr. were isolated and analysed (Fig. 33) only very minor amounts of the smaller oligonucleotides apparent after 22 hr. were present after 1 hr. Had the nuclease been purely exonucleolytic in action, some mononucleotides would have been expected among the products, even after a short digestion time. Thus, the nuclease would seem to be endo- rather than exonucleolytic. None of the peaks from the 22 hr. digestion inhibited the DNA nucleotidyltransferase reaction, and, in fact, some caused a slight stimulation. On the other hand, oligonucleotides isolated from the 1 hr. digest inhibited the transferase reaction in exactly the same way as those tested in the experiment shown in Fig. 33.

Oligonucleotides bearing a 3'-phosphoryl terminal group are known to inhibit DNA nucleotidyltransferase (Keir, 1962), and it is also known that this inhibition can be reduced by treating the oligonucleotides with bacterial alkaline phosphatase. The oligonucleotides obtained by digestion of DNA with the liver nuclease were therefore treated with bacterial alkaline phosphatase, which has been shown by Heppel, Harkness and Hilmoe (1962) to be capable of removing phosphate from deoxyribooligonucleotides. However,

incubation of the inhibitory oligonucleotides with the phosphatase did not alter the inhibition (Fig. 34). It seemed unlikely that the bacterial alkaline phosphatase was unable to remove the terminal phosphate group, since in preliminary experiments with the deoxyribonucleoside monophosphates, the enzyme removed 100% of the residues. Furthermore, Keir (1962) has been successful in destroying the inhibitory effect of the 3'-phosphoryl terminal group of deoxyribooligonucleotides by incubating them with bacterial alkaline phosphatase.

Further investigations on the products of the digestion were carried out to determine whether any specific feature of these could be correlated with the inhibitory activity. When a "limit" digest of DNA was prepared, involving the periodic addition of purified inhibitor preparation over several days, only deoxyribonucleosides were detected after paper chromatography of the reaction products. No deoxyribonucleotides could be detected by paper chromatography in ethanol/ammonium acetate/EDTA, which separates nucleosides from monophosphates completely (Fig. 37). It is clear from this that there is phosphatase activity present in the purified inhibitor preparation which is capable of removing phosphate groups from deoxyribonucleotides and

perhaps from oligonucleotides. A shorter digestion time (24 hr.) was therefore tried so that the products could be examined before they had been degraded as extensively as in the previous experiment (Fig. 38). Table 13 indicates that the early peaks again consisted chiefly of the deoxyribonucleosides, however, some deoxyribonucleoside monophosphates were also present, suggesting that the phosphatase may have only limited activity. The only other derivatives which were noted were probably dinucleotides, as judged from their position on chromatograms, but the identities of these were not determined. The most outstanding feature of the digest was the presence of IdR. The origin of this nucleoside was not clear, but GdR was absent from the deoxyribonucleosides whereas AdR was present. dIMP was not identified, but dAMP, dGMP, dCMP and TMP were. From this information, it seemed that the IdR was produced from the deoxyribonucleosides rather than from the deoxyribonucleoside monophosphates. Mager and Magasanik (1957) found that in extracts of E. coli, GMP can undergo reductive deamination to IMP in the presence of NADH, with the production of NH_3 and NAD^+. However, this enzyme is not known in mammalian systems, and, in any case, it is unlikely that there would be sufficient NADH present in the purified inhibitor preparation to allow the reaction

to take place. Liver is known to contain the enzyme adenosine deaminase (Conway and Cook, 1939) which deaminates AdR to give IdR. However, the fact that the digest contained a reasonably large amount of AdR would suggest that IdR was not produced by the action of the enzyme.

The alternative to the IdR being the product of enzyme action is that it was produced by chemical deamination during the processes involved in the isolation of the products from the column. This again seemed unlikely since AdR might also have been expected to be deaminated, and since none of the treatments employed seemed to be sufficiently harsh to cause this type of chemical degradation. Furthermore, none of the methods used have previously been reported to give rise to degradation of oligonucleotides, nucleotides or nucleosides.

In a further fractionation experiment on a 2 hr. digest (Fig. 39), the reaction products were treated first with bacterial alkaline phosphatase and then with purified snake venom diesterase. The oligonucleotides should give rise to a mixture of deoxyribonucleosides and deoxyribonucleoside monophosphates, according to the equations :

(a) d-pXpYpZ $\rightarrow$ d-XpYpZ + P_i $\rightarrow$ dX + d-pY + d-pZ

(b) d-XpYpZp $\rightarrow$ d-XpYpZ + P_i $\rightarrow$ dX + d-pY + d-pZ

The above equations show the reactions catalysed by the two enzymes. They represent the reactions when the oligonucleotide has (a) a 5'-phosphoryl terminal group and (b) a 3'-phosphoryl terminal group. The results shown in Table 14 are incomplete, since some of the fractions examined contained only small amounts of oligonucleotides and when these were split up into several constituents some of the component parts were too small to be identified spectrophotometrically. Only spots which were identified from their position on the chromatograms and from their spectra are included in the Table. However, the yield of nucleotides and nucleosides from fractions 3 and 4 was sufficiently large to allow their spectra to be determined unequivocally. It can be seen (Table 14) that all four nucleosides are present in fraction 3, suggesting that all four are present at the phosphoryl terminal ends of oligonucleotides. No nucleosides were found in fraction 4. The probable explanation for this is that the oligonucleotides had a higher average chain length, and hence the relative number of terminal groups was fewer.

No dIMP was present in any of the fractions, indicating that it is not present in non-terminal positions of the oligonucleotides, although some IdR was found in fraction 1,

indicating that there might be terminal deamination of the oligonucleotides. Since neither deoxyribonucleosides nor dIMP inhibited the DNA nucleotidyltransferase reaction (Tables 15 and 16), the possibility remained that the presence of a dIMP unit in the phosphoryl terminal position of an oligonucleotide might inhibit the incorporation of ^{32}P-TMP into DNA. It is known that dITP can replace dGTP in the DNA nucleotidyltransferase system from E. coli to an extent of 25% of the incorporation obtained in the presence of dGTP (Lehman, 1959), but that dITP is not able to replace any of the other deoxyribonucleoside triphosphates. This indicates that dIMP can be incorporated and is able to form hydrogen bonds with dCMP in a manner analagous to dGMP. However, since dIMP is not a normal constituent of DNA, it is possible that its presence in the terminal position of an oligonucleotide might inhibit the DNA nucleotidyltransferase system.

In summary, therefore, it seems clear that the inhibition of the DNA nucleotidyltransferase by the purified inhibitor preparation is due to the presence of a nuclease. The nuclease seems to inhibit by two mechanisms: the simple breakdown of primer to fragments which prime only at a slower rate, or do not prime at all, and the production of

inhibitory oligonucleotides. These inhibitory oligonucleotides seem to contribute an appreciable proportion of the inhibitory effect. This effect does not appear to be due to small fragments acting as primers, but not being detected by the assay, nor does the inhibition by the oligonucleotides appear to depend on the presence of a terminal phosphate group, since this can be removed by a phosphatase without reversing the inhibition.

4. 8 The possible significance of the inhibitory nuclease.

The mechanism by which the growth of a particular tissue is controlled is not understood. Many studies have been carried out on normal rat liver but the reasons for its alteration from a non-growing to a rapidly growing tissue following partial hepatectomy are not clear. Various theories have been suggested, such as the activity of a "humoral agent" (Buchner, Scott and Aub, 1951) and the congestion of the remaining liver tissue due to diminution of the vascular bed through which the portal blood flows (Mann, 1940) but neither of these theories has been proved, and, in fact, there is evidence against both. Many biochemical alterations during liver regeneration have been demonstrated, but one of the more interesting in connection

with the present study has been described by Brody (1958), who noted that in the non-malignant growth of rat liver the increase in DNA synthesis was accompanied by an increase in DNase activity. This activity was measured at a low pH in the presence of a high salt concentration, and so it would seem that it was chiefly DNase II activity that was measured. Evidence of this type would appear to implicate DNase in the synthesis of DNA. However, it is not clear how the DNase examined in the present study, or the other DNases, are concerned with DNA replication, since they are present in normal liver. The function of the various types of DNases which appear to be present in most tissues, whether they are proliferating or not, is equally puzzling. The presence of intracellular inhibitors of DNase, which have already been described, may limit the activities of the enzymes <u>in vivo</u>. Unfortunately, the presence of these inhibitors invalidates certain studies on the presence and activity of DNase I and DNase II in different tissues, and without more detailed understanding of the occurrence of such inhibitors it would seem to be difficult to compare proliferating and non-proliferating tissues with respect to their DNase activities.

The effect of bovine liver extract on the growth of rat

ascites tumour cells in vitro has been examined by Hori and Ukita (1962). They found that such an extract inhibited the growth of the cells, and fractionated the extract using ammonium sulphate, heat treatment, organic solvents and DEAE-cellulose columns. The inhibitory principle seems to be quite different from the liver nuclease, since it is much less heat-labile. The mode of action of the inhibitory principle was not elucidated, but it is unlikely that a nuclease could be responsible, since this would require the penetration of the enzyme, possibly into the nucleus of the cell. It would seem more probable that the explanation for the inhibition of the growth of the ascites cells lies in the enzymic destruction of some essential factor in the growth medium.

Goutier and Bologna (1962) investigated the inhibitory factor of rat liver after the preliminary report of its presence by Gray et al. (1960). The method employed for assay was based on that of Bollum and Potter (1958) using the incorporation of ^{3}H-TdR into DNA by an extract of rat liver, regenerating for 25 hr. They found that an aqueous extract of rat liver inhibited the incorporation of ^{3}H-TdR, while an extract in 0.25M sucrose did not, and they therefore carried out subcellular fractionations to determine the intracellular location of the inhibitor. It was found

that only the microsomal fraction caused inhibition, and Goutier and Bologna concluded that DNase did not cause the inhibition for the following three reasons:

I. More alkaline DNase was observed in the mitochondrial than in the microsomal pellet, but the mitochondria did not inhibit. The probable explanation of this finding lies in the fact that native DNA was used as primer, and, as shown in Table 8, the nuclease does not inhibit the incorporation of ^{32}P-TMP into DNA under these conditions. This explanation is confirmed by the fact that the mitochondria were found to stimulate slightly the incorporation of TdR into DNA.

II. Heating of the microsomes to 56° for 15 min. completely inactivated the DNase, but the inhibitory activity was only reduced to 35 to 50% of its unheated level. The findings presented in this thesis are similar for the DNase activity, but the inhibitory activity would have been completely abolished by heating to 56° for 15 min. (Fig. 18).

III. On addition of 0.1 µg. of crystalline DNase I to the assay (an amount which corresponded to the activity of the microsomal pellet) the incorporation was increased. This again is probably explained by the fact that native DNA was used as primer. However, the results of Table 7

indicate that direct comparisons of this type cannot be made, since the enzymes of pancreas and liver have different properties. It is probable, therefore, that the inhibition observed by Goutier and Bologna operates at some point in the synthesis of TTP from TdR, and since it is stated that phosphatase action is not the cause, the inhibition of TTP formation may be analagous to that observed by Gray et al. (1960) with extracts of rabbit liver.

The significance of the presence of the inhibitory nuclease in normal liver is not clear, and since the mechanism of DNA replication in vivo is not understood, it is difficult at present to postulate a role for a nuclease which attacks denatured DNA more rapidly than native DNA. Only denatured DNA can be used as a primer by the purified calf thymus DNA nucleotidyltransferase system of Bollum, but other systems such as those from regenerating rat liver and ascites tumour cells can use native DNA at a reduced rate. Until the in vivo requirements for primer DNA are completely elucidated, it remains impossible to propose a definite function for nucleases present in normal and proliferating cells.

Summary.

1. Some characteristics of the enzyme DNA nucleotidyltransferase in crude extracts of calf thymus glands have been examined. The enzyme has a requirement for magnesium ions, and its activity is enhanced by, although not dependent on, the presence of 2-mercaptoethanol and EDTA. The optimal concentrations for primer DNA and deoxyribonucleoside triphosphates were determined and the time course of incorporation of ^{32}P-TMP into DNA was examined. The addition of NaCl to the medium was found to inhibit the reaction.

2. It was confirmed that calf serum and extracts of rat liver and kidney inhibit the DNA nucleotidyltransferase reaction.

3. It is postulated that the inhibitory effect of calf serum is due primarily to its content of monovalent cations, since the inhibitory factor is dialysable and heat-stable, and since monovalent cations are known to have an inhibitory effect on the reaction. The inhibition by extracts of rat liver and kidney is due to a non-dialysable, heat-labile component.

4. Fractionation of extracts of rat liver showed that the hydrolysis of the deoxyribonucleoside 5'-triphosphates by phosphorolytic enzymes is not the cause of the inhibition.

5. The inhibitory factor from rat liver was purified about 10 to 15-fold with a yield of 20%. A large amount of the phosphatase activity was removed by this procedure.

6. The inhibitory activity was found to be due, at least in part, to the action of a nuclease on the DNA primer. The DNase and inhibitory activities parallelled one another closely during the purification procedure, and showed a similar sensitivity to heat treatment.

7. Part of the inhibition of the transferase was found to be due to the products of the action of the nuclease on the DNA primer, and not merely to the breakdown of the newly synthesised portions of the DNA chains to acid-soluble fragments, although this did contribute to the overall inhibitory activity.

8. It was found that a relatively high concentration of pancreatic DNase I was required to inhibit the incorporation of ^{32}P-TMP into DNA, and that a concentration of this enzyme which produced a similar release of acid-soluble products from DNA as the purified inhibitor caused a much smaller decrease in the incorporation of ^{32}P-TMP. The addition of an amount of splenic DNase II which produced only a small amount of acid-soluble products did inhibit the reaction strongly, however.

9. When the inhibitor preparation was added to a DNA nucleotidyltransferase assay primed with native DNA, a 105% stimulation of incorporation was observed instead of the 35% inhibition seen when denatured DNA was used as primer.

10. The nuclease in the purified inhibitor preparation was shown to have an optimum pH range of 6.5 to 8.5 and a requirement for a divalent cation, either Mn^{++} or Mg^{++}. Ca^{++} cannot activate the nuclease alone, nor can it act synergistically in the presence of Mg^{++}. Increasing the ionic strength of the medium inhibits the liver nuclease, and while the preparation contains a small

amount of RNase activity, it is not clear whether this is due to the enzyme itself or to a contaminating enzyme. The nuclease hydrolyses thermally denatured DNA approximately 2.5 times more rapidly than native DNA. No evidence could be found for the presence of a coexistent inhibitor of the nuclease in extracts of rat liver.

11. These properties of the nuclease appear to distinguish it from any hitherto described and well-defined nuclease.

12. The activities of various enzymes hydrolysing DNA were determined in subcellular fractions of rat liver. The relative activities of enzymes hydrolysing native and denatured DNA did not appear to differ significantly in the fractions, but the recovery of enzymes hydrolysing denatured DNA was appreciably lower than that of those hydrolysing native DNA.

13. The products of a 6 hr. digestion of DNA by the purified inhibitor preparation were chromatographed on a DEAE-cellulose column, and found to be

inhibitory when added to the DNA nucleotidyltransferase assays.

14. The products of the digestion of DNA for 1 and 22 hr. were chromatographed and the elution patterns compared. Only very minor amounts of the smaller products seen after 22 hr. were detected after 1 hr., which suggests that the enzyme is not an exonuclease. Oligonucleotides from the 1 hr. digestion inhibited the DNA nucleotidyltransferase, but those from the 22 hr. digest did not. When treated with the bacterial alkaline phosphatase to remove the terminal phosphate groups, the inhibitory effect of the oligonucleotides was not reduced.

15. Examination of the products of a "limit digest" of DNA by the liver nuclease revealed the presence of nucleosides and no other derivatives. A 24 hr. digest contained a small amount of dinucleotides, dGMP, dCMP, dAMP, TMP, AdR, CdR, TdR and IdR. These results indicate the presence of a phosphatase in the inhibitor preparation. The source of the IdR is not known.

16. The products of a digestion of DNA with the liver nuclease for 2 hr. were chromatographed on DEAE-cellulose and then treated with bacterial alkaline phosphatase and snake venom diesterase. All four bases seemed to be present at the phosphoryl terminal ends of the oligonucleotides. IdR also appeared to be present at this end, but no evidence was obtained for its occurrence in non-terminal positions.

17. AdR, GdR, CdR, TdR and dTMP do not inhibit the DNA nucleotidyltransferase.

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