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ASPECTS OF RIBONUCLEIC ACID BIOSYNTHESIS IN MAMMALIAN CELLS

BY Margaret N. I. Barclay

Thesis presented for the degree of

Doctor of Philosophy

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APRIL, 1968

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ABBREVIATIONS

A - Adenine

G - Guanine

C - Cytosine

U - Uracil

AMP, GMP, CMP, UMP ribonucleoside 5'-phosphates of adenine,
guanine, cytosine and uracil respectively

ADP, GDP, CDP, UDP ribonucleoside 5'-(pyro) diphosphates of
adenine, guanine, cytosine and uracil
respectively

ATP, GTP, CTP, UTP ribonucleoside 5'-(pyro) triphosphates of
adenine, guanine, cytosine and uracil
respectively

dAMP, dGMP, dCMP, TMP deoxyribonucleoside 5'-phosphates of adenine,
guanine, cytosine and thymine

dADP, dGDP, dCDP, TDP deoxyribonucleoside 5'-(pyro) diphosphates
of adenine, guanine, cytosine and thymine

dATP, dGTP, dCTP, TTP deoxyribonucleoside 5'-(pyro) triphosphates
of adenine, guanosine, cytosine and thymine

Pi, PPi inorganic orthophosphate and pyrophosphate

RNA ribonucleic acid

DNA deoxyribonucleic acid

mRNA messenger RNA

rRNA ribosomal RNA

tRNA transfer RNA

| | |
|--------------------|---|
| RNA polymerase | ribonucleoside triphosphate: RNA nucleotidyl transferase. 2.7.7.6. |
| DNA polymerase | deoxyribonucleosidetriphosphate: DNA deoxynucleotidyltransferase. 2.7.7.7. |
| RNase | ribonuclease |
| DNase | deoxyribonuclease |
| DNP | deoxyribonucleoprotein |
| tris | 2-amino-2-hydroxymethylpropane-1,3-diol |
| DEAE cellulose | diethylaminoethyl cellulose |
| MPB | 2-mercapto-1(β -4 pyridethyl) benzimidazole |
| DMS | dimethyl sulphoxide |
| DOC | sodium deoxycholate |
| SSS | streptomycin sulphate supernatant fraction |
| NCS | Nuclear Chicago solvent |
| EDTA | ethylenediamine-tetraacetic acid |
| K phosphate buffer | potassium phosphate buffer, prepared by titrating a solution of AnalaR KH_2PO_4 with AnalaR KOH |
| d.p.m. | disintegrations/minute |

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

1. Introduction

Although the nucleic acids were recognised by Friedrich Miescher in 1870, most of the major developments in the elucidation of their chemistry and biochemistry have been made in the last fifteen years. In 1953 Watson and Crick put forward their description of the macromolecular organisation of DNA from consideration of existing information and focussed attention in particular on the biological significance of this physical structure. Following on this, Kornberg et al. in 1956 discovered the enzyme DNA polymerase which showed that DNA could be replicated enzymatically. Around 1960, several groups of workers Weiss (1960), Stevens (1961), Hurwitz (1962) and Chamberlin (1962) demonstrated the presence of the enzyme DNA-dependent RNA polymerase which could account for the formation of RNA by the transcription of DNA. Cell-free amino acid incorporating systems have also been developed by Zamecnik, Gros, Tissières and others. (Zamecnik (1959) and Tissières et al. (1960)).

Although until recently all the DNA of a eukaryote cell was considered to be contained in the nucleus, evidence has accumulated in the last few years that small amounts of DNA (around 5% of the total) are also present in the cytoplasm localised in the mitochondria, centrioles, and in plant cells, in the plastids (Granick and Gibor (1967)). Here however mention of DNA will be restricted to nuclear DNA.

RNA has been shown to exist in several forms in both the cell

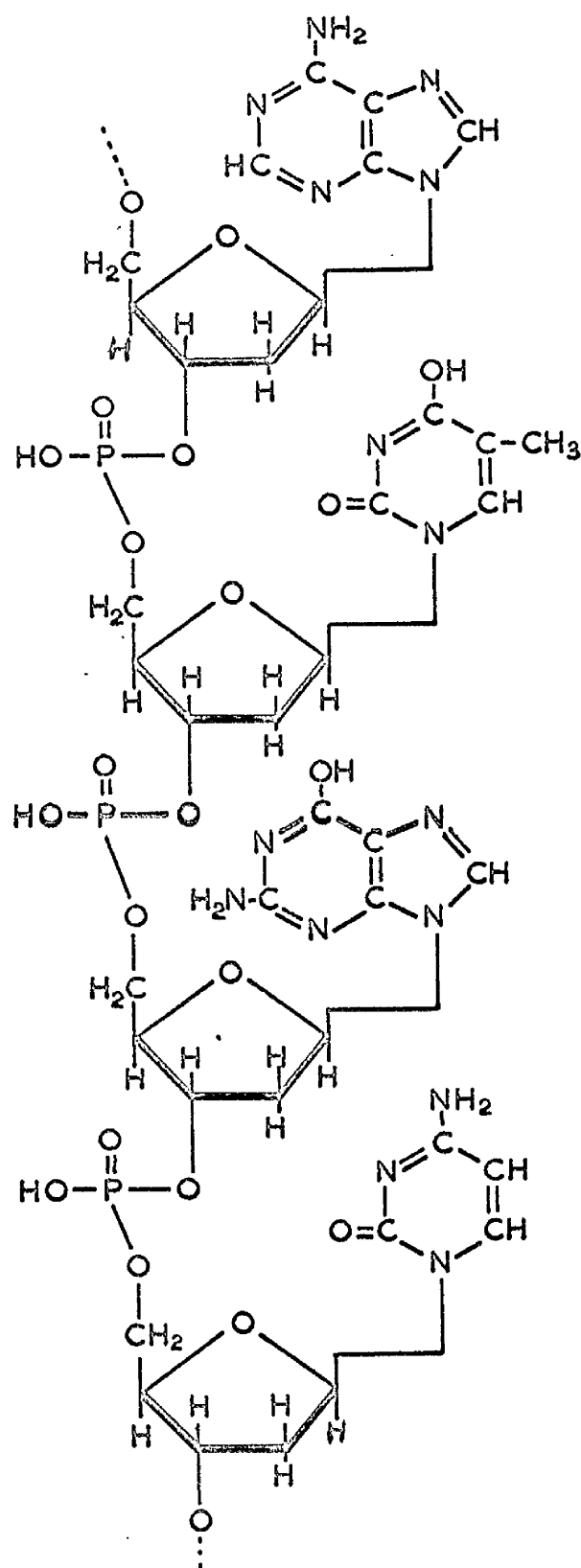
nucleus and cytoplasm. The major part of the cytoplasmic RNA is found in submicroscopic particles of ribonucleoprotein known as ribosomes, while soluble RNA forms a minor portion. A third form is mRNA which in the cytoplasm is thought to act as a template for protein synthesis. The synthesis of RNA itself seems largely restricted to the nucleus, the evidence for this being discussed later.

2.i. Structure of DNA

DNA exists mainly in the cell nucleus in the form of a deoxy-ribonucleoprotein complex. The molecule itself has been shown to consist of long unbranched chains of deoxyribonucleotides, which are in turn composed of phosphate and deoxyribose linked to adenine, guanine, cytosine or thymine. Fig. A shows a diagram of the polynucleotide structure, with the sugar residues joined by 3',5' phosphodiester linkages.

In 1950, Chargaff pointed out the equivalence of adenine and thymine and of guanine and cytosine in DNAs from many sources. Gradually it became clear that the molecule consisted of two polynucleotide chains and that these chains were held in a double helical configuration in part by the steric arrangement of bases within each chain and in part by hydrogen bonds between complementary base pairs. The double helical structure proposed for DNA by Watson and Crick (1953) and later modified by Wilkins (1956) was based mainly on X-ray diffraction data, but also explains many of

FIGURE A



The structure of the polydeoxyribonucleotide DNA.

its other physical properties. The constituent polynucleotide strands are antiparallel and wound round a common axis to form a right-handed double helix with a narrow groove and a wide groove running along the length of the molecule. There are ten bases per turn of the helix in the so called 'B configuration', and the double helical structure is stabilised by hydrogen bonding between the adenine of one strand and the thymine of the other or by similar base pairing of cytosine and guanine. The geometry of the mutual arrangement of DNA molecules has also been studied. In fibres having the 'B' configuration and in wet gels of DNA (15-60% DNA) the double helices tend to form hexagonal or near-hexagonal arrays (Luzzati, Nicolaieff and Masson (1961)) while gels of nucleohistone appear to have protein crosslinks between the DNA molecules, keeping them at fixed distances from each other and in regular arrays (Itzhaki (1966)).

ii. Replication

The specific pairing of bases in the double helix suggested that the sequence of bases along any one strand might provide a template for the synthesis of complementary strands, and when new molecules of DNA were formed one strand might be derived from the parental DNA while the second strand was newly synthesised. The classic experiments of Meselson and Stahl (1958) using $[N^{15}]$ labelled E.coli showed the existence of this semiconservative form of replication.

The enzyme DNA polymerase which has been extracted from both bacterial and animal cells can carry out the synthesis of new DNA in vitro (Smellie (1965)). This enzyme requires the presence of all 4 deoxyribonucleoside triphosphates, Mg^{2+} ions, and a source of DNA as primer. Both bacterial and mammalian DNA polymerase preparations will promote the terminal addition of a deoxyribonucleotide residue at the 3'-hydroxyl end of the primer chain if the incubation is carried out in the presence of only one deoxyribonucleoside-5'-triphosphate at a time. This reaction is limited and there does not seem to be any restriction exerted by the primer on the nucleotide being incorporated. With calf thymus enzymes the conditions for this reaction vary significantly from those of the true DNA polymerase reaction and the two activities may be separated (Keir and Smith (1963)), (Bollum, Groeniger and Yoneda (1964)). The physical properties of the product of the DNA polymerase reaction closely resemble those of natural DNA and suggest a polymer with a molecular weight of about 5 million in the form of a double-stranded rodlike structure which collapses on heating in a manner similar to native DNA (Lehman (1959)). It has also been shown that the chemical composition of the reaction product is characteristic of any given DNA primer (Josse, Kaiser and Kornberg (1961)). A particularly convincing example of this dependence can be seen in experiments with poly dA-T as primer where, despite the availability of all four deoxyribonucleoside 5'-triphosphates, the product contains

exclusively deoxyadenosine and deoxythymidine residues. Exact sequences of nucleotides in DNA have not been worked out but Josse, Kaiser and Kornberg (1961) have devised a procedure called 'nearest neighbour frequency analysis' which permits measurement of the frequency with which any one deoxyribonucleotide occurs adjacent to any of the four major deoxyribonucleotides in a newly synthesised strand of DNA. In this procedure, four independent reaction mixtures are prepared, each containing DNA, Mg^{2+} ions, DNA polymerase and the four deoxyribonucleoside 5'-triphosphates, one of which is labelled with $[P^{32}]$ in the α -phosphate. The labelled deoxyribonucleoside 5'-triphosphate is different in each reaction mixture. After incubation the product is isolated and degraded with micrococcal nuclease (E.C. 3.1.4.7.) so that the phosphorus originally attached to the 5'-hydroxyl of the incorporated nucleoside residue is released in ester linkage with the 3'-hydroxyl of the adjacent nucleoside residue. The deoxyribonucleoside 3'-phosphates so formed are separated from one another, and their radioactivity measured. The results of such experiments are consistent with a mechanism by which the polymerase forms new polydeoxyribonucleotide strands which are complementary to both strands of the DNA primer. Moreover if the product of a reaction, whose nearest neighbour frequency has been determined, is used as a primer in a second synthetic reaction, the frequencies observed in the second product match those in the first.

There are several interesting topics being pursued at present in connection with DNA replication e.g. repair mechanisms, initiation of strand replication, and more detailed work on the normal replicative action (Jehle (1965)).

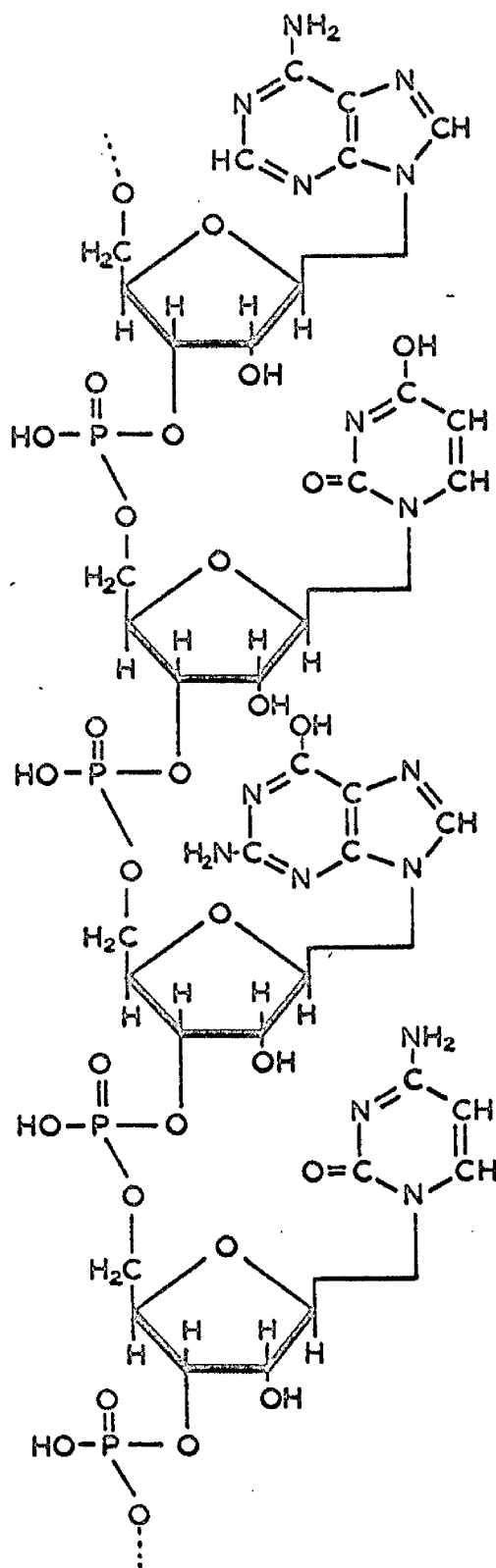
3. Structure of RNA

RNA unlike DNA exists, in the cell, in several forms. Chemically they all consist of unbranched chains of ribonucleotides (see Fig. B) joined by 3',5'-phosphodiester linkages. In size however they vary widely.

i. Ribosomal RNA (rRNA)

The ribosomal RNA from Escherichia coli has been widely studied (Littauer and Eisenberg (1959) and Boedtker, Möller and Klemperer (1962)). It has been shown to consist of two basic components whose sedimentation coefficients are around 23 and 16 Svedberg Units. Their respective molecular weights, according to Kurland (1960) are 1.12×10^6 and 0.56×10^6 . Intact 70s ribosomes from E. coli are known to consist of two subunits 50s and 30s (Tissières and Watson (1958)) and Kurland (1960) showed that each 30s subunit contained one RNA molecule whose molecular weight was 0.56×10^6 (16s RNA). Of the 50s subunits, some were found to contain one RNA molecule each, molecular weight 1.12×10^6 (23s RNA) while others contained two RNA molecules each, molecular weight 0.56×10^6 . Thus the original 70s ribosomal particle contains two molecules of RNA (23s + 16s) or three 16s molecules. Green and Hall (1961) showed however

FIGURE B



The structure of the polyribonucleotide RNA.

that the 50s particles incorporated into ribosomes each contained a 23s molecule in nearly every case. The values quoted for ribosomes prepared from other tissues vary a little e.g. 28s and 18s were obtained by Hall and Doty (1959) from calf's liver. There has also recently been found a 5s RNA component of the 50s ribosome (Monier et al.(1967)).

ii. Transfer RNA (tRNA)

Several distinct species of tRNA have been recognised. These are much smaller structures, than rRNA, having molecular weights in the range 25,000 to 30,000, corresponding to not more than 80-100 nucleotides (Tissières (1959), Brown and Zubay (1960) and Cox and Littauer (1960)). In all biologically active molecules of this RNA, nucleotides at the 3'-hydroxyl end are arranged in a characteristic sequence - pCpCpA.

iii. Messenger RNA (mRNA)

The molecular weight of mRNAs have not been accurately established. Early measurements gave values of about 100,000 for mRNA from bacterial sources (Nomura, Hall, Spiegelman (1960)), but much larger molecules have also been described (Monier, Naoro, Hayes, Hayes, Gros (1962) and Spiegelman (1961)). It might be expected that there will be a considerable range of sizes for mRNA molecules.

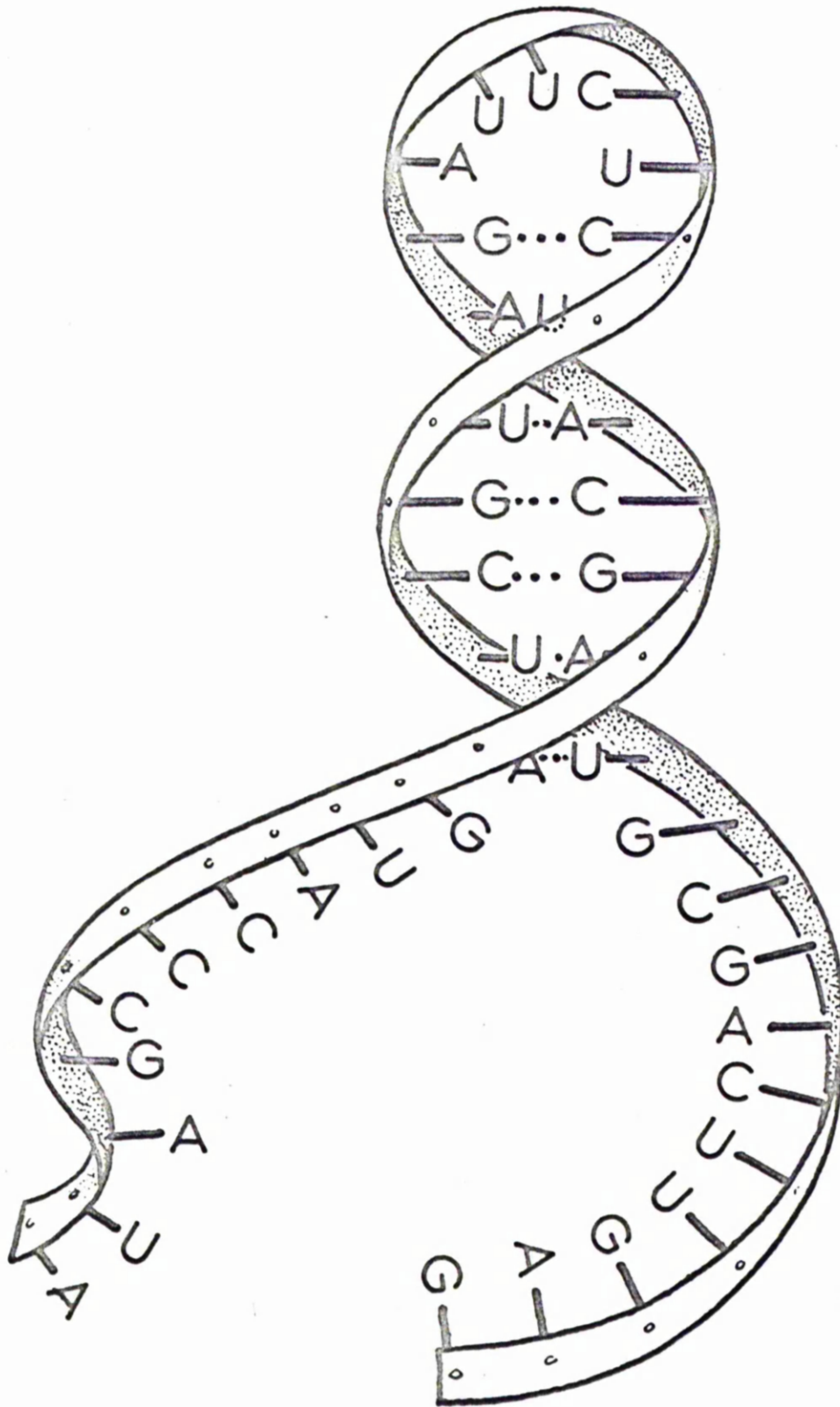
iv. Possible secondary and tertiary structures of RNA

Unlike DNA which is a continuous, perfectly shaped double helix, which imparts rigidity to the molecular structure along its

FIGURE C

A diagrammatic representation of RNA showing the existence of areas of base pairing within a single strand.

FIGURE C



entire length, RNA is much less rigid and consists of only one continuous polynucleotide chain. Thus any helical regions in an RNA chain could arise only as a result of interactions between bases which are confined to one chain. Helical regions do exist and while these dissociate on heating this process appears to be readily reversible. Denaturation of RNA takes place over a far wider range of temperatures than is the case with DNA. This indicates a successive melting of a number of individual short helical regions (Doty, Boedtker, Fresco, Haselkorn and Litt (1959); Fresco and Alberts (1960)). The base pairing in RNA occurs between adenine and uracil on the one hand, and guanine and cytosine on the other, and because the helical regions arise as a result of the chain folding back on itself, sections of the helical regions are antiparallel. Spirin in his book 'Macromolecular Structure of Ribonucleic Acids' (1964) stresses the fluidity of the RNA structure which may unfold and reform to some more stable conformation depending on the prevailing conditions. Whenever these conditions are altered, the structure will be modified accordingly.

From studies with ribosomal RNA Spirin (1963) suggests that the loops which occur in the RNA from 30S ribosomes are of the order of 30 nucleotide units in size with up to 10 units between the loops. In RNA from 50S ribosomes he suggests that these loops are larger - around 40 units with up to 12 units between them.

Much more is known about the structure of tRNAs, although

even here there is a great deal of speculation. All tRNA is thought to have a clover leaf structure with base pairing across each segment of the leaf. There would seem to be evidence to suggest that such a structure would not remain flat but fold over on itself in solution (Zachau (1967)).

5s RNA is thought to have a structure akin to that of tRNA with at least two main regions of base pairing (Monier (1967)).

4. The synthesis of RNA (in vivo)

i. Site of synthesis

The hypothesis regarding the nucleus as the site of cellular RNA synthesis is not new. It postulates the transfer of the RNA from the nucleus to the cytoplasm and there is considerable evidence for its support at present. Several groups of workers (Zalokar, Goldstein) have studied the time course of RNA labelling with radioactive precursors in intact cells and found consistently that initial labelling takes place in the nucleus. After a lag the labelled RNA appears in the cytoplasm. This lag varies from a minute in Neurospora (Zalokar (1960)) to an hour with human amnion cells in culture (Goldstein and Micou (1959)) i.e. the lag varies with the length of the cell life cycle. It has been suggested that the nuclear RNA is degraded and that the fragments migrate to the cytoplasm where they are re-used to synthesise cytoplasmic RNA (Harris & Watts (1962)). This remains a possibility as detailed kinetic analysis of the experiments has proved difficult, but it is

not thought likely that it is the major pathway.

Experiments in which RNA synthesis in Amoeba proteus or Acetabularia was observed following enucleation or transplantation of labelled nuclei have also been carried out (Brachet (1955)). If it can be contrived that there is no subsidiary source of DNA in the cytoplasm e.g. in chloroplasts, then RNA production drops when the nucleus is removed (Prescott (1959)).

Thus it would seem that the major portion of cellular RNA is synthesised in the nucleus and must be transferred to the cytoplasm.

ii. Mechanism of RNA production

a. RNA synthesis and actinomycin D

Actinomycin is a bacteriostatic antibiotic which has been found to inhibit the synthesis of nucleic acids in intact bacterial and mammalian cells and in enzymic preparations. The most widely used form is actinomycin D. Low concentrations of actinomycin selectively suppress cellular (Reich, Franklin, Shatkin and Tatum (1961)) and enzymic RNA synthesis (Hurwitz, Furth, Malamy & Alexander (1962)), DNA synthesis being affected only at much higher concentrations (Kirk (1960); Keir, Omura and Shepherd (1963)).

The inhibition of RNA synthesis requires the binding of actinomycin to DNA (Hurwitz, Furth, Malamy and Alexander (1962)). When DNA is added to solutions of the antibiotic, the maximum value of the optical density in the visible region (425m μ) is reduced and shifted to a longer wavelength. This effect has been used by

Kersten to find which constituents of DNA would bind to the inhibitor (Kersten (1961)). He found that spectral changes could be reproduced using various purine derivatives in the following order of effectiveness:- deoxyguanosine > guanosine > adenine =AMP=ATP=adenosine=deoxyadenosine. The pyrimidine derivatives were inactive. Kersten concluded that actinomycin reacts with the deoxyguanosine residues in DNA.

However since actinomycin does not appear to bind to RNA the conformation in which the guanine is held must be significant. It was then found that the maximum binding capacity of single-stranded or heated DNA is less than native DNA with a similar base composition (Goldberg, Rabinowitz and Reich (1962) and Kahan, Kahan and Hurwitz (1963)). Dissociation of the actinomycin-DNA complex begins at the onset of strand separation when the complex is being heated (Reich and Goldberg (1964)). However helical DNA which contains no guanine fails to bind the antibiotic.

Actinomycin is not thought to intercalate between successive base-pairs in helical DNA (Reich and Goldberg(1964)). A model for the structure of actinomycin-DNA complexes, based on X-ray diffraction data has been proposed (Hamilton, Fuller and Reich (1963)). According to this model, actinomycin is visualised as bound to guanine or G-C pairs, in the minor groove of helical DNA.

Many experiments with actinomycin have been performed using a purified bacterial RNA polymerase in vitro. In such systems the

susceptibility of DNA-directed RNA synthesis depends on the capacity of the template to bind the antibiotic (Goldberg et al. (1962), Kahan et al. (1963)). Actinomycin does not inhibit the enzyme directly nor does it compete with the nucleotide precursors or cofactors, inhibition only being overcome if increasing amounts of DNA are added to the system (Goldberg and Rabinowitz (1962)). No inhibition of polyribonucleotide synthesis occurs if the bacterial polymerase is supplied with an RNA template.

In vivo it has been found that almost all cellular RNA synthesis can be stopped in the presence of low levels of actinomycin D (Reich, Franklin, Shatkin and Tatum (1962), Mach and Tatum (1963), Merits (1963) and Franklin (1963)). The small residual incorporation of radioactive precursors into RNA that is resistant to actinomycin appears to be caused by terminal group turnover of the pCpCpA sequence in tRNA. (Eason, Cline and Smellie (1963)).

Not all classes of RNA exhibit the same susceptibility to actinomycin. Perry (1963) found that the incorporation of tritiated nucleotides into nucleolar and cytoplasmic RNA was suppressed by very low concentrations of the drug, whereas the incorporation into chromosomal, 'DNA-like', RNA was unaffected. The reason for this varying susceptibility is not certain. Both the base composition and length of the individual DNA templates seem critical.

b. Hybridisation experiments

Further information on the source of RNA can be obtained using

the technique of specific hybridisation. When heat denatured or single-stranded DNA and RNA are heated together and cooled slowly, they will interact to form a hybrid, provided the sequence of bases in the RNA molecule is complementary to that in regions of the DNA (Yankofsky and Spiegelman (1962)). Hybrid molecules containing a strand of DNA and one of RNA form a peak intermediate between denatured DNA and free RNA when the treated RNA and DNA is run on CsCl equilibrium gradients. This peak when isolated and freed from CsCl can be shown to contain RNA but to be resistant to RNase digestion which suggests that the RNA is present in a bound form.

This technique has been used to demonstrate a sequence homology between DNA and rRNA in bacterial (Yankofsky and Spiegelman (1962b)), plant (Chapchase, Birnstiel (1963)) and animal cells (McConkey and Hopkins (1965) and Attardi, Huang and Kabat (1965)). The results suggest that the rRNA molecules are each derived from distinct sequences of the DNA. These sequences are thought to account for about 0.3% to 0.4% of the total DNA present (Yankofsky and Spiegelman (1962b) and (1963) and Attardi, Huang and Kabat (1965)).

5s and tRNA have also been shown to form specific hybrids with their homologous DNAs. Here the proportion of DNA thought to be involved is 0.1% (Goodman and Rich (1962), Giacomoni and Spiegelman (1962) and Monier (1967)).

The property of rapidly labelled RNA to combine with homologous DNA was first investigated by Hall and Spiegelman (1961) using a

system of phage infected E. coli. These observations were then extended to rapidly labelled RNA from uninfected bacterial cells (Hayashi, Hayashi and Spiegelman (1964)). Perry et al. (1964) showed that rapidly labelled RNA from L cells in culture can form hybrids with L cell DNA.

The experiments on hybridisation can be interpreted as demonstrating that all known classes of RNA may ultimately be derived from sequences on DNA.

c. Asymmetric synthesis

RNA could be formed by transcription of one or both strands of DNA. Marmur and Greenspan (1963) demonstrated that in vivo only one DNA strand is copied. Because of the different buoyant density of the two DNA strands of bacteriophage SP8, it was possible to separate the two strands and perform hybridisation experiments between each of the complementary strands and the new RNA induced in Bacillus subtilis, in response to infection with the bacteriophage SP8. They found that the RNA hybridised with the heavy pyrimidine rich strand of DNA, and therefore had the same base sequence as the light, purine rich, DNA strand. Similarly it was found that in both uninfected and T2 phage infected E. coli rapidly labelled RNA could hybridise to only one half of the homologous DNA (McCarthy, Bolton (1964) and Hall, Green, Nygaard and Boezi (1963)). These are all phage systems however and as yet no evidence has been presented showing a similar phenomenon occurring in mammalian cells.

Early experiments on the synthesis of RNA in vitro showed that when native double-stranded DNA was employed as a template both strands were transcribed (Chamberlin and Berg (1962) and Hurwitz, Furth, Anders and Evans (1962)). In subsequent experiments, Hayashi, Hayashi and Spiegelman (1964) studied the RNA product obtained in vitro using DNA from bacteriophage ϕ X174 and the double-stranded, circular replicative form (RF) of this DNA that is synthesised in infected cells. They found that when single-stranded DNA of ϕ X174 was employed the RNA had a composition complementary to that of the DNA template. On the other hand when intact circles of the double-stranded replicative form were used, the RNA product was transcribed from one strand only. This RNA had a nucleotide composition corresponding to that of the mature virus DNA. Since breakage of the circular replicative form of ϕ X174 DNA resulted in transcription of both strands of the primer it seemed that the mechanism of strand selection by the RNA polymerase depended on the physical integrity of the DNA primer.

Further experiments have been carried out with phage α in Bacillus megatherium by Geiduschek et al. (1964). They were also able to show asymmetric transcription with homologous DNA but found that this was not dependent on the integrity of the DNA primer as native degraded DNA produced similar results. It is perhaps significant that these fragmented portions of DNA are still larger than the whole of ϕ X174 DNA but it would seem experimental technique

will have to be further refined before the exact mechanism of transcription will be known.

iii. Messenger RNA biosynthesis

Volkin and Astrachan (1956) were the first to demonstrate a rapidly labelled RNA which resembled DNA in base composition. It has been possible to show by autoradiography that the earliest accumulation of RNA precursors occurs on the chromosomes (Pelling (1959)). A number of facts point to this being mRNA. The RNA formed on the chromosomes reflects the base composition of the DNA present as does mRNA. Lerman et al. (1963) have shown that very low doses of actinomycin D selectively inhibit the synthesis of ribosomal RNA while RNA synthesised in the presence of small doses of actinomycin consists exclusively of DNA-like RNA (Reich, Franklin, Shatkin & Tatum (1962)). In autoradiographic experiments, similar doses of actinomycin completely suppress the incorporation of labelled precursors into nucleolar RNA and inhibit the synthesis of chromosomal RNA only slightly (Perry (1962)). Embryonic cells having no nucleoli up to the gastrula stage as well as anucleolar mutants do not synthesise rRNA while DNA-like RNA synthesis proceeds normally (Brown and Gurdon (1964) and Brown and Littau (1964)). As will be subsequently discussed the bulk of the cellular RNA polymerase is contained in the chromatin fraction of the nucleus. Newly formed chromosomal DNA-like RNA differs from other cellular RNA classes in respect to its behaviour during protein removal.

DNA must be liberated or the chromatin treated with hot phenol before this RNA can be extracted (Georgiev (1967)). This is probably due to a peculiar characteristic of its association with chromosomal nucleoprotein. Thus the study of the interrelation between template, enzyme and product during RNA biosynthesis is of obvious interest.

In lampbrush chromosomes, it is possible to differentiate chromocenters into areas where deoxyribonucleoprotein (DNP) is aggregated and areas where so called loops - areas of loosely arranged chromatin material - occur. Autoradiographic experiments show that RNA is synthesised solely in the loops (Gall and Callan (1962)). Frenster et al. (1963) have isolated chromatin and extended 'unwound' chromatin from homogenates of ultrasonically disrupted lymphocyte nuclei. The major part of the newly formed RNA is found in association with the extended form of the chromatin but this may be an artefact of isolation procedures. The exact difference between these two forms of chromatin seems obscure.

While histones are known to inhibit glycolysis and synthesis of ATP in nuclei (Hnilica and Billen (1964) and McEwen, Allfrey and Mirsky (1963)), there is also evidence that they influence the synthesis of DNA and RNA (Huang and Bonner (1962)). Stedman and Stedman (1947) were the first to postulate a role for histones in the regulation of RNA production. Experimental evidence for this was published by Huang and Bonner (1962) and Allfrey, Littau and

Mirsky (1963). They showed that the removal of histones from isolated cell nuclei or chromatin stimulates RNA synthesis. Huang and Bonner (1962) isolated chromatin from pea seedling nuclei and showed its limited ability to serve as primer in RNA synthesis. They then split off the histones (separating the fractions on a 4M-CsCl gradient) and found the RNA polymerase activity of the DNP increased some 20-25 fold. When histone was again added to the incubation mixture, inhibition of RNA polymerase was noted. Allfrey treated calf thymus nuclei with trypsin and found a 2-3 fold increase in the labelled RNA formed.

It would seem that the histones act not on the RNA polymerase but on the DNA template since the addition of an excess DNA to the incubation mixture containing a purified RNA polymerase, DNA and histone, restores the synthesis of RNA (Bonner and Huang (1963)). This inhibitory action is not solely due to the insolubility of the nucleohistone as soluble preparations of nucleohistone are still low in priming activity (Huang, Bonner and Murray (1964), Butler and Chipperfield (1967)). Furthermore chromatin preparations from which some protein has been removed, by for example, salt extraction, but which are still insoluble, can function as templates for RNA synthesis (Georgiev (1966)).

Allfrey, Faulkner and Mirsky (1964) also showed that the inhibitory effect of histones might be modified by acetylation or methylation and postulated a control of histone inhibition during

RNA synthesis.

Addition of histone to lampbrush chromosomes causes the loops to disappear i.e. the DNP condenses (Izawa, Allfrey, Mirsky (1963)) and removal of histone causes disaggregation of this fraction (Littau, Burdick, Allfrey and Mirsky (1965)). Thus there would seem to be two classes of DNP - one histone rich and the other histone poor. Frenster's analyses of these two types of chromatin however seem to show no great quantitative difference in histone level (Frenster (1965)). Thus there is no direct evidence for the function of histone in chromosomes.

Bonner et al. (1963) using a cell free protein synthesising system and DNP from various plant tissues were able to show a variation in the protein produced. About 1.5% of the new protein formed using DNP originating from the cotyledon had the characteristics of globulin which is actively produced in those cells. If DNP from the apical bud is used then little globulin is formed. Thus DNP would seem to contain the factors controlling gene activity while this has been lost in purified DNA.

Recently the role of the non-histone protein has been tested by Gilmour and Paul (1967). Using a purified bacterial RNA polymerase and calf thymus chromatin as primer, RNA was synthesised and hybridised with homologous DNA. It was found that a large percentage of sites on the chromatin were unavailable for transcription (Paul and Gilmour (1966)). The chromatin was then further

fractionated and the nucleoproteins obtained used as primers with the same polymerase system. The removal of histone showed a 4-6 fold increase in activity, which was reversed on adding back excess histone. When DNA was recombined with histone alone, no synthesis of RNA could be obtained but when it was recombined with histone in the presence of a non-histone chromosomal fraction, the material showed the same properties as the original chromatin. This would suggest that the non-histone protein fraction of DNP plays a significant part in regulation of RNA synthesis.

iv. Ribosomal RNA biosynthesis

Data indicating the presence of ribosomal RNA in the nucleolus have been presented. Incorporation of labelled precursors, followed by nuclear fractionation has shown 45s RNA associated with the nucleolar fraction. It is thought this 45s fraction is a precursor of normal 28s and 18s ribosomal RNA (Penman (1966)). A number of authors have studied the distribution of DNA capable of hybridising with rRNA between chromatin and nucleolus. Chipchase and Birnstiel (1963) have shown that in pea seedlings this form of DNA (about 1% of the total) is scattered throughout the nucleus and not restricted to the nucleolus. However experiments with HeLa cells have shown a higher concentration of this DNA in the nucleoli (McConkey and Hopkins (1964)). Separation of the nucleoli is technically difficult and so the above results may yet be explained. Ritossa and Spiegelman (1965) attempted to overcome this by studying the DNA

complementary to rRNA in Drosophila larvae having 1, 2, 3 or 4 nucleoli. The quantity of complementary DNA varied in direct proportion to the number of nucleoli.

v. Transfer RNA biosynthesis

There would seem to be little known about the detailed kinetics of tRNAs syntheses in animal cells. Like other forms of RNA they are synthesised in the nucleus, and nucleoli have been shown to contain considerable amounts (Chipchase and Birnstiel (1963b)). Birnstiel has shown further that 5,6-dichloro- and 4,5,6-trichloro-1-(β -D-ribofuranosyl) benzimidazole which inhibits chromosomal but not nucleolar RNA synthesis, does not alter the rate of incorporation of precursor into tRNA (Sirlin, Jacob & Birnstiel (1965)).

The synthesis of tRNA is inhibited by the presence of actinomycin D but the inhibition is not so great as in the case of mRNA or rRNA. In rat liver and L cells it has been shown that most of the resistant label could be accounted for in the terminal - pCpCpA nucleotide sequence (Merits (1963) Franklin (1963)). Turnover of this sequence in vivo has been noted in various tissues (Cannon (1964), Burny and Chantrenne (1963)).

vi. Viral RNA biosynthesis

This form of RNA is distinct in that it is formed in a host cell under the direction of a viral template. This template is characteristic of the virus and may be either DNA or RNA. Much

attention has been paid to the polymerases involved in viral replication as these would seem to be separate enzymes from those of the host (Keir and Gold (1963), and Spiegelman and Hayashi (1963)). The role of virus specific RNA in the replication of DNA viruses is somewhat similar to that of mammalian mRNA. It is transcribed from the viral DNA template by RNA polymerase and associates with ribosomal particles to translate the coding of the DNA into viral specific proteins.

Bacterial and animal cells infected with an RNA virus develop RNA-dependent synthesising systems for the production of new viral RNA. This could occur by the mechanism proposed for the single-stranded DNA virus ϕ X174. On infection, the viral DNA is converted to a double-stranded form (Sinsheimer, Starman, Naglor & Guthrie (1962)). This serves as a template for the synthesis of virus specific mRNA. It has been shown that the RNA is complementary to a second or 'minus' strand of the DNA (Hayashi, Hayashi & Spiegelman (1963)). Ochoa et al. proposed a similar mechanism as the first step in the replication of viral RNA (Ochoa, Weissman, Borst, Burdon & Billeter (1964)) while Montagnier and Sanders (1963) were the first to describe the formation of double-stranded virus specific RNA *in vivo*.

Spiegelman et al. have produced evidence which seems in conflict with the above hypothesis. The double-stranded (RF) RNA can be found in infected cells but this poses the question of whether

it is necessary for RNA replication. An enzyme has been isolated from the phages Q β and MS2 which can synthesise mature viral RNA in vitro (Haruna and Spiegelman (1965a)). The RNA produced is infectious and exhibits the same template properties in the polymerase reaction as the original viral RNA. No heat sensitive ribonuclease-resistant material could be detected during the reaction. However if the purified Q β polymerase was supplied with fragmented template RNA the reaction was slow and incomplete, and the product biologically inactive (Haruna and Spiegelman (1965b)). Here the product was ribonuclease resistant.

This would mean proposing the existence of a polymerase which could act from a single strand of template to produce not a 'Watson-Crick' image but an exact replica A for A and G for G.

A further paper by Mills, Pace and Spiegelman (1966) modifies their earlier views. They report the finding of a non-infectious complex having a sedimentation coefficient of 15s. It has been possible by double labelling, to establish that this complex contains one strand of template and another of product. Further, on heat denaturation the complex gives rise to infectious plus strands of RNA.

Other groups working with the RNA-containing bacteriophages R17 and M12 of E. coli (Francke and Hofschneider (1966), Erikson (1966), Erikson and Franklin (1966), Franklin (1966) and Granboulan and Franklin (1966)) have recognised two infectious RNA fractions

in addition to mature virus RNA in infected cells. One of these behaves as double-stranded RNA and appears in infected cells prior to new single-stranded viral RNA. The other species of RNA behaves as if it were double-stranded RNA with a tail of single-stranded RNA attached.

In conclusion it might be said that some evidence has been obtained for a double-stranded replicative form of RNA in all the viral systems examined.

5. The synthesis of RNA (in vitro)

i. Polynucleotide phosphorylase

This enzyme was the first to be isolated which could catalyse the synthesis of ribonucleic acids. The initial work was done by Grunberg-Manago, Ortiz and Ochoa (1956) using extracts of Azotobacter vinelandii. Since then this enzyme has been detected in a wide variety of organisms. Ribonucleoside diphosphates are required as substrates and are incorporated into polyribonucleotides in the presence of Mg^{2+} . Incorporation will take place in a reaction mixture containing one or more of the diphosphates. The requirement for diphosphate seems absolute as neither mono- nor triphosphates serve as substrates. The crude enzyme preparation is not dependent on the addition of RNA but purified preparations show primer requirements.

Initially this enzyme was thought to be responsible for RNA biosynthesis in vivo but gradually it was shown that the base

composition of the RNA product reflected merely the base composition of the ribonucleoside diphosphates in the reaction mixture, rather than that of the primer (Grunberg-Manago (1963) gives a review). It seems that the reaction proceeds by the addition of monomer units to the 3' hydroxyl end of the primer in a chain extension mechanism (Singer, Heppel and Hilme (1957 and 1960)). The reaction requires a free-OH at the 3' position in the primer and stoichiometric amounts of inorganic orthophosphate are released.

Although no longer thought to play a major role in the synthesis of RNA the enzyme has been useful in preparing various artificial homo- and hetero-polymers as even without a primer, polymer formation can occur.

There are comparatively few references in the literature to polynucleotide phosphorylase being found in mammalian cells. It has been suggested that this may be due to destruction of the nucleoside diphosphates by adenylate kinase and there is an account of the phosphorylase in rat liver nuclei which contain very little adenylate kinase (Siebert, Villabos, Suk Ro, Steele, Lindemayer, Adams and Busch (1966)).

ii. Transfer RNA cytidylyl-adenylyl transferase

Heidelberger et al. (1956) first showed that a cytoplasmic fraction from rat liver could incorporate AMP into RNA. If the enzyme is first incubated in the absence of RNA, its ability to incorporate AMP is reduced. This ability can be restored by the

presence of CTP. The two nucleotides are incorporated in the ratio of one A to two C but no other nucleotides are incorporated (see Smellie 1963 for a review).

iii. Homo-polyribonucleotide formation

There have been several reports of enzyme fractions which incorporate single ribonucleoside triphosphates into homopolymers. These systems are characterised by the lack of stimulation when the other ribonucleoside-5'-triphosphates are added to the reaction either singly or together.

An extract from calf thymus nuclei has been found to bring about the synthesis of poly A (Edmonds and Abrams (1960), (1962)). A system synthesising poly A has also been prepared from chick embryos (Chung, Mahler and Enrione (1960)). This enzyme appears to require Mg^{2+} and additional RNA as primer. Abrams et al. (1962) have described the purification of extracts from calf thymus nuclei which incorporate CMP from CTP into RNA. A significant proportion of the CMP units is located in terminal sites of the polyribonucleotide chain and although this enzyme does not utilise GTP or UTP, it does catalyse a very limited incorporation of AMP residues from ATP, mainly into terminal positions.

The incorporation of uridine nucleotides into RNA by enzyme systems derived from Ehrlich and Landschutz ascites tumour cells as well as a number of other animal tissues has been described by Burdon and Smellie (1960, 1961a,b,c) and by Smellie (1962). In

experiments with $[^{14}\text{C}]$ UTP, 40% of the uridine residues are located on 3'-OH ends of polyribonucleotide chains. A similar system has been purified from the soluble fraction of rat liver by Klemperer and Kammen (1962). This enzyme incorporates UMP residues into RNA and the reaction is not stimulated by the addition of ATP, GTP and CTP.

A number of reports have described particulate fractions from a wide variety of cells. An enzyme from E. coli ribosomes catalyses the RNA-dependent incorporation of ribonucleotides into RNA. The enzyme has been purified and requires Mg^{2+} and added RNA (August, Ortiz and Skalka (1962) and August, Ortiz and Hurwitz (1962)). Extensive polymer synthesis was observed only with ATP although the other nucleotides were incorporated to a small extent. Cytoplasmic ribosome preparations have been shown to support the RNA-dependent incorporation of nucleotides into RNA (Baltimore and Franklin (1963) and Wykes and Smellie (1966)). In the case of the Landschutz ascites cells the incorporation was predominantly terminal.

There is also an enzyme system which incorporates ribonucleoside triphosphates into polydeoxyribonucleotides (Krakow, Kammen and Canellakis (1961) and Canellakis and Krakow (1962)). Any of the four ribonucleoside triphosphates are incorporated terminally into DNA. The enzyme is distinct from DNA polymerase and adds only one residue onto the ends of the DNA chain. The product will then act as primer in the rat liver system which incorporates UMP

residues into poly U. It has also been demonstrated that under special conditions, in the presence of Mn^{2+} DNA polymerase will incorporate ribonucleoside triphosphates (Berg(1964)).

The biological function of the enzymes described above remains obscure.

iv. RNA polymerase

This enzyme has been studied in a great variety of organisms. It was initially discovered in rat liver (Weiss (1960)) but to that must be added bovine lymphosarcoma (Furth & Ho (1965)), rat testes (Ballard & Williams-Ashman (1966)) and ascites tumour cells (Burdon and Smellie (1961b), Eason and Smellie (1964)) among others. In plants, RNA polymerase has been found in maize seedlings (Mans and Novelli (1964)) and in nuclei from pea embryos (Huang, Makeshwan and Bonner (1960)). Most of the work has in fact been done using a purified bacterial enzyme from E. coli (Chamberlin and Berg (1962)), Micrococcus lysodeikticus (Weiss and Nakamoto (1961 a and b)) or A. vinelandii (Krakow & Ochoa (1963)). There are also many instances of experiments involving the enzyme from viral sources. For a review see D. Elson in the Annual Review of Biochemistry 1965.

RNA polymerase appears to be found closely attached to chromatin material and is often in fact in an aggregate form when isolated. There are a few instances of a soluble mammalian enzyme e.g. Ballard and Williams-Ashman (1966) have found one in rat testes. RNA polymerase activity has also been detected in mito-

chondria (Kalf (1964)) and chloroplasts (Kirk (1964)).

The properties of the comparatively crude mammalian preparation appear similar, insofar as they may be tested, to those of the enzyme purified from bacterial sources. DNA is required as a template, and incorporation is markedly stimulated by the presence of all four nucleoside triphosphates. Mn^{2+} and Mg^{2+} both stimulate the enzyme but Mn^{2+} to a higher level of incorporation. The reaction is markedly enhanced by the presence of ammonium sulphate but inhibited by actinomycin D, DNase and RNase.

The product of the DNA-dependent RNA polymerase reaction satisfies all the normal criteria for RNA and analysis of the product has shown it to consist of single-stranded polyribonucleotides of high molecular weight containing all four ribonucleoside residues linked by 3',5'-phosphodiester bonds (Hurwitz and August (1963) and Fox and Weiss (1964)).

DNA primers from a variety of sources have been employed. The base composition of the RNA product clearly reflects that of the DNA primer. This is best seen by the results obtained with poly dA-T primer where the product contains only the nucleotides to be expected if a base-pairing mechanism were concerned. The results, with single stranded DNA of ϕ X174 also show equivalence between dA and U, dT and A, dG and C and dC and G. The question of whether only one or both strands is copied has already been discussed under asymmetric synthesis in section 4.ii.

In the absence of any added template and after a variable lag period, RNA polymerase catalyses the formation of poly A and poly U from ATP and UTP respectively. The reaction appears to require Mn^{2+} specifically being inhibited if only Mg^{2+} is present. The homopolymer formation is also inhibited by the presence of any of the other ribonucleoside triphosphates (Smith, Ratliff, Williams and Martinez (1967)).

The mammalian RNA polymerase has not yet been purified significantly but that of E. coli has been prepared and found to have a sedimentation coefficient of 20s and a molecular weight of 600,000 (Doerfler, Zillig, Fuchs and Albers (1962)). Treatment by agents such as streptomycin or detergents causes dissociation of the enzyme into subunits which may or may not be of regular size (Colvill et al. (1966)). Fuchs et al. (1964) have studied highly purified preparations of RNA polymerase under an electron microscope and have shown that there are six subunits, linked side by side in such a way, that the native enzyme appears as a cylinder with a height of 95\AA , external diameter of 125\AA and internal diameter of 40\AA .

It has been found possible to saturate DNA from T7 bacteriophage with RNA polymerase. The amount required suggests that the saturation value cannot reflect occupation of the entire DNA surface with enzyme. Instead there must be a limited number of sites on the T7 molecule which can bind the enzyme (Richardson (1966)). Crawford

et al.(1965) and Stayter (1965) have calculated that there probably are 8 sites of attachment per polyoma DNA molecule.

Bonner et al.(1961) have found that newly synthesised RNA of pea seedling nuclei is bound to DNA and is resistant to RNase action. As judged by thermal dissociation data which showed the complex to melt at 50-60°C at any ionic strength investigated, the authors came to the conclusion that RNA is bound to DNA by a protein 'linker'.

Experiments involving actinomycin D have been carried out by Goldberg, Reich and Rabinowitz (1963) to examine the relationship between the binding site of actinomycin on DNA to the surface of the template on which the RNA polymerase functions. They find that actinomycin bound to DNA may displace some of the polymerase suggesting that some part of the actinomycin binding sites is occupied by the enzyme under normal circumstances. This would mean the enzyme would lie in the minor groove of the DNA molecule.

The nature of the binding sites remains obscure, but may be specific sequences of nucleotides in the DNA. The sites are likely to be similar throughout the bacterial world, since asymmetric synthesis of RNA has been observed in a number of heterologous enzyme-template systems (Colvill et al.(1965)). When the DNA template is heat denatured, the number of sites available for the initiation of RNA synthesis, as measured by the number of growing chains of RNA per DNA molecule, increases greatly (Maitra and Hurwitz (1965)).

Under these circumstances much shorter RNA chains are formed, loss of asymmetric transcription is observed, and it is considered that the selectivity of the attachment mechanism has been lost.

Fox et al. (1965) have shown that inhibition of RNA synthesis by tRNA involves the binding of the RNA to the polymerase. This inhibition by RNA may involve competition with DNA for the active site on the molecule of RNA polymerase. There is, however a separate site for product RNA, as the growing RNA chains and template DNA can remain attached to the polymerase simultaneously (Hayashi (1965) and Bremer et al. (1966)).

From the kinetics of inhibition of RNA polymerase by acridines at varying concentrations of DNA and ribonucleoside triphosphates Nicholson and Peacock (1966) concluded, that the inhibitor could combine reversibly with the polymerase and occupy sites with which both the free nucleotide bases and the DNA bases normally combine. There might thus be a case for considering three types of binding sites on the enzyme, one for DNA bases, one for RNA bases and one for free nucleotide bases.

Aims of the present work

Several purified systems have been mentioned which catalyse incorporation of ribonucleoside triphosphates into RNA. The most highly purified preparations have been of bacterial origin where there appears to be a lower degree of association between DNA and the RNA polymerase protein, than there is in mammalian cells. The aim of this present work was to investigate the possibility of freeing a mammalian RNA polymerase preparation from DNA. The mammalian source used was that of Landschutz ascites tumour cells.

METHODS AND MATERIALS

METHODS

1. The growth of Landschutz ascites tumour cells

The Landschutz ascites tumour was propagated by serial transplantation in Porton strain, albino mice from the departmental colony.

Tumour for transplantation was withdrawn aseptically by syringe from mice inoculated seven days previously. 0.3 ml. of this cell suspension, was inoculated intraperitoneally into young adult mice and was allowed to grow for seven days when about 4-5 ml. of tumour suspension was present in each mouse.

2. Preparation of the crude polymerase enzyme

Mice were killed by cervical dislocation, the skin of the abdomen was swabbed with absolute alcohol and the peritoneal wall exposed. Tumour was withdrawn with a syringe and transferred to a chilled 50 ml. conical centrifuge tube containing 10 ml. ice cold 0.9% sodium chloride to prevent clotting. The cells were washed in 0.9% saline to remove ascitic plasma and collected by centrifuging at 600g for 5 minutes at 0°C. Any erythrocytes contaminating the cells were removed by suspending the cells in 20 ml. 0.9% saline and centrifuging at 200g for 5 min. at 0°C when the heavier tumour cells sedimented and the erythrocytes in suspension could be removed by a Pasteur pipette. The tumour cells were washed again and the packed cells exposed to osmotic shock by suspending in 10 volumes of 0.01M-tris/HCl pH 7.5, 0.02M with respect to 2-mercapto-ethanol.

The suspension was allowed to sit in ice for 10 min. as this was found to minimise the force necessary to rupture the cells. The cells were ruptured by gentle homogenising for 1 minute in a pre-cooled Sireica homogeniser with a tightly fitting pestle. Disruption of the cells, but not nuclei was confirmed microscopically after staining with 1% crystal violet in 0.1M-citric acid. The suspension was centrifuged at 600g for 5 min. at 0°C and the supernatant cellular debris decanted. The pellet was washed in 5 volumes 0.9% saline and recentrifuged at 600g for 5 minutes at 0°C. The resulting pellet was suspended in three volumes 0.01M-tris/HCl pH 7.5, 0.02M with respect to 2-mercapto-ethanol and rapidly frozen by immersion in a bath of industrial spirit containing solid CO₂. No more than 4 ml. was used in any 50 ml. tube. The frozen suspension was rapidly thawed, This was achieved by dipping the tube in a bath of water at approximately 60°, removing it and agitating the suspension with a vortex mixer. This procedure was repeated until the suspension thawed, care being taken to expose the thawed nuclei to the minimum of heat. The freeze/thawing process was repeated two or three times until microscopic examination of samples stained with crystal violet showed that the thawed nuclei were disrupted. The suspension was then centrifuged at 10,000g for 10 min. at 0°. The supernatant fluid was discarded and the washed pellet routinely suspended in 4 volumes 0.01M-tris/HCl pH 7.5, 0.02M with respect to 2-mercapto-ethanol and stored in 2 ml. batches at -60°C until required.

It was found that the preparation could be stored in this way for at least 6 months with less than a 20% drop in RNA polymerase activity.

3. Enzyme assays

i. RNA polymerase assays

Polymerase activity was measured by a modification of the method of Weiss and Nakamoto (1961). The volume of the incubation mixtures was 0.6 ml. and the tubes, usually 10 ml. round bottomed centrifuge tubes, were incubated with shaking at 37°C. After incubation the reaction was terminated by freezing the tubes in a mixture of solid CO₂ and ethanol. The tubes could then be stored at -10° until required. Alternatively 5 ml. of 5% ice cold trichloroacetic acid were added and the samples processed immediately. All assays were performed in duplicate.

Preparation of samples for scintillation counting.

The usual procedure consisted of precipitating the RNA with 5% trichloroacetic acid, followed by repeated washing of the precipitate to remove unincorporated nucleotides. The level of any residual unincorporated nucleotides was measured by estimating the radioactivity in the unincubated samples.

The procedure employed is a modification of that described by Baltimore and Franklin (1963). All the operations were carried out at 4°. To the frozen tube was added 5 ml. of 5% ice cold trichloroacetic acid, 0.2 ml. of ice cold saturated sodium pyrophosphate and

0.5 ml. (2 mg./ml.) carrier yeast RNA or bovine serum albumin. The contents of the tube were mixed using a Vortex mixer and allowed to sit until fully thawed (20 min.). Where incubation mixtures were not frozen before processing, 0.2 ml. of ice cold saturated sodium pyrophosphate and 0.5 ml. of bovine serum albumin (2 mg./ml.), as carrier, were added directly to the samples following the addition of trichloroacetic acid. The samples were mixed, allowed to stand for 15 min., centrifuged at 600g for 15 min. and the supernatant fluid discarded.

The sediment so obtained was dissolved in 0.2 ml. of 0.5N-NaOH, then immediately precipitated with 5 ml. of cold trichloroacetic acid and 0.2 ml. sodium pyrophosphate as before. The tubes were mixed and allowed to stand for 10 min. for equilibration. The precipitates were collected by centrifugation for 10 min. at 600g. The pellets were resuspended in 5 ml. of trichloroacetic acid and 0.2 ml. of sodium pyrophosphate and after 10 min. recentrifuged at 600g for 10 min.. They were finally washed in 5 ml. of trichloroacetic acid and the subsequent drained pellets suspended in 0.2 ml. 98% formic acid. The samples were heated at 100° in a water bath for 30 min. to hydrolyse the RNA and to assist solution of the RNA and protein in the formic acid.

Measurement of radioactivity

2.9 ml. of absolute alcohol were added to the tritiated samples. The samples were mixed and added to scintillation vials containing

6.8 ml. of toluene based scintillation fluid. This fluid consists of 4 g. of P.P.O. (2,5 diphenyloxazole) and 200 mg. of P.O.P.O.P. (1,4-di-[2-(5-phenyloxazolyl)]-benzene) dissolved in 950 ml. of AnalaR toluene. The samples were then counted in either a Nuclear Chicago Model 725 liquid scintillation spectrometer, or a Packard series 4000 liquid scintillation spectrometer. The efficiency of counting was determined by the channels ratio method and ranged from 15-25% for tritium.

3.ii. RNA polymerase assay - 2nd method of preparation of samples for scintillation counting.

The precipitated RNA after incubation was sometimes collected and washed on a teflon coated glass fibre disc (diam. 2.5 cm. - Fiberfilm filter material) clamped in a Millipore filter holder. The sample treated with 0.2 ml. saturated sodium pyrophosphate and carrier, as before, was rinsed onto the disc with a further 10 ml. of cold 5% trichloroacetic acid. The material on the disc was washed with 10 ml. of cold 5% trichloroacetic acid containing 4% saturated sodium pyrophosphate and the disc was finally washed with 10 ml. absolute alcohol and allowed to dry. The dry disc was transferred to a scintillation vial and 0.5 ml. of hyamine hydroxide added. The vial was incubated at 57° for 20 min. and 10 ml. of the toluene scintillation fluid added. Radioactivity in the samples was determined as described above.

3.iii. DNA polymerase assay

This was assayed in a similar fashion. The assay volume was 0.25 ml. and the tubes were shaken in a water bath at 37°. The incubation was terminated by pipetting 0.05 ml. of the sample onto a Whatman No. 1 paper disc (2.0 cm.) previously impregnated with 0.05 ml. of (2 mg./ml.) bovine serum albumin and dried. The discs were washed in ice cold 5% trichloroacetic acid containing 4% saturated sodium pyrophosphate solution, taking about 15 ml. washing solution/disc. The discs were given 4 washes of about 10 min. and were then washed in alcohol and ether and allowed to dry. The samples were counted in 10 ml. toluene scintillation fluid as previously described.

3.iv. DNase assay

The method used depends on the degradation of DNA to acid soluble fragments which are estimated spectrophotometrically. The reaction mixture (0.2 ml.) containing 0.1 ml. calf thymus DNA (0.5 mg./ml.), 0.02 ml. tris/HCl buffer (1M pH 8.5), 0.02 ml. $MgCl_2$ (0.1M) and 0.06 ml. of the enzyme fraction to be assayed, was incubated at 37°C for 30 min. 0.2 ml. of carrier bovine serum albumin (2 mg./ml.) were added and 0.5 ml. of ice cold 1N-perchloric acid to precipitate nucleic acid and proteins. The sample was cooled at 4° for 5 min. and the precipitate removed by centrifugation. The supernatant was diluted with an equal volume of water and the optical density at 260 mμ measured.

3.v. RNase assay

This method depends on the degradation of yeast RNA to acid soluble fragments which are assayed spectrophotometrically. A solution of yeast RNA (2 mg./ml. in 0.9% NaCl) was prepared and dialysed for 24 hours against a large excess of 0.9% NaCl. The reaction mixture (1.0 ml.) containing 0.25 ml. of this RNA solution, 0.15 ml. M-tris/HCl buffer pH 8.5 and 0.6 ml. of the enzyme fraction to be assayed, was incubated for 30 min. at 37°. 0.5 ml. of ice cold 1N-perchloric acid was added to precipitate nucleic acid and proteins, which were removed by centrifugation in the cold. The supernatant fluid was diluted with 5 volumes of distilled water and the optical density at 260 mμ determined.

3.vi.

a. Alkaline phosphatase

The assay depends on the estimation of inorganic phosphate released from ribonucleoside triphosphates by phosphatase. The reaction mixtures (1.0 ml.) contained 1 μmole each of UTP, CTP, ATP and GTP, 2 μmoles MgCl₂, 0.6 ml. of the enzyme fraction to be assayed and was 0.1M with respect to tris/HCl buffer pH 7.5. Incubation was carried out at 37° for 30 min. after which 0.5 ml. 1N-perchloric acid was added. The precipitated nucleic acids and proteins were removed by centrifugation. The inorganic phosphate present in the supernatant fluid was measured by a modification of the method of Allen (1940) giving an estimate of phosphatase activity.

b. Inorganic phosphate

A sample of the solution to be assayed containing 4-30 $\mu\text{g.}$ phosphorus was pipetted into a test tube and 0.24 ml. of 10N-sulphuric acid were added, followed by 0.2 ml. 8.3% (W/V) solution of ammonium molybdate and 0.4 ml. 1% (W/V) solution of 2,4-diaminophenol hydrochloride (AnalaR) in 20% (W/V) sodium metabisulphite solution. The volume was made up to 5.0 ml. with water and the mixture allowed to stand for 10 min. at room temperature before the optical density was measured at 720 $\text{m}\mu$ against a reagent blank. A standard phosphorus solution was prepared by dilution of a stock phosphate standard (AnalaR - 2.193 g. KH_2PO_4 in 500 ml. water i.e. 1 ml. \equiv 1 mg. phos.).

3.vii. Micrococcal nuclease assay

The principle of the assay is again the measurement of optical density of acid soluble material released from nucleic acid by the enzyme. The reaction mixture consisted of 0.1 ml. of tris/HCl buffer (0.1M pH 8.8), 0.1 ml. DNA (2.0 mg./ml.), 0.05 ml. of calcium chloride (0.01M) and 0.1 ml. of the enzyme fraction. The tubes were incubated at 37° for 30 min. and the reaction stopped by adding 0.5 ml. 1N-perchloric acid. The tubes were chilled in ice for 10 min., 2.7 ml. of water were added and the precipitate was removed by centrifugation. The optical density of the supernatant samples was measured at 260 $\text{m}\mu$.

4. Preparation of DNA

DNA was prepared routinely from both calf thymus and Landschutz ascites tumour by a modification of the method of Kay, Simmons and Dounce (1952). The freshly excised calf thymus gland was frozen in solid CO₂/ethanol and stored at -60° until required. 50 g. of frozen thymus, broken into large pieces were blended at 4° with 200 ml. of 0.9% NaCl/0.01M-sodium citrate in a Waring Blendor. The homogenate was centrifuged for 15 min. at 600 g and the supernatant fluid discarded. The sediment was resuspended and the procedure repeated three times. The pellet from the last extraction was homogenised at 4° with 1 litre of 0.9% NaCl and the homogenate allowed to come to room temperature. To it were added with stirring 90 ml. of a 5% solution of sodium lauryl sulphate in 45% ($\frac{W}{V}$) ethanol. The mixture was stirred vigorously for 2 hours during which time the viscosity rose markedly. 55 g. of solid NaCl were added, bringing the NaCl concentration to 1M. When the NaCl had completely dissolved the material was centrifuged for 1 hour at 35,000 g. To the supernatant from this step 2 volumes of ice cold ethanol were added with stirring. The crude DNA came out of solution as a gelatinous mass which slowly lost water to form a white fibrous precipitate which was gathered by winding round a glass stirring rod. The precipitate was removed from the glass rod, washed three times with absolute ethanol, three times with acetone and dried.

The DNA was purified further. The crude preparation was cut

into small pieces with scissors and added to 700 ml. of 0.0001 M-NaCl. The suspension was stirred at room temperature until all the pieces had dissolved, when 63 ml. of 5% sodium lauryl sulphate in 45% ethanol were added and stirring continued for an hour. 45 g. of solid NaCl were then added, bringing the NaCl concentration to 1M. After complete solution of the NaCl the mixture was centrifuged at 35,000g for 30 min. The supernatant was treated as before and the precipitate dried. This step was repeated until there was no visible protein precipitated when NaCl added; usually twice.

DNA from ascites tumour cells was prepared in a similar fashion but usually on a smaller scale. The tumour cells were removed and washed nuclei prepared as already described. These nuclei were then homogenised in 5 volumes 0.9% NaCl and 9 ml./100 ml. suspension, 5% sodium lauryl sulphate added as before. The same steps as already described when using calf thymus were then carried out, scaling down the quantities as required.

5. Measurement of the 'melting temperature' of DNA

The sample of DNA to be examined was prepared in 0.01M-tris/HCl buffer pH 7.5 and the optical density measured at 260 m μ against a blank containing the other constituents of the system in tris/HCl e.g. MPB and DMS. The sample was diluted to give around 0.3 optical density units/ml. The spectrophotometer used was the SP800 (Unicam) with the temperature controlled cell accessory (SP870). During heating the temperature was noted on the chart at 2° intervals. The

temperature at the mid-point of the increase in absorbancy is the T_m or 'melting temperature' of the DNA.

6. Preparation of RNA from incubation mixtures

50 ml. of RNA polymerase incubation mixture were prepared and incubated in a round bottomed flask at 37° in a shaking water bath for 30 minutes.

RNA was extracted from this by a modification of the method described by Penman (1966). All the samples were chilled in ice before fractionation.

The solution containing the RNA to be extracted was added to a chilled tube containing 0.05 ml. of washed 4% bentonite suspension. The solution was made 1% with respect to sodium dodecyl sulphate (SDS) and allowed to stand for 5 min.. One volume of a 90% aqueous solution of redistilled phenol containing 0.1% 8-hydroxy-quinoline, was added. The mixture was shaken vigorously at room temperature for 5 minutes. One volume (with respect to the original RNA solution) of chloroform containing 1% isoamyl alcohol was then added and the mixture shaken vigorously for a further 5 minutes.

The solution was then resolved into an aqueous phase and a phenol-chloroform phase by centrifugation at $1500 \times g$ for 5 minutes. The phenol-chloroform layer was discarded and any interphase material retained, and the phenol-chloroform extraction was repeated. The extraction of the aqueous phase was repeated another two times, using the chloroform-isoamyl alcohol mixture alone.

The RNA was precipitated with a 1% solution of cetyl-triammonium bromide and the precipitate collected by centrifugation at 20,000g for 15 min.. The precipitate was washed three times with 70% ethanol containing 0.1M-sodium acetate to convert the RNA into the sodium salt.

7. Density gradient centrifugation

These were carried out in varying concentrations of sucrose or glycerol. A mixing device (Britten, Roberts (1960)) was used to deliver 4.5 ml. of a solution of sucrose in water linearly graded from 40% (W/V) to 5% (W/V). 0.5 ml. of enzyme preparation were layered on the gradient and the tubes were centrifuged in the swinging bucket rotor (SW 39) of a Spinco Model L preparative ultracentrifuge for varying times and speeds (e.g. Table 4).

Around 30 fractions were collected from each tube by puncturing the base with a hypodermic needle and allowing the contents to drip out into the test tubes which contained 0.5 ml. of water. The optical density of the fractions was measured at 260 and 280 mμ.

Experiments were run using bands of sucrose or glycerol of graded densities e.g. 1 ml. of 50% sucrose was placed at the foot of a 5 ml. centrifuge tube, followed by 1 ml. of 40%, then 1 ml. of 30% and lastly 1 ml. of 20% solution. 0.5 ml. sample would then be layered on top as before. The tubes were then centrifuged and fractionated as described in Table 4.

8. Preparation and use of DEAE cellulose

The cellulose was added with stirring to three times its own volume of 0.5 N-NaOH. The suspension was stirred gently as vigorous stirring was found to break up otherwise stable agglomerates releasing very fine particles which tend to clog a column. Stirring was stopped after 30 min. and the cellulose allowed to settle for 10 min.. The supernatant liquid was removed by suction together with any fine particles that had remained in suspension. The alkali washing was repeated until the supernatant liquid was colourless. The cellulose was then suspended in water, stirred and allowed to settle as before. The supernatant liquid and 'fines' were again removed by suction. This was repeated until the supernatant liquid was relatively free of 'fines'. The suspension was then transferred to a Buchner funnel and washed with water until free of alkali. The cellulose was resuspended in 0.1 N-HCl which was removed by filtration and the cellulose washed to pH 7.

Finally the washed cellulose was suspended in either 1M-K phosphate buffer pH 7.5 or 0.3M-K phosphate buffer/30% with respect to glycerol (AnalaR) and allowed to equilibrate at 4° overnight.

Various sizes of column were used. All were poured and run at 4°. Addition of the crude polymerase preparation to the top of a column in the normal manner was found to reduce the flow rate. However if the sample to be fractionated on the column (about 4 ml.) was mixed with the cellulose prior to pouring the column and the

slurry allowed to sediment under gravity, the flow rate could be maintained at 1 ml./min.. The column was then eluted with approximately ten bed volumes of 0.3M-K phosphate buffer pH 7.5. Various concentrations (from 0.05M to 1.0M) of KCl in 0.3M-K phosphate were then passed through the column. The volume of the fractions collected varied with the column used. The fractions containing material absorbing at 280 mμ were assayed for RNA polymerase and protein. Each column was only used once.

After use the cellulose was removed from the column and stored under water saturated with toluene to inhibit bacterial growth. When a sufficient quantity had been collected the whole batch was regenerated by the procedure described above.

Experiments were also run using DEAE cellulose in 0.3M-K phosphate buffer pH 7.5/30% with respect to glycerol. Usually because of extremely slow flow rates the polymerase preparation was mixed with a small quantity of cellulose (5 ml. packed volume) and after equilibration the cellulose removed by centrifugation at 600g for 5-10 min.. The cellulose would then be resuspended in fresh buffer and the procedure repeated. The supernatant fractions were assayed for RNA polymerase and protein.

9. Estimation procedures

a. Protein estimation

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951).

Reagent A contained 2% (W/V) Na_2CO_3 in 0.1N-NaOH. Reagent B contained 0.5% (W/V) $CuSO_4 \cdot 5H_2O$ in 1% (W/V) sodium potassium tartrate. Reagent C was an alkaline copper solution (50 ml. reagent A were mixed with 1 ml. reagent B). Reagent D consisted of a diluted Folin reagent (Folin-Ciocalteu phenol reagent was titrated to a phenolphthalein end-point and diluted to make it 1N in acid).

To 1 ml. of a suitable dilution of the protein solution (50 $\mu g./$ ml.) 5 ml. reagent C was added and after 10 min., 0.5 ml. reagent D was rapidly added. The mixture was allowed to stand for at least 30 min. and was read against a reagent blank in a Unicam SP 600 spectrophotometer at 750 $m\mu$. A standard solution of bovine serum albumin (0-500 $\mu g./ml.$) was used to calibrate the assay.

b. Estimation of DNA

DNA was estimated by the Burton procedure (Burton (1956)). diphenylamine reagent was prepared by dissolving 1.5 g. of AnalaR diphenylamine in 100 ml. of glacial acetic acid and adding 1.5 ml. of concentrated sulphuric acid. Immediately before use, 0.1 ml. of a solution of 16 mg. of redistilled acetaldehyde (b. p. 21°) in 1 ml. water was added.

1 ml. of the sample to be assayed containing about 50 $\mu g./ml.$, (in 1N-perchloric acid) was added to 2 ml. of the diphenylamine reagent. The sample was mixed and incubated at 30° overnight. The optical density of the solution was measured at 600 $m\mu$ against a

reagent blank. A standard solution of DNA (0-200 μ g. DNA/ml.) was used for calibration.

10. Materials

Adenine, guanine, cytosine, uracil, thymine, the ribonucleosides, deoxyribonucleosides, ribonucleoside 5'-mono- and triphosphates and deoxyribonucleoside 5'-mono- and triphosphates were purchased from Sigma Chemical Company or Calbiochem. [^3H]ribonucleoside 5'-triphosphates were obtained from Schwartz Bioresearch Inc., Orangeburg, N.Y.. Crystalline pancreatic ribonuclease and deoxyribonuclease were purchased from the Sigma Chemical Company. Actinomycin D was a gift from Merck, Sharp and Dohme, Inc.. Phosphocreatine kinase, phospho-enol-pyruvate and pyruvate kinase were purchased from the Sigma Chemical Company. MPB was a gift from I.C.I. (Pharmaceuticals division). Calsolene oil was a gift from I.C.I. (Dyestuffs Division, Grangemouth). Of the materials used in scintillation counting, teflon coated glass fibre paper (Fiberfilm material) was purchased from Joymar Scientific Incorp., New York and Hyflo-Super-Cel, P.P.O. and P.O.P.O.P. from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

R E S U L T S

I. Crude RNA Polymerase preparation

i. Investigation of conditions of assay

Studies by Burdon and Smellie (1962) have demonstrated the existence in Landschutz ascites tumour cells of RNA-dependent and DNA-dependent systems for incorporation of ribonucleotides into RNA. Experiments were carried out on a crude nuclear preparation which was shown to possess DNA-dependent RNA polymerase activity. The system was supplied with equimolar amounts of ribonucleoside 5'-triphosphates, DNA as a template, Mn^{2+} ions and ammonium sulphate. Initially an ATP generating system, phosphocreatine and phosphocreatine kinase was included in the reaction mixture.

Fig. I shows the time course of incorporation of $[^3H]$ UMP into acid insoluble material by the crude enzyme preparation. This was assumed to represent predominantly RNA polymerase activity. The rate of incorporation appears to be relatively constant up to 30 min. and incubations were usually carried on for 30 min. or 60 min. The relationship between activity and protein concentration is shown in Fig. 2 from which it can be seen that this is almost linear over the range 50 $\mu g.$ to 500 $\mu g./assay$.

The requirements of the enzyme were investigated with respect to Mn^{2+} and Mg^{2+} ions, in the presence and absence of ammonium sulphate. A summary of the results is shown in Fig. 3. In the absence of ammonium sulphate there is a marked reduction in nucleotide incorporation. Mn^{2+} ions appear to cause a greater stimulation

FIGURE 1

The time course of the incorporation of $[^3\text{H}]$ UMP into
RNA by the crude ascites enzyme

- A. zero to 15 min.
- B. zero to 240 min.

The reaction mixture contained 50 μ moles tris-HCl buffer, pH 7.5; 1 μ mole MnCl_2 ; 6 μ moles 2-mercapto-ethanol; 100 μ moles EDTA; 80 μ g. native Landschutz DNA; 240 μ moles $(\text{NH}_4)_2\text{SO}_4$; 100 μ moles each of ATP, GTP, CTP and UTP; $[^3\text{H}]$ UTP (1×10^7 d.p.m./ μ mole) and 0.1 ml. of enzyme (3 mg. protein/ml.) in a total volume of 0.6 ml.

1.4 μ moles phosphocreatine and 70 μ g. phosphocreatine kinase were added as energy source in the 0.6 ml. reaction mixture.

The reaction was carried out at 37° .

FIGURE 1.

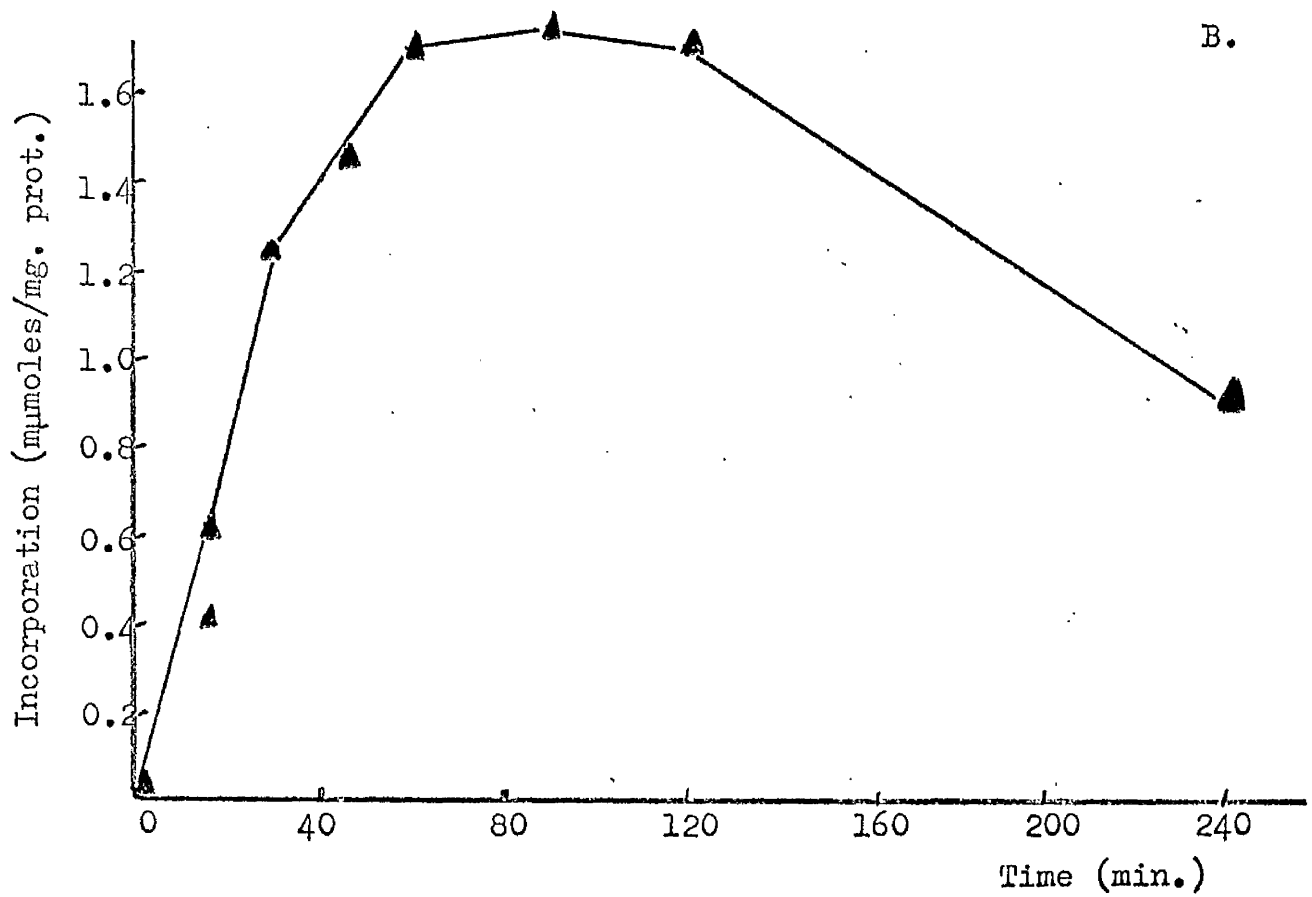
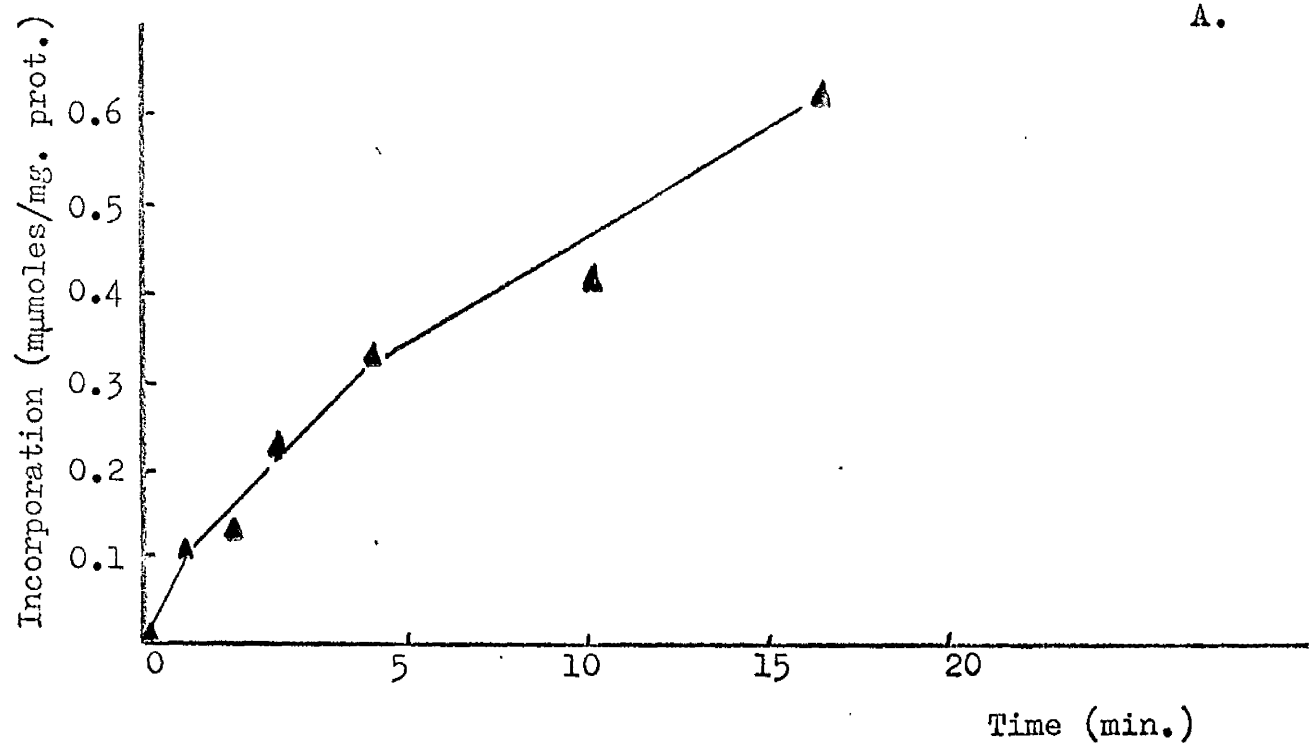


FIGURE 2

The effect of enzyme protein concentration on the
incorporation of $[^3\text{H}]$ UMP into RNA by the crude RNA polymerase

The reaction mixture was identical to that used in Figure 1 except that the concentration of enzyme protein added, was varied as shown.

The samples were incubated at 37° for 30 min.

FIGURE 2.

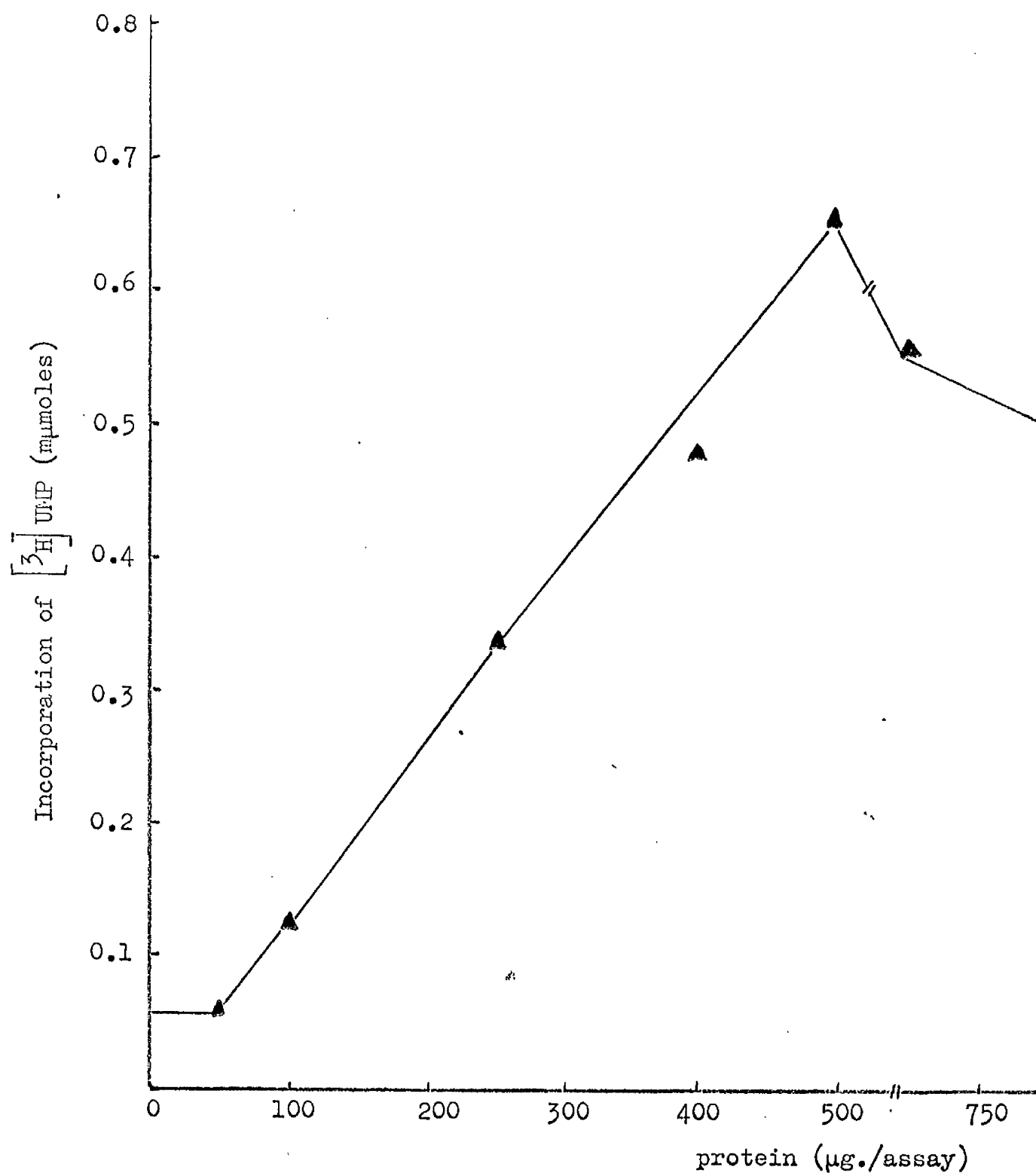


FIGURE 3

The effect of various ions on the crude ascites

RNA polymerase preparation

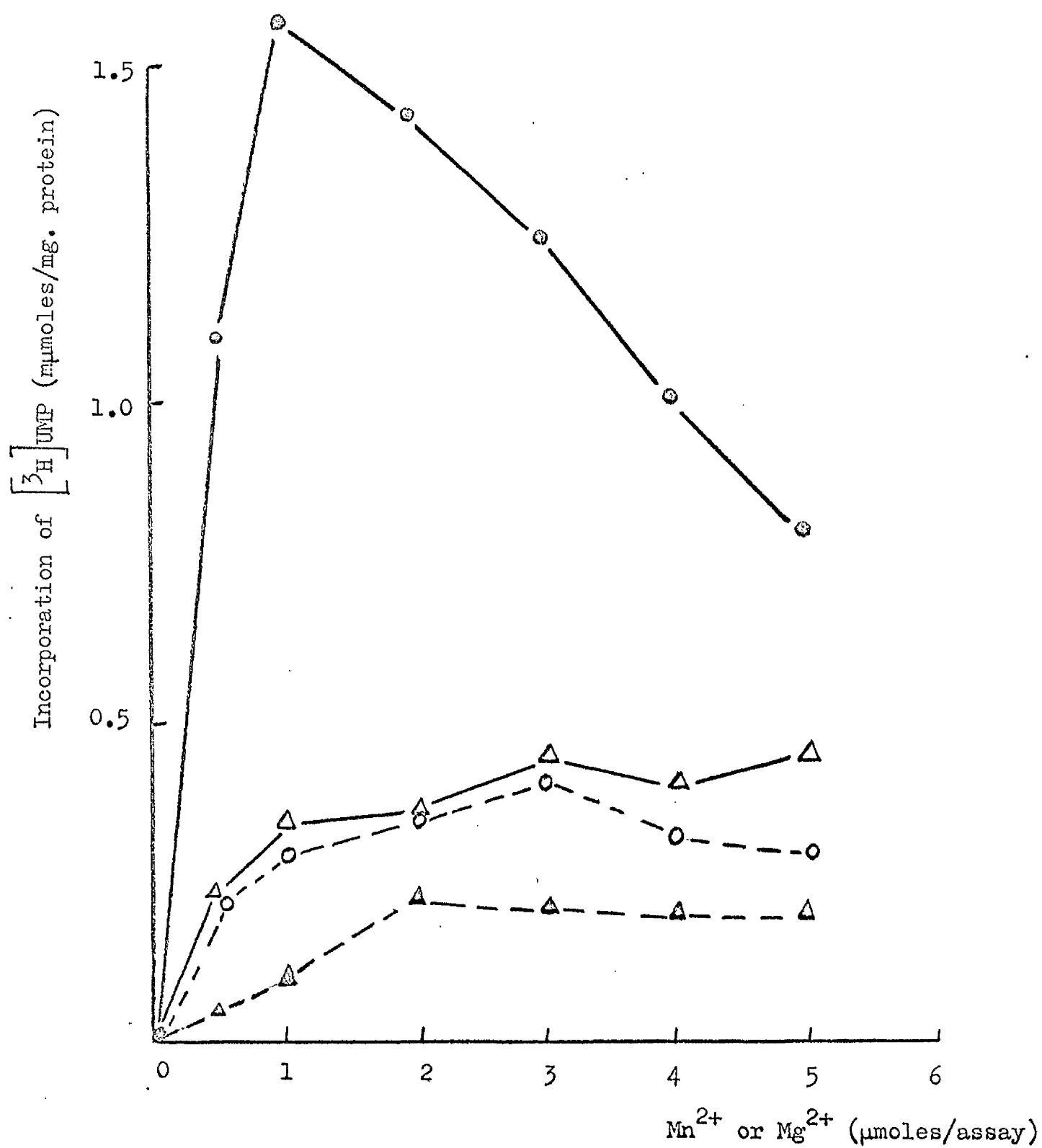
- 1) The effect of addition of Mn^{2+} or Mg^{2+} to the assay, in the presence and absence of ammonium sulphate.

The reaction mixture of 0.6 ml. contained 50 μ moles tris-HCl buffer, pH 7.5; 6 μ moles 2-mercapto-ethanol; 100 μ moles of EDTA; 80 μ g. native Landschutz DNA; 100 μ moles each of ATP, GTP, CTP and UTP; $[^3H]$ UTP (1×10^7 d.p.m./ μ mole) and 0.1 ml. of enzyme (3 mg. protein/ml.). Phosphocreatine and phosphocreatine kinase were added as in Figure 1.

| | |
|-----------|---|
| —●— | contained in addition varying amounts of Mn^{2+} as shown, and 240 μ moles $(NH_4)_2SO_4$ |
| —△— | contained in addition varying amounts of Mg^{2+} as shown, and 240 μ moles $(NH_4)_2SO_4$ |
| - - 0 - - | contained in addition varying amounts of Mn^{2+} as shown |
| - - ▲ - - | contained in addition varying amounts of Mg^{2+} as shown. |

The samples were incubated at 37° for 30 min.

FIGURE 3.



of enzyme activity than Mg^{2+} , with an optimum of 3 $\mu\text{moles/assay}$. In the presence of 0.4M-ammonium sulphate, the maximum activity on addition of Mn^{2+} ions was about three times that observed with Mg^{2+} ions, the optimum concentration of Mn^{2+} occurring at about 1 $\mu\text{mole/assay}$. This led to an investigation of incorporation of ribonucleoside 5'-triphosphates into RNA in the presence of varying concentrations of $(\text{NH}_4)_2\text{SO}_4$ (Fig. 4) with the Mn^{2+} concentration maintained at 1 $\mu\text{mole/assay}$. The graph shows a maximal incorporation at 240 $\mu\text{moles } (\text{NH}_4)_2\text{SO}_4/\text{assay}$. Since it seemed possible that these changes were caused by alterations in the ionic strength of the reaction mixture various substitutes for $(\text{NH}_4)_2\text{SO}_4$ were tested. Fig. 5 shows that at an ionic strength of 0.4M-ammonium sulphate produced greater stimulation than Na_2SO_4 , or 0.8M- NH_4Cl , - NaCl or - KCl .

The optimum pH of the reaction was examined, using triethanolamine/HCl buffer. As can be seen from Fig. 6 this lies in the region of pH 7.5. The effects of EDTA on this system were also examined and the activity at various concentrations is shown in Fig. 7. It can be seen that the maximal activity occurred at a concentration of 0.1 $\mu\text{mole/assay}$ and this concentration was employed in subsequent assays. The addition of 2-mercapto-ethanol up to 6 $\mu\text{moles/assay}$ was found to increase the incorporation of ribonucleotides (Fig. 8). 6 $\mu\text{moles per assay}$ were normally added in subsequent experiments. Care had to be taken to use freshly prepared solutions of 2-mercapto-ethanol as it readily became oxidised in dilute

FIGURE 4

The effect of various ions on the crude ascites

RNA polymerase preparation

- 2) The effect of varying concentrations of $(\text{NH}_4)_2\text{SO}_4$ in the presence of Mn^{2+} .

The reaction mixture contained 50 μmoles tris-HCl buffer, pH 7.5; 1 μmole MnCl_2 , 6 μmoles 2-mercapto-ethanol, 100 μmoles EDTA; 80 $\mu\text{g.}$ native Landschutz DNA; 100 μmoles each of ATP, GTP, CTP and UTP; $[\text{}^3\text{H}]$ UTP (1×10^7 d.p.m./ μmole) and 0.1 ml. of enzyme (3 mg. protein/ml.) in a total volume of 0.6 ml.. Phosphocreatine and phosphocreatine kinase were added as in Fig. 1.

Various concentrations of $(\text{NH}_4)_2\text{SO}_4$ were added as indicated.

The samples were incubated at 37° for 30 min.

FIGURE 4.

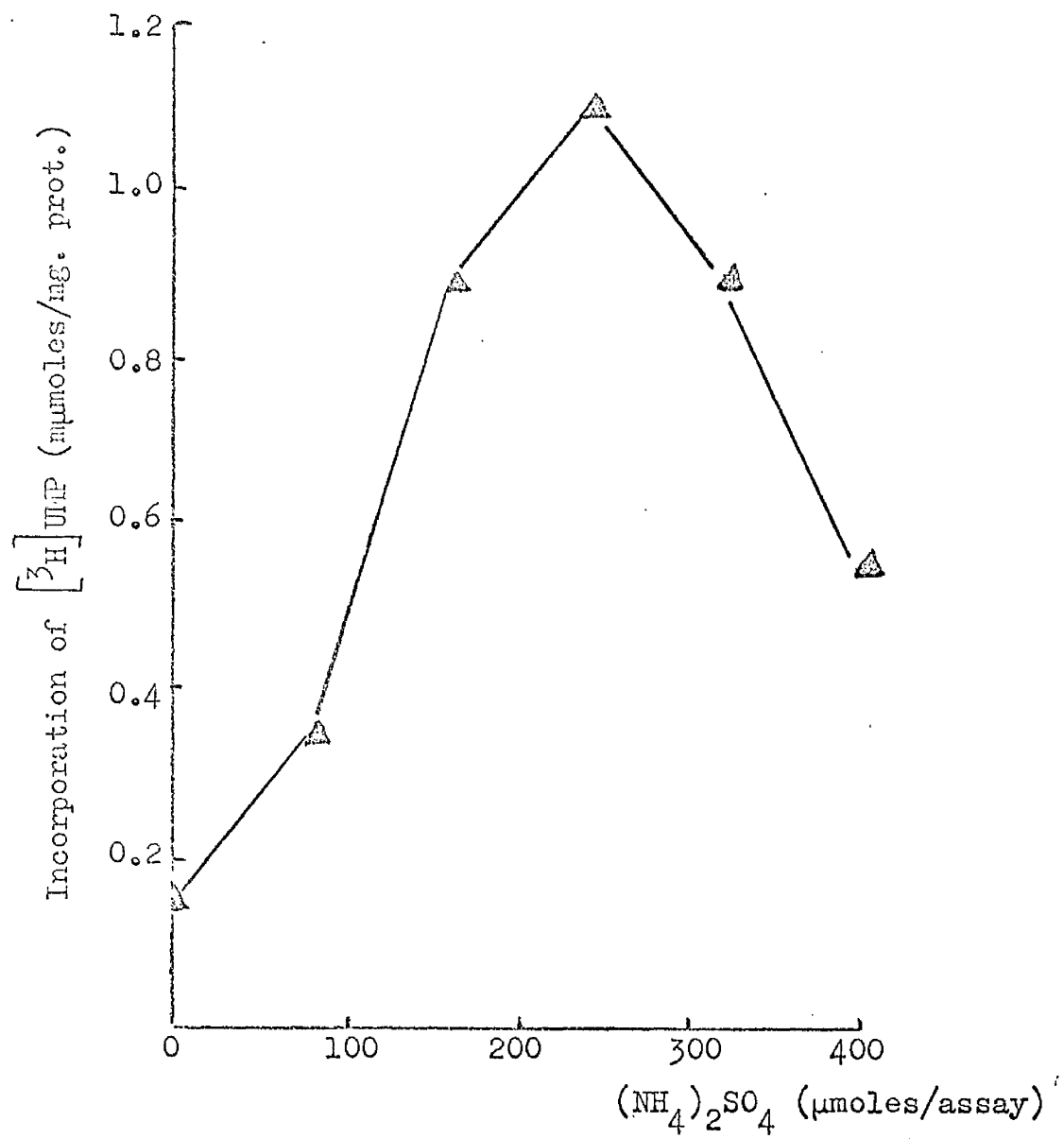


FIGURE 5

The effect of various ions on the crude ascites

RNA polymerase preparation

- 3) Various ions were tested as replacements for $(\text{NH}_4)_2\text{SO}_4$, in the presence of 1 μmole of Mn^{2+}

The reaction mixture contained 50 μmoles tris-HCl buffer, pH 7.5; 1 μmole MnCl_2 ; 6 μmoles 2-mercapto-ethanol; 100 μmoles EDTA; 80 $\mu\text{g.}$ native Landschutz DNA; 100 μmoles each of ATP, GTP, CTP and UTP; $[^3\text{H}]$ UTP (1×10^7 d.p.m./ μmole); 1.4 μmoles phosphocreatine and 70 $\mu\text{g.}$ phosphocreatine kinase, and 0.1 ml. of enzyme (3 mg. protein/ml.) in a total volume of 0.6 ml.

240 μmoles of $(\text{NH}_4)_2\text{SO}_4$ or Na_2SO_4 ; 480 μmoles of KCl, NH_4Cl or NaCl were added as indicated.

The samples were incubated at 37° for 30 min.

| | | | |
|-------|---|-------|-----------------------------------|
| — ■ — | represents $(\text{NH}_4)_2\text{SO}_4$ | — □ — | represents NH_4Cl |
| — ▲ — | represents Na_2SO_4 | — △ — | represents NaCl |
| — ● — | represents KCl | | |

FIGURE 5.

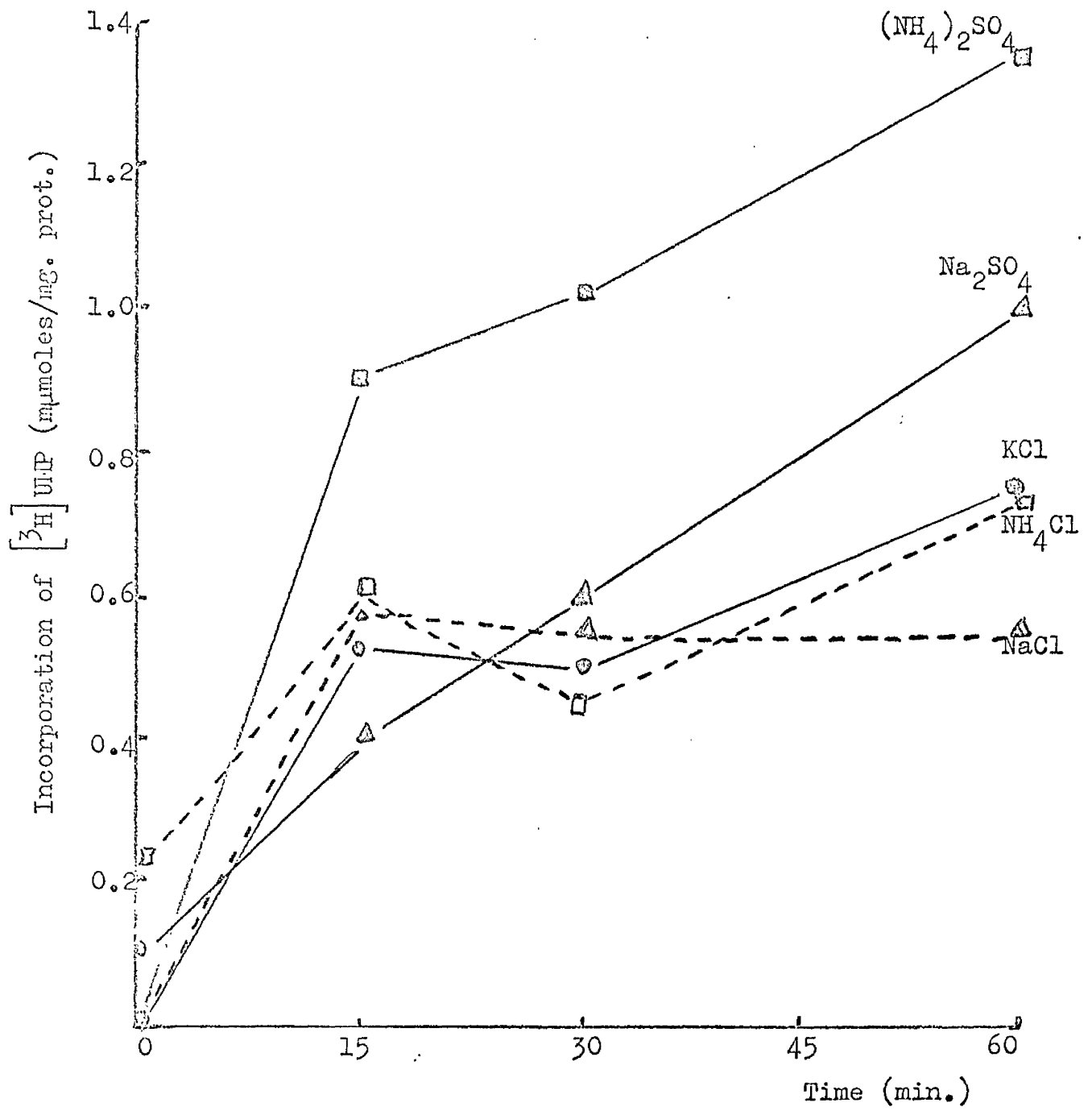


FIGURE 6

The effect of pH on the crude RNA polymerase activity

The reaction mixture was identical with that described in Fig. 1 except that the pH of the triethanolamine buffer used was varied as indicated.

The samples were incubated at 37° for 30 min.

FIGURE 6.

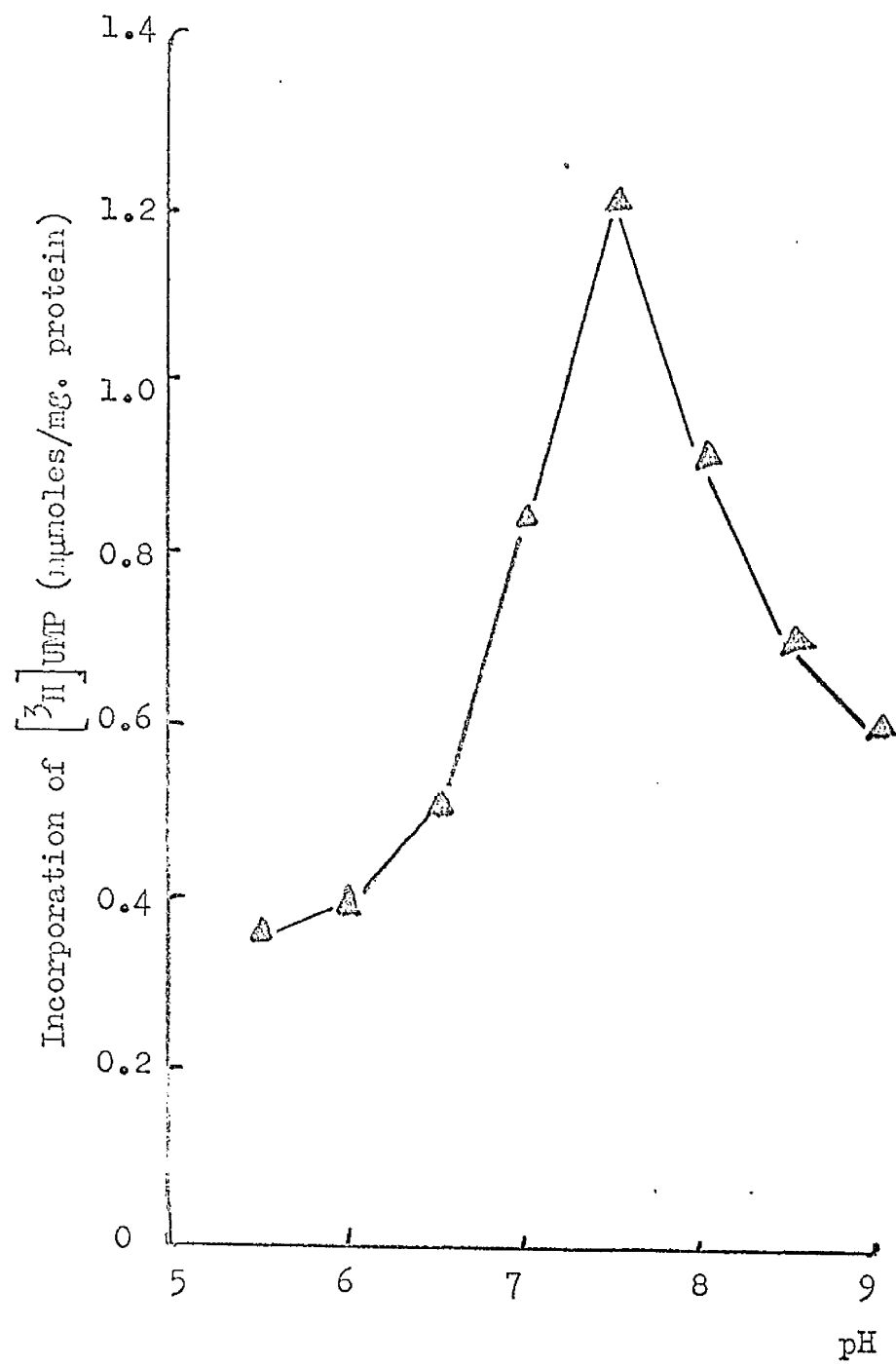


FIGURE 7

The effect of varying EDTA concentrations on
the crude ascites RNA polymerase activity

The reaction mixture was identical with that described in Fig. 1 except that the concentrations of EDTA were varied as indicated.

The samples were incubated at 37° for 30 min.

FIGURE 7.

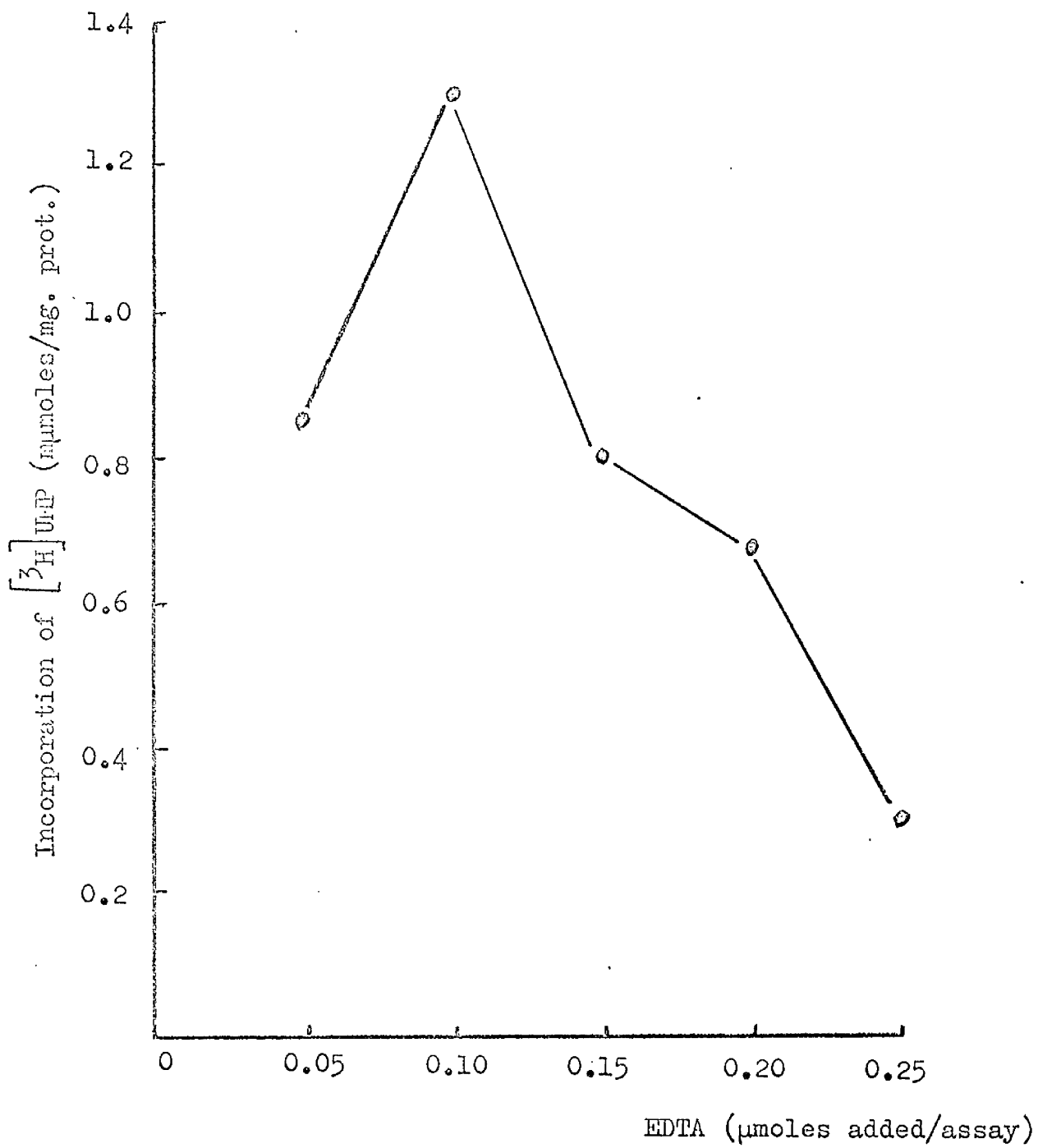


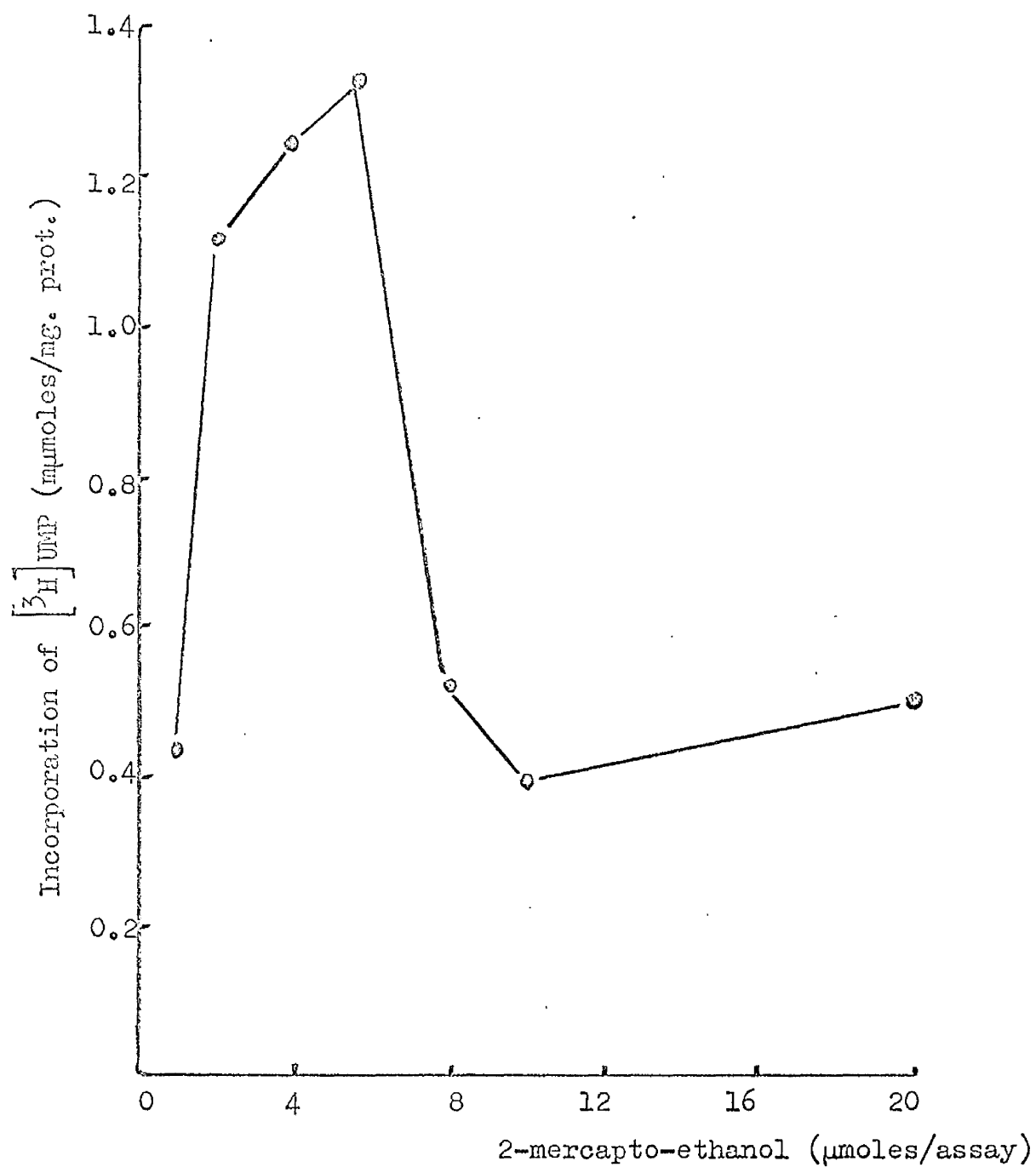
FIGURE 8

The effects of varying the concentration of 2-mercapto-ethanol
present on the activity of the crude ascites RNA polymerase

The reaction mixture was identical with that described in Fig. 1 except that the concentrations of 2-mercapto-ethanol added were varied as indicated.

The samples were incubated at 37° for 30 min.

FIGURE 8.



solution.

ii. Enzyme stability

The specific activity of the enzyme was reduced when the enzyme was incubated at 20° or 37° for varying times before assay. Fig. 9 shows the time course of incorporation following prior incubation under the above conditions. There is a small decrease in activity after incubation at 20° for 30 min., but at 37° the decrease is much more marked. Even when stored at 4° the specific activity was found to decrease appreciably with time (Fig. 10). In these experiments the incubation time was constant at 30 min. Activity is stabilised however when the preparation was stored in 0.02M-2-mercapto-ethanol at -60°C (Fig. 11). Some preparations were stored for up to six weeks in 30% glycerol at -60°C without loss of activity.

II. Fractionation of RNA polymerase

In the crude polymerase preparation the active protein is in aggregate form. This proved a major difficulty during subsequent fractionation. The activity of the enzyme in various concentrations of tris/HCl and potassium phosphate buffers is shown in Table 1. The preparation becomes soluble under conditions of high ionic strength. 0.3M-K phosphate pH 7.5 was used in a number of subsequent experiments.

i. Pancreatic DNase I

The enzyme appears to be very firmly bound to the DNA in the

FIGURE 9

The effects of a prior incubation of
the crude ascites RNA polymerase

The reaction mixture was identical with that described in Fig. 1. The samples were given a prior incubation as shown. The ribonucleoside triphosphates were then added and the time course of the subsequent incorporation at 37° followed.





| | |
|---|--|
|  | represents control (no prior incubation) |
|  | represents 30 min. at 20.5° |
|  | represents 15 min. at 37° |
|  | represents 30 min. at 37° |

FIGURE 9.

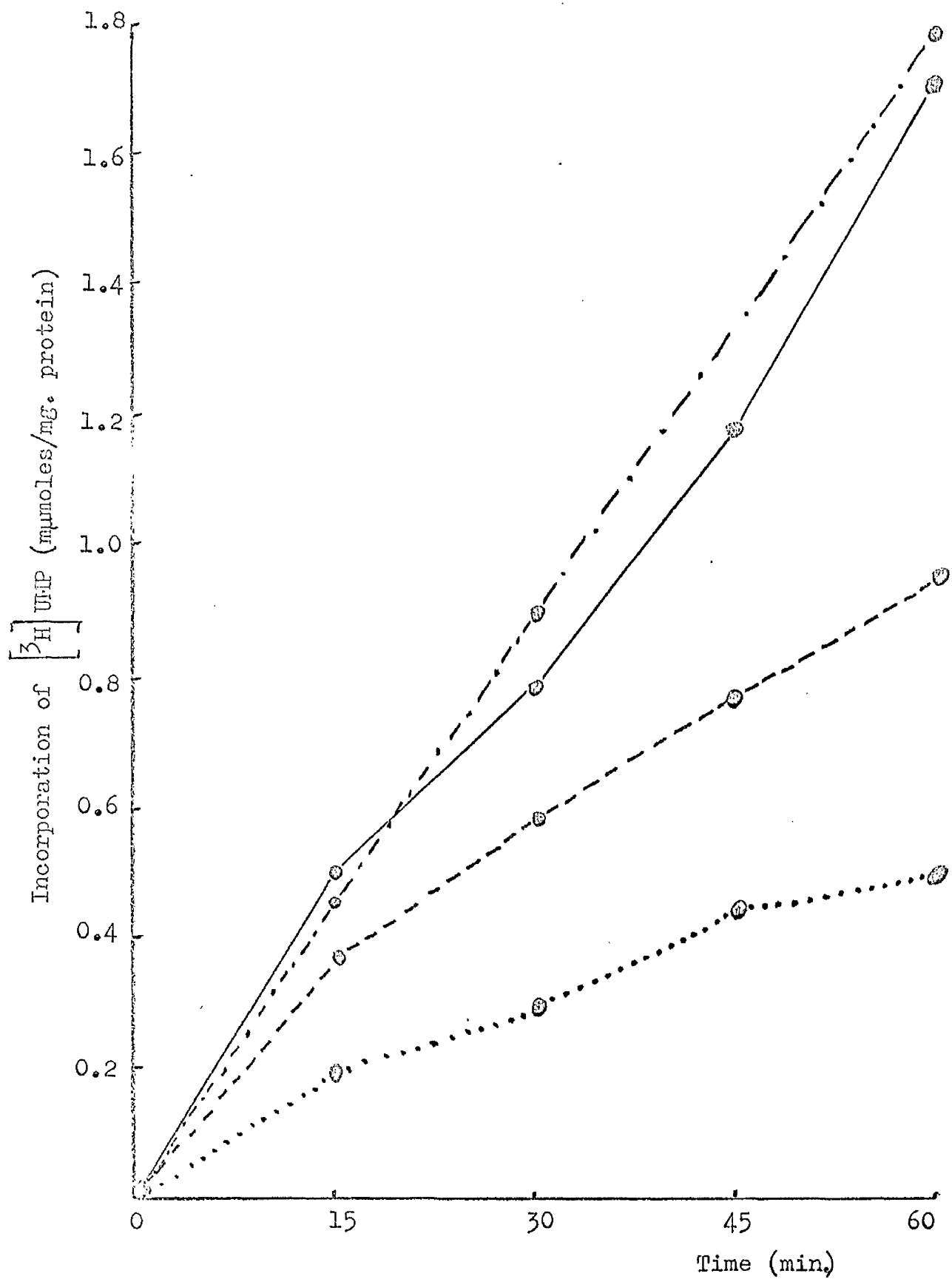


FIGURE 10

A study of storage conditions of the crude RNA polymerase

a) at 4°C.

The crude preparation was stored for varying times at 4°C, prior to assay. The reaction mixture used was identical to that described in Fig. 1.

The samples were incubated at 37° for 30 min.

FIGURE 10.

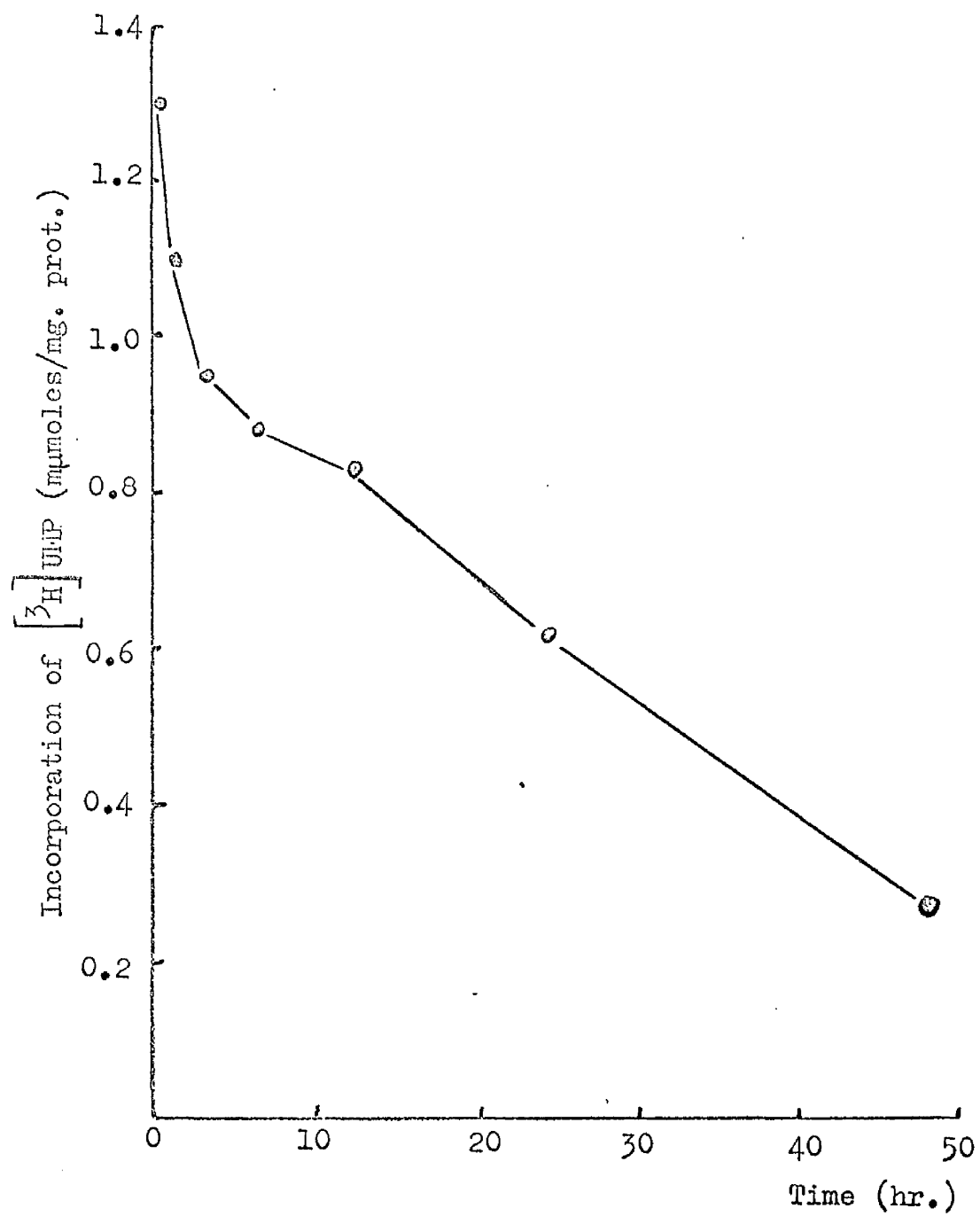


FIGURE 11

A study of storage conditions of the crude RNA polymerase

b) at -60°C .

The crude preparation was stored for varying times at -60°C , prior to assay. The reaction mixture used was identical to that described in Fig. 1.

The samples were incubated at 37° for 30 min.

FIGURE 11.

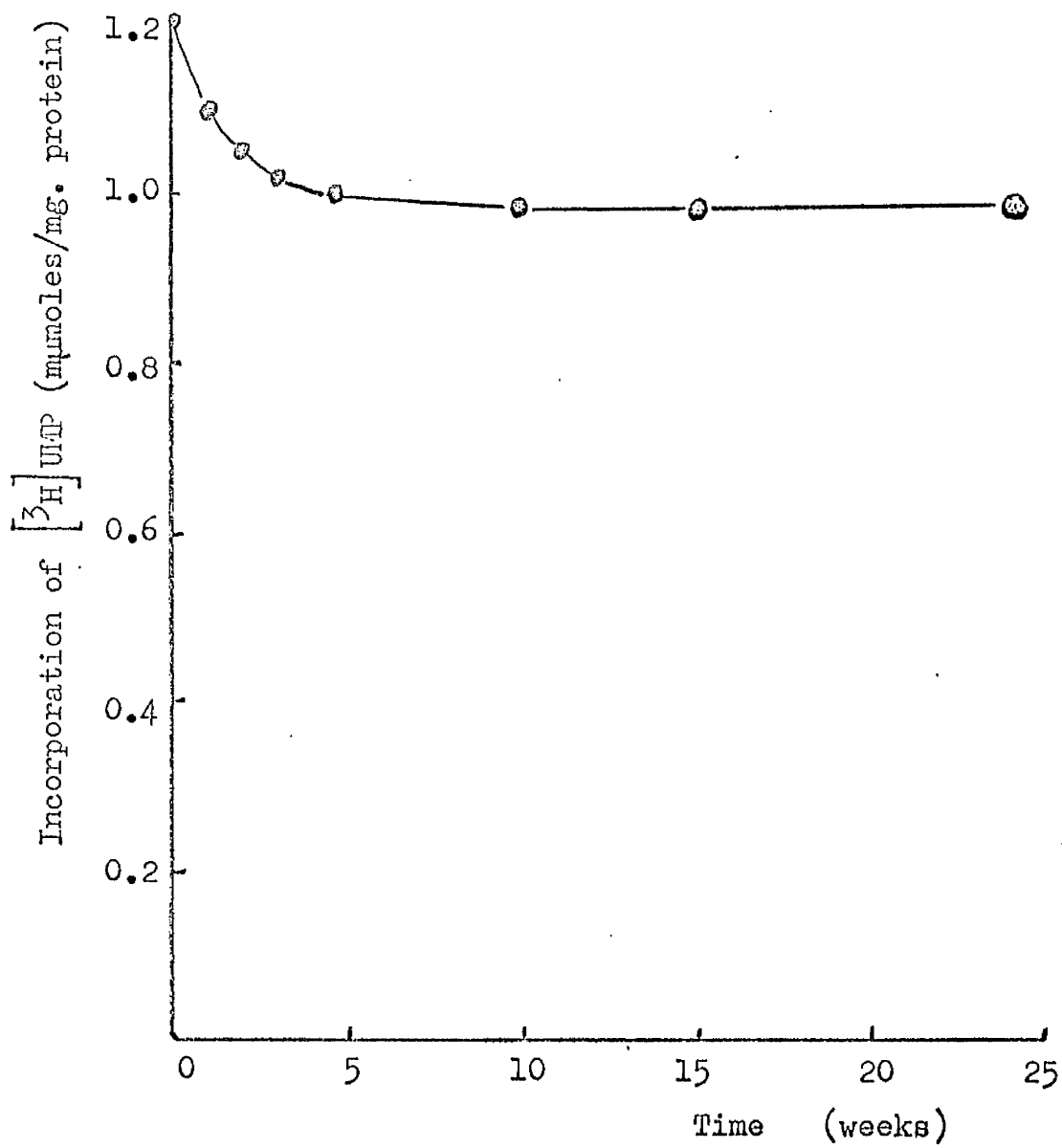


TABLE 1.

The effects of various concentrations of tris/HCl and phosphate buffers at pH 7.5, on the crude RNA polymerase

| Buffer pH 7,5 | Incorporation μmoles [³ H] UMP/mg. prot. | Description of System |
|-------------------|---|---|
| tris/HCl 0.01M | 1.25 | Aggregate material in suspension. |
| tris/HCl 0.1M | 1.27 | Aggregate material in suspension. |
| tris/HCl 0.3M | 1.51 | Rise in viscosity apparent solution. |
| tris/HCl 0.5M | 1.68 | Viscous solution. |
| tris/HCl 1.0M | 1.09 | Viscous solution. |
| phosphate 0.01M | 1.32 | Aggregate material in suspension. |
| phosphate 0.1M | 1.35 | Aggregate material in suspension. |
| phosphate 0.3M | 1.84 | Viscous solution. |
| phosphate 0.5M | 1.75 | Viscous solution. |
| phosphate 1.0M | 0.98 | Viscous solution. |

The crude enzyme pellet was suspended in the appropriate buffer to give a protein concentration of 3 mg./ml.. The phosphate buffer used was prepared from AnalaR KH_2PO_4 and AnalaR KOH.

The treated enzyme was assayed as in Fig. 1.

The samples were incubated at 37° for 30 min.

preparation. Experiments were carried out to determine whether the DNA could be removed by treatment with DNase without destroying the polymerase. Various conditions of digestion were investigated. Usually 100 μ g. DNase were added for each 3 mg. of DNA estimated to be present. Fig. 12 shows the release of nucleotides with time at different temperatures. Incubation at 0° reaches maximal digestion at about 24 hr.. A balance had to be drawn between optimum conditions for DNase attack and preservation of the polymerase activity. Normally 10 min. at 37° was chosen.

Having treated the crude polymerase preparation with DNase an attempt was made to separate out the DNase, polynucleotide and active polymerase fractions. The RNA polymerase was still in an aggregate form. Initially the products of hydrolysis were filtered through Whatman No. 42 filter paper on a ground glass Buchner funnel to remove large particulate material. Various other filters, including Millipores of pore size 0.45 μ 2.5 cm. diam., were tested but either the filtrate was cloudy or the filter was soon clogged. The solid material once removed from the filter and suspended in 0.01M-tris pH 7.5/0.02M with respect to 2-mercapto-ethanol, showed an increased dependence on DNA but a marked decrease in activity. This suggested that DNase was being retained on the filter paper. In order to test the retention of DNase by the preparation a washing procedure was devised. The crude preparation after treatment with DNase was centrifuged at 10,000g for 10 min.. The supernatant

FIGURE 12

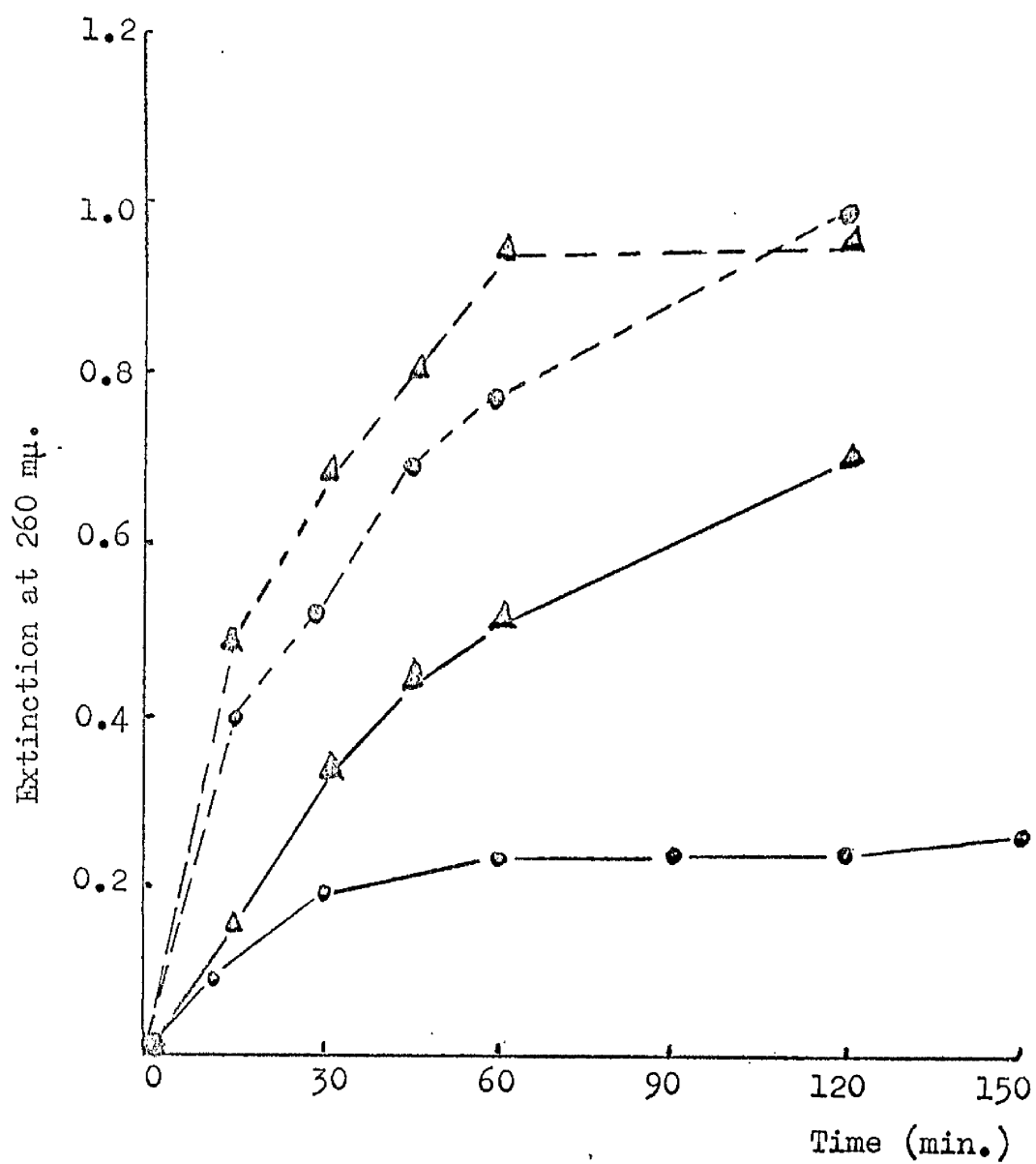
The effect of temperature on the action of pancreatic DNase I,
using the crude enzyme preparation as substrate

The reaction mixture contains 100 μ g. pancreatic DNase I for each 3 mg. of DNA estimated to be present.

The samples were incubated as shown and the concentration of nucleotide liberated measured at 260 m μ .

- represents - incubation carried out at 0°C.
- ▲— represents - incubation carried out at 20°C.
- - ● - - represents - incubation carried out at 30°C.
- - ▲ - - represents - incubation carried out at 37°C.

FIGURE 12.



liquid was decanted and retained. The pellet was resuspended in 5 ml. 0.01M-tris/HCl pH 7.5, 0.02M with respect to 2-mercapto-ethanol. The new suspension was then recentrifuged as before. This was repeated several times. All the supernatant and pellet fractions were assayed for RNA polymerase and DNase activities. Table 2 shows a summary of these results. There was little polymerase activity in any of the fractions that had been treated with DNase, while a control sample showed a loss of activity as the experiment proceeded. DNase activity on the other hand was remarkably constant in the pellet fractions.

ii. Fractionation on DEAE cellulose

When the crude enzyme preparation was treated with DNase and the resultant milky suspension applied to a DEAE cellulose column in 0.3M-phosphate pH 7.5, most of the DNase was eluted in the starting buffer (Fig. 13). Protein was eluted from the column with increasing concentrations of KCl in 0.3M-phosphate buffer pH 7.5. When the enzyme material was not treated with DNase polymerase activity was found in the 0.1M-KCl/0.3M-phosphate fraction. After treatment with DNase, the results were variable. In several experiments extremely active samples were obtained from elution of the column with 0.1M-KCl/0.3M-phosphate buffer. The loss in activity from this fraction may be due to variation in the composition of the chromatin present or to some residual DNase being eluted with the active fraction. This latter idea implies an

TABLE 2.

Pancreatic DNase - Washing Procedures

The RNA polymerase samples were assayed as described in Fig. 1.

The DNase samples were assayed as described under 'Methods' p.39 and the results obtained compared with those obtained with known amounts of pancreatic DNase I.

The samples were incubated at 37° for 30 min.

TABLE 2.

Pancreatic DNase - Washing Procedures

| Fraction | Untreated RNA polymerase $[^3\text{H}]$ UMP/mg. prot. mmoles | RNA polymerase treated with DNase $[^3\text{H}]$ UMP/mg. prot. mmoles | DNase present $\mu\text{g.}/\text{ml.}$ |
|-----------------|---|--|--|
| Initial Extract | 1.16 | 1.16 | - |
| Supernatant 1. | 0.01 | 0.03 | 15.6 |
| Supernatant 2. | 0.02 | 0.05 | 2.4 |
| Supernatant 3. | 0.02 | 0.01 | 0.08 |
| Supernatant 4. | 0.01 | 0.02 | 0.04 |
| Supernatant 5. | 0.02 | 0.02 | 0.02 |
| Supernatant 6. | 0.04 | 0.03 | 0.03 |
| Pellet 1. | 0.98 | 0.15 | 5.6 |
| Pellet 2. | 0.82 | 0.10 | 4.0 |
| Pellet 3. | 0.76 | 0.08 | 3.4 |
| Pellet 4. | 0.70 | 0.15 | 3.0 |
| Pellet 5. | 0.67 | 0.12 | 2.8 |
| Pellet 6. | 0.61 | 0.22 | 2.7 |

FIGURE 13

The elution of crude RNA polymerase from DEAE cellulose

- A. The sample was applied directly to the DEAE cellulose as described in text p.46. The column was eluted with increasing concentrations of KCl in 0.3M-phosphate buffer pH 7.5.
- B. The sample was pretreated with 100 μ g. pancreatic DNase I per 3 mg. DNA, at 37^o for 10 min. before application to the cellulose. The column was eluted again with increasing concentrations of KCl in 0.3M-phosphate buffer pH 7.5.

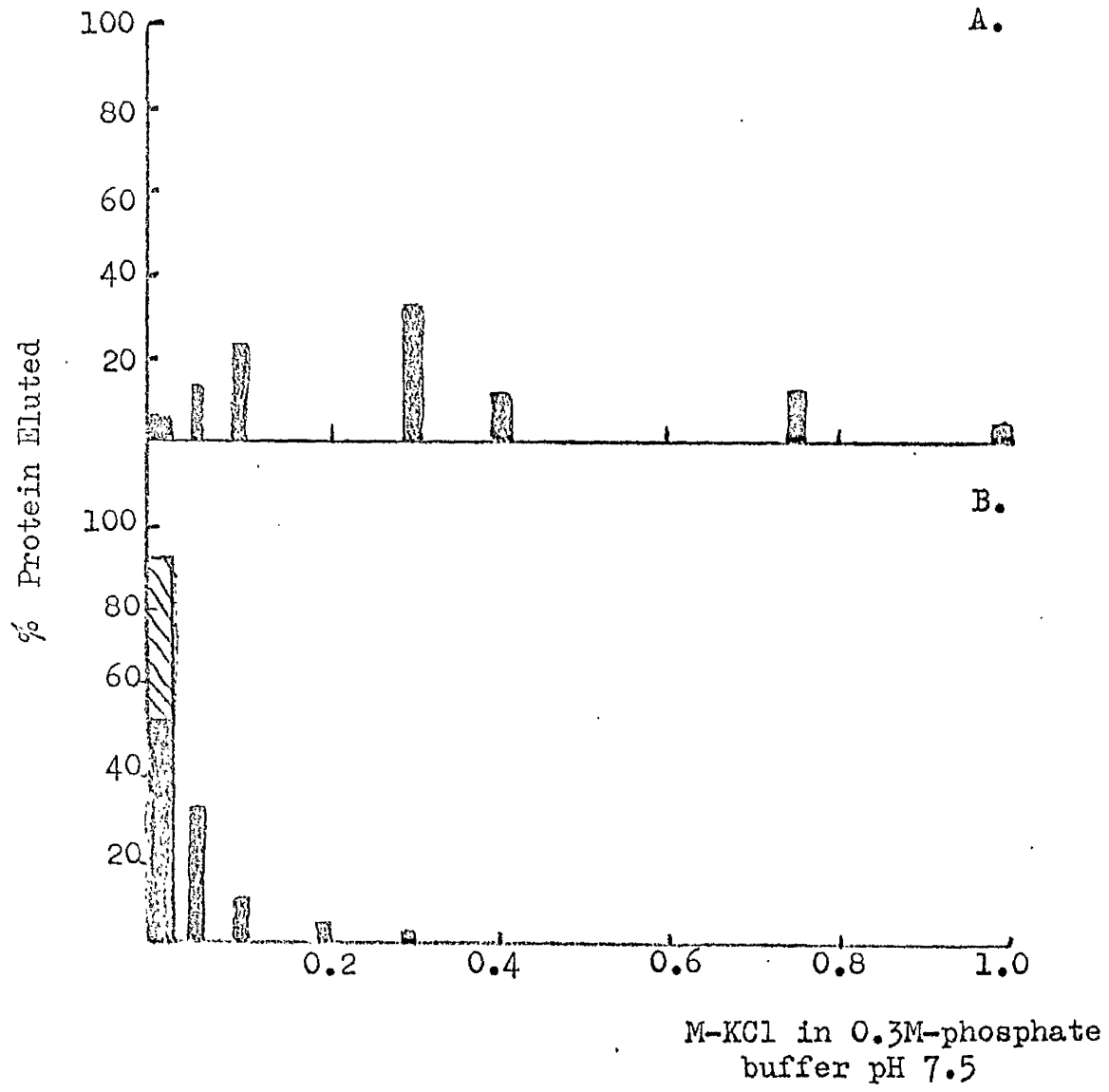


- represents total protein



- represents pancreatic DNase

FIGURE 13.



increased sensitivity of the purified fraction to the action of DNase.

The action of micrococcal nuclease (from Staphylococcus aureus) on the polymerase preparation was tested. This enzyme has been shown to require the presence of $0.001\text{M}-\text{Ca}^{2+}$. It seemed possible that treatment of the crude RNA polymerase preparation with nuclease, followed by fractionation on DEAE cellulose might separate the RNA polymerase from associated DNA and the nuclease. 160 $\mu\text{g.}$ of micrococcal nuclease were added to an RNA polymerase preparation containing 100 $\mu\text{g.}$ of DNA. The pH of the preparation had previously been adjusted to 8.5 with $1.0\text{M}-\text{tris}/\text{HCl}$ and a calculated volume of $0.01\text{M}-\text{CaCl}_2$ added. The sample was incubated at 37° for 15 min.. After 15 min. the mixture was chilled in ice and mixed with 20 ml. of DEAE cellulose which had previously been equilibrated with $0.3\text{M}-$ phosphate buffer pH 7.5 in 30% glycerol. The mixture was allowed to stand in ice for 10 min. and centrifuged at 10,000g for 10 min.. The supernatant liquid was decanted and retained. The pellet was resuspended in 10 ml. of $0.3\text{M}-$ phosphate buffer with glycerol as above and the new suspension centrifuged again. This procedure was repeated four times; for the 5th and final wash the precipitate was resuspended in $0.5\text{M}-$ buffer. All the supernatants were assayed for RNA polymerase and micrococcal nuclease activities. Table 3 shows a summary of these results. There is little polymerase activity again in any of the fractions that were treated with

TABLE 3.

Fractionation of crude RNA polymerase on DEAE cellulose
following treatment with micrococcal nuclease

| Fraction | RNA polymerase μmoles [³ H] UMP/mg. prot. | Micrococcal nuclease E ₂₆₀ released |
|-------------------|--|---|
| Crude Preparation | 1.24 | - |
| Supernatant 1. | 0.02 | 0.120 |
| Supernatant 2. | 0.04 | 0.075 |
| Supernatant 3. | 0.00 | 0.051 |
| Supernatant 4. | 0.02 | 0.020 |
| *Supernatant 5. | 0.00 | 0.015 |

*Previous supernatants were in 0.3M-phosphate buffer pH 7.5, 30%
with respect to glycerol.

Supernatant 5 in 0.5M-phosphate buffer pH 7.5, 30% with respect
to glycerol.

RNA polymerase

These samples were assayed as described in Fig. 1.

Micrococcal nuclease

These samples were assayed as described in 'Methods' p.41.

The samples were incubated at 37° for 30 min.

micrococcal nuclease. The nuclease seems to appear mainly in the initial supernatant but trace amounts would be sufficient to mask the presence of the RNA polymerase. The presence of an enzyme such as DNase or micrococcal nuclease as a contaminant of the RNA polymerase preparation could interfere seriously with its assay and it becomes difficult therefore to interpret the results obtained. Moreover since only 70% of the protein applied to the column was recovered it is possible that the RNA polymerase is retained on the column.

iii. Density gradient centrifugation

Attempts were made to fractionate the enzyme preparation using various types of sucrose and glycerol gradients (Table 4). The chief difficulties arose from the aggregate nature of the preparation at low ionic strengths. In the aggregate form there was a tendency to pack closely either at the bottom of the tube or at the interface of two bands. This pellet was difficult to resuspend for assay. As collection of fractions from these gradients by the dropwise procedure proved unreliable, the centrifuge tubes containing a band of highly viscous material were cut at the appropriate level and the required fraction removed with a Pasteur pipette. In preparations containing 5% sodium deoxycholate however, it was possible to harvest the fractions in the normal manner. When the extinction of these samples was measured at 280 m μ only one protein peak was observed. The position of this peak in the gradient can

TABLE 4a.

Fractionation of the crude RNA polymerase using
density gradient centrifugation through sucrose

The sucrose gradients were prepared in two ways.

- i) A linear gradient of sucrose in water from 40% (W/V) to 5% (W/V) was formed using the mixing device of Britten and Roberts (1960). Any further constituents e.g. 0.01M-tris/HCl were uniform throughout the gradient. A 0.5 ml. sample was applied to the surface of the gradient (4.5 ml.) and the material centrifuged in the SW39 head of the Spinco Model L ultracentrifuge at 20,000 r.p.m. for 6 hr.. The sample was fractionated by puncturing the base of the tube with a hypodermic needle and allowing the content to drip out.
- ii) An 0.5 ml. sample was centrifuged through a series of layers of sucrose in tris/HCl, phosphate buffer or 5% DOC of increasing density. Here the material was centrifuged at varying speeds for 30 min. and the gradient fractionated by means of cutting the centrifuge tube at the interfaces between adjacent layers of sucrose. A summary of these experiments is shown opposite.

Fractionation of the crude RNA polymerase using
density gradient centrifugation through sucrose

| <u>Material</u> | <u>Centrifuge</u> <u>Speed</u> <u>(r.p.m.)</u> | <u>Time</u> | <u>Means of fractionating</u> <u>gradient</u> | <u>Result</u> |
|--------------------------------------|--|-------------|--|--|
| 5%-40% sucrose/ 0.01M-tris/HCl | 20,000 | 6 hr. | Dropwise | Pellet present. Regular distribution of UV absorbing material in supernatant. |
| 20%-80% sucrose/ 0.01M-tris/HCl | 30,000 | 30 min. | Tube cut at interface of layers of sucrose. | Bands ^x observed at interfaces of 40% and 45% and 45% and 50% sucrose. |
| 5%-40% sucrose/ 1M-Na perchlorate | 20,000 | 6 hr. | Dropwise | Pellet present. Regular distribution of UV absorbing material in supernatant. |
| 20%-60% sucrose/ 0.5M-phosphate | 30,000 | 30 min. | Tube cut at interface of layers of sucrose. | Band of UV absorbing material observed in 20% sucrose layer. |
| 20%-60% sucrose/ 5% DCC | 30,000 | 30 min. | Tube cut at interface of layers of sucrose. | Band ^x observed at interface of 10% and 20% sucrose. |
| | 40,000 | 30 min. | | Band ^x observed at interface of 20% and 30% sucrose. |
| | 50,000 | 30 min. | | Band ^x observed at interface of 40% and 50% sucrose. |

Band^x = band of UV absorbing material

TABLE 4a.

TABLE 4b.

Fractionation of the crude RNA polymerase using
density gradient centrifugation through glycerol

The glycerol gradients were prepared and fractionated in the same way as the sucrose gradients described in Table 4a. A summary of these experiments is shown opposite.

Fractionation of the crude RNA polymerase using
density gradient centrifugation through glycerol

| <u>Material</u> | <u>Centrifuge</u> | | <u>Means of fractionating</u> | <u>Result</u> |
|---|--------------------------|-------------|--|---|
| | <u>Speed</u> (r.p.m.) | <u>Time</u> | <u>gradient</u> | |
| Glycerol 5%-40%/0.01M-tris/HCl | 20,000 | 6 hr. | Dropwise | Pellet present. Regular distribution of UV absorbing material in supernatant. |
| Glycerol (20%-60%) 0.3 sat. with respect to (NH ₄) ₂ SO ₄ | 20,000 | 30 min. | Tube cut at interface of layers of sucrose. | Band ^x observed in 30% glycerol layer. |
| | 25,000 | 30 min. | | Band ^x observed at the interface of 30% and 40% glycerol. |
| Glycerol (20%-60%) 0.5M-phosphate | 25,000 | 30 min. | Tube cut at interface of layers of sucrose. | Band ^x observed in 30% glycerol layer. |
| | 35,000 | 30 min. | | Band ^x observed in 30% glycerol layer. |
| Glycerol (20%-50%) 5% DOC | 25,000 | 30 min. | Dropwise | Band ^x observed in 20% glycerol layer. |
| | 35,000 | 30 min. | | Band ^x observed in 30% glycerol layer. |
| | 45,000 | 30 min. | | Band ^x observed at the interface of 30% and 40% glycerol. |

Band^x = band of UV absorbing material

TABLE 4b.

be varied with rate and time of centrifugation.

iv. Effects of detergents on crude RNA polymerase preparations

Several detergents were tested for their ability to aid the solution of the polymerase aggregate while retaining enzymic activity. For these studies it was desirable that the detergent should not absorb at 260 or 280 m μ , interfere with the method employed for protein estimation or cause any side reactions with other components of the system.

In the earliest experiments, sodium deoxycholate (DOC) was tested. A suspension of polymerase containing 0.5% or a higher concentration of DOC gradually forms a homogeneous gel. Fig. 14 shows the effect of varying concentrations of DOC on the incorporation of [^3H]UMP into RNA by the crude RNA polymerase. After an initial drop, the activity of the preparation appears to increase as the concentration of deoxycholate increases up to about 10%. Controls were run for each concentration to ensure that the incorporation was genuine and not due to adsorption of nucleotides on the viscous DNA protein. Subsequent experiments were carried out using 5% or 10% deoxycholate. Fig. 15 shows a time curve of incorporation using a polymerase preparation made 10% with respect to DOC. The rate of incorporation appears to remain constant up to 60 min.. The spectrum of sodium deoxycholate in water shows a maximum at 227 m μ (Fig. 16) and DOC does not appear to interfere in the estimation of protein by the method of Lowry et al. (1951).

FIGURE 14

The effect of increasing concentrations of DOC on
the incorporation of ribonucleoside triphosphates
by the crude RNA polymerase

—●— represents test samples
--●-- represents control samples

Test reaction mixture

This was identical to that of Fig. 1. The enzyme was suspended in varying concentrations of DOC as shown.

Control reaction mixture

This was identical to that of Fig. 1 but no Mn^{2+} or unlabelled ribonucleotides were added prior to incubation. All the samples were incubated at 37° for 30 min.

FIGURE 14.

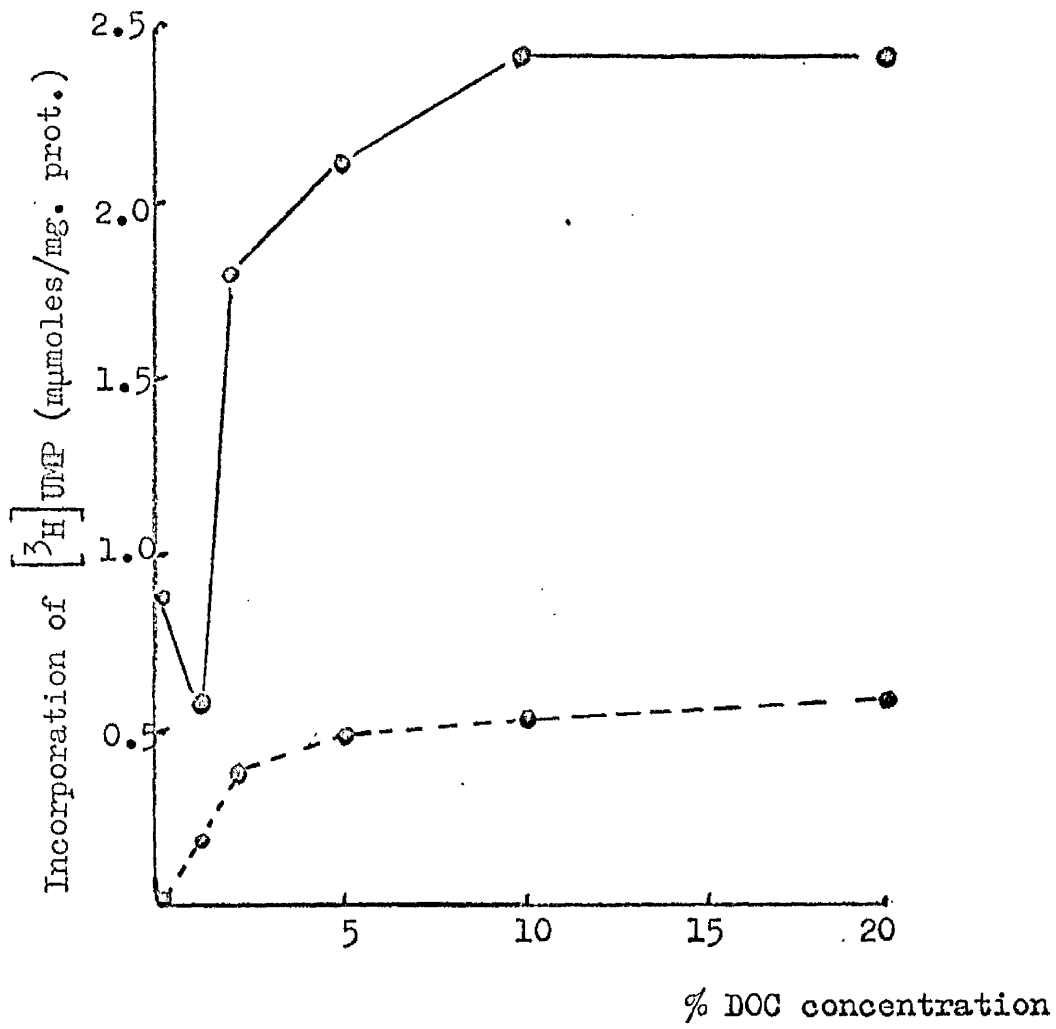


FIGURE 15

The time course of the incorporation of [3 H]UMP into
RNA by DOC treated RNA polymerase

The reaction mixture was identical to that in Fig. 1. The enzyme was dissolved in 10% DOC and the incubation times at 37° were as indicated.

FIGURE 15.

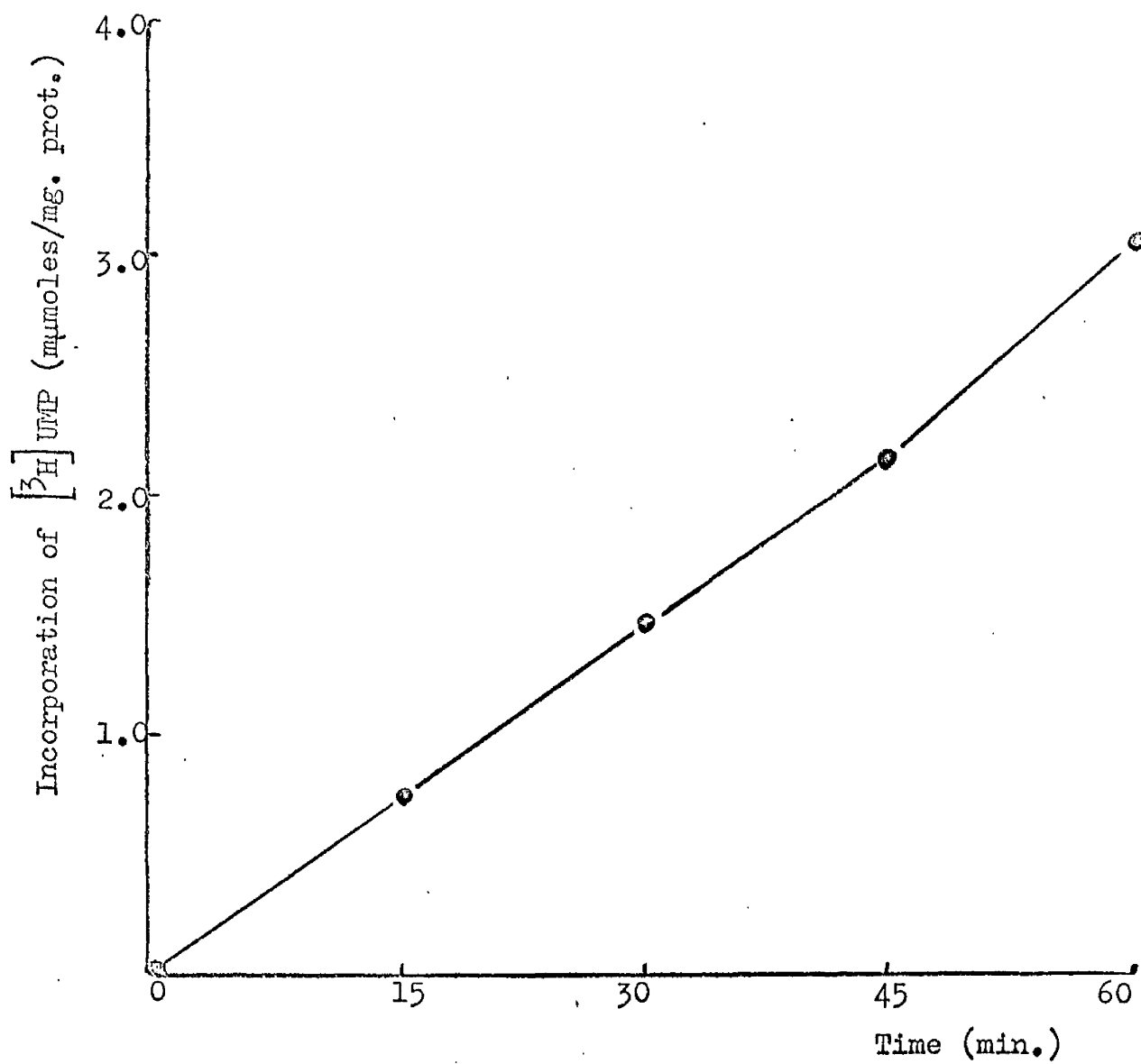
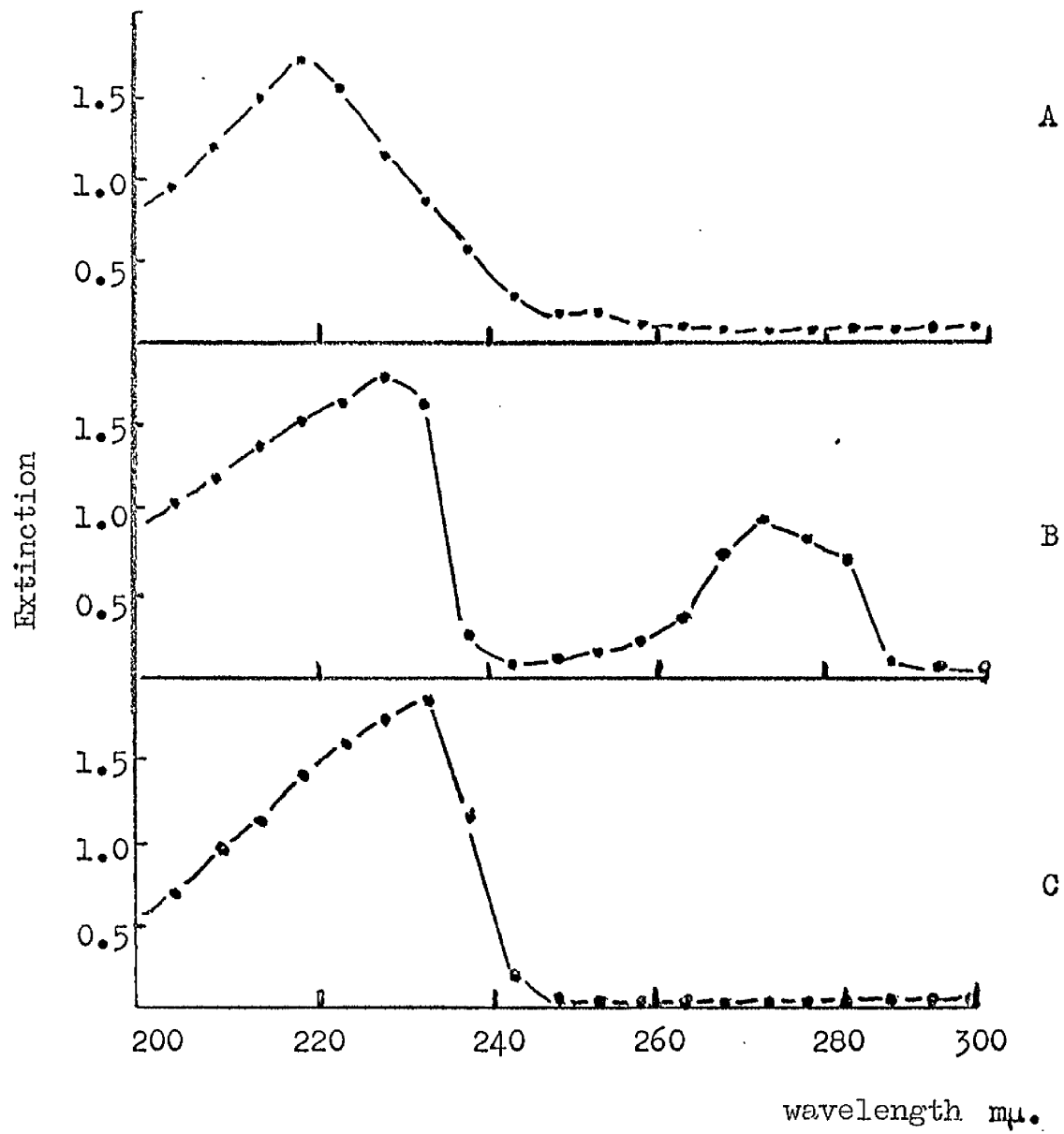


FIGURE 16

- A. The spectrum of sodium deoxycholate in water.
- B. The spectrum of Triton X-100 in water.
- C. The spectrum of sodium-N-lauroyl sarcosinate.

FIGURE 16.



The polymerase preparation is soluble in a 10% solution of Triton X-100 in water. Fig. 17 shows the effect of various concentrations of the detergent on activity. There is no dramatic rise in activity as there was with DOC but a slight increase was noted. The spectrum of Triton X-100 shows an absorption peak around 280 m μ and it was found to interfere with Lowry protein reagents to give very high protein values. The detergent could not be removed even by extensive dialysis and consequently it was not considered suitable for further study.

The enzyme will also dissolve in 10% sodium-N-lauroyl sarcosinate. Fig. 18 shows the time course of incorporation of labelled UMP by the crude RNA polymerase in the presence of this detergent. However sodium-N-lauroyl sarcosinate causes considerable inhibition and although its spectrum (Fig. 16C) shows no marked absorption in the 260/280 m μ region and there is no side reaction when estimating protein, no further experiments were carried out.

Calsolene oil HS is an amber anionic liquid. The crude enzyme preparation dissolved in a 10% solution but activity was almost entirely lost. Fig. 19 shows spectrum of this detergent in water. Due to its absorption in the 260/280 m μ region it seemed likely it would interfere with protein estimation. The detergent was not subsequently tested.

Cetyl-trimethyl-ammonium bromide was also examined. The poly-

FIGURE 17

The effect of increasing concentrations of Triton X-100 on
the incorporation of [3 H]UMP into RNA by the
crude ascites RNA polymerase

The reaction mixture was identical to that in Fig. 1. The enzyme was suspended in varying concentrations of Triton X-100 as shown. The samples were incubated at 37° for 30 min.

FIGURE 17.

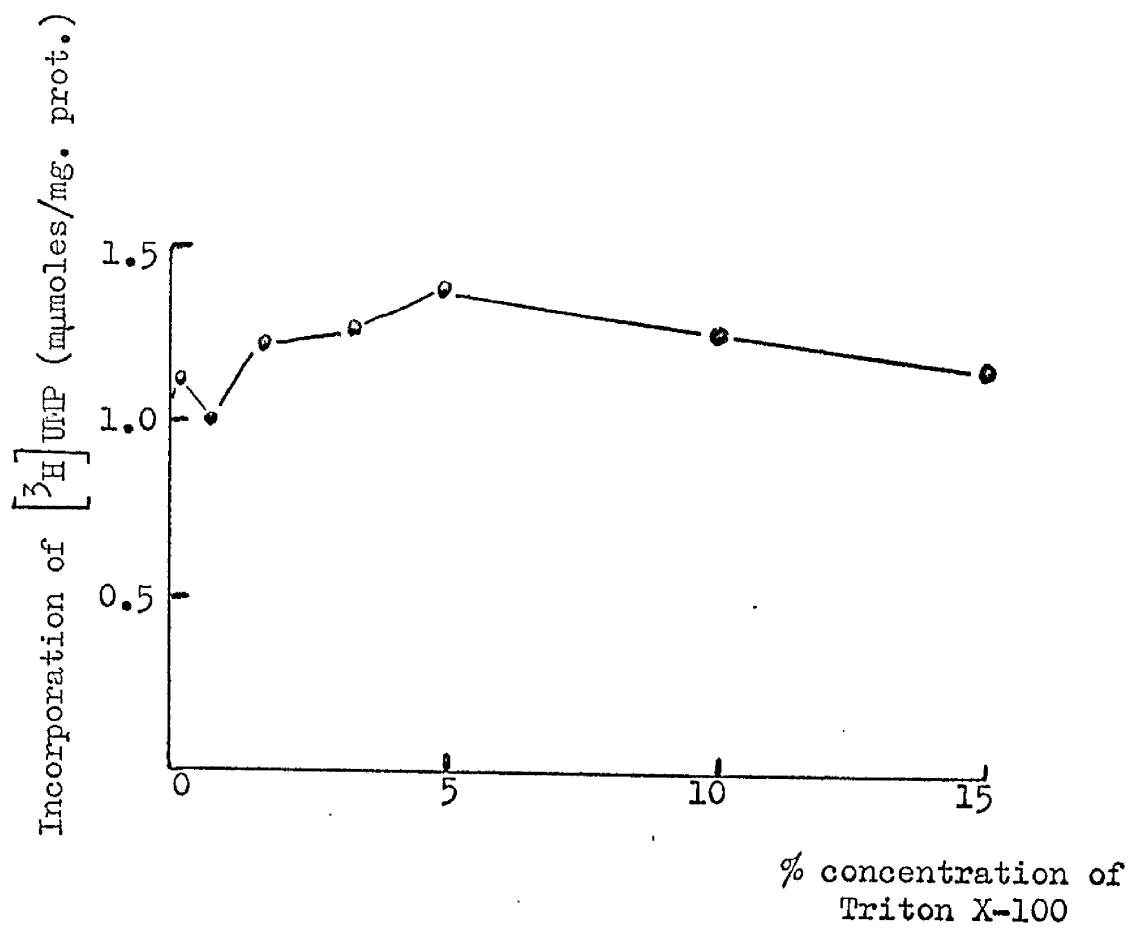


FIGURE 18

The time curve of the incorporation of $[^3\text{H}]$ UMP into RNA by
the crude RNA polymerase suspended in
16% sodium-N-lauroyl sarcosinate

The reaction mixture was identical to that in Fig. 1. The samples were incubated at 37° for times shown.

—●—

represents untreated RNA polymerase

- - ● - -

represents RNA polymerase, 16% with respect to
sodium-N-lauroyl sarcosinate

FIGURE 18.

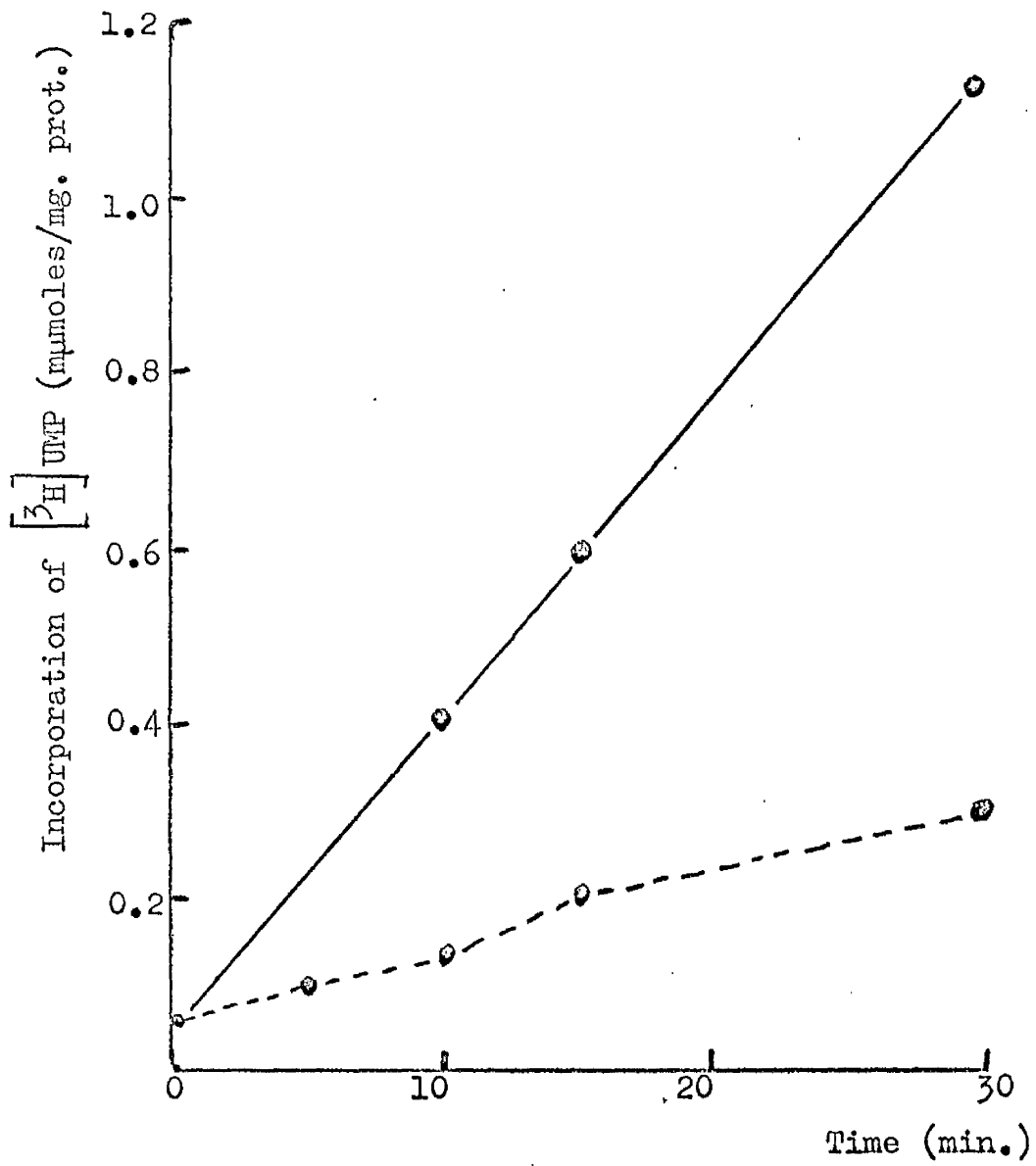
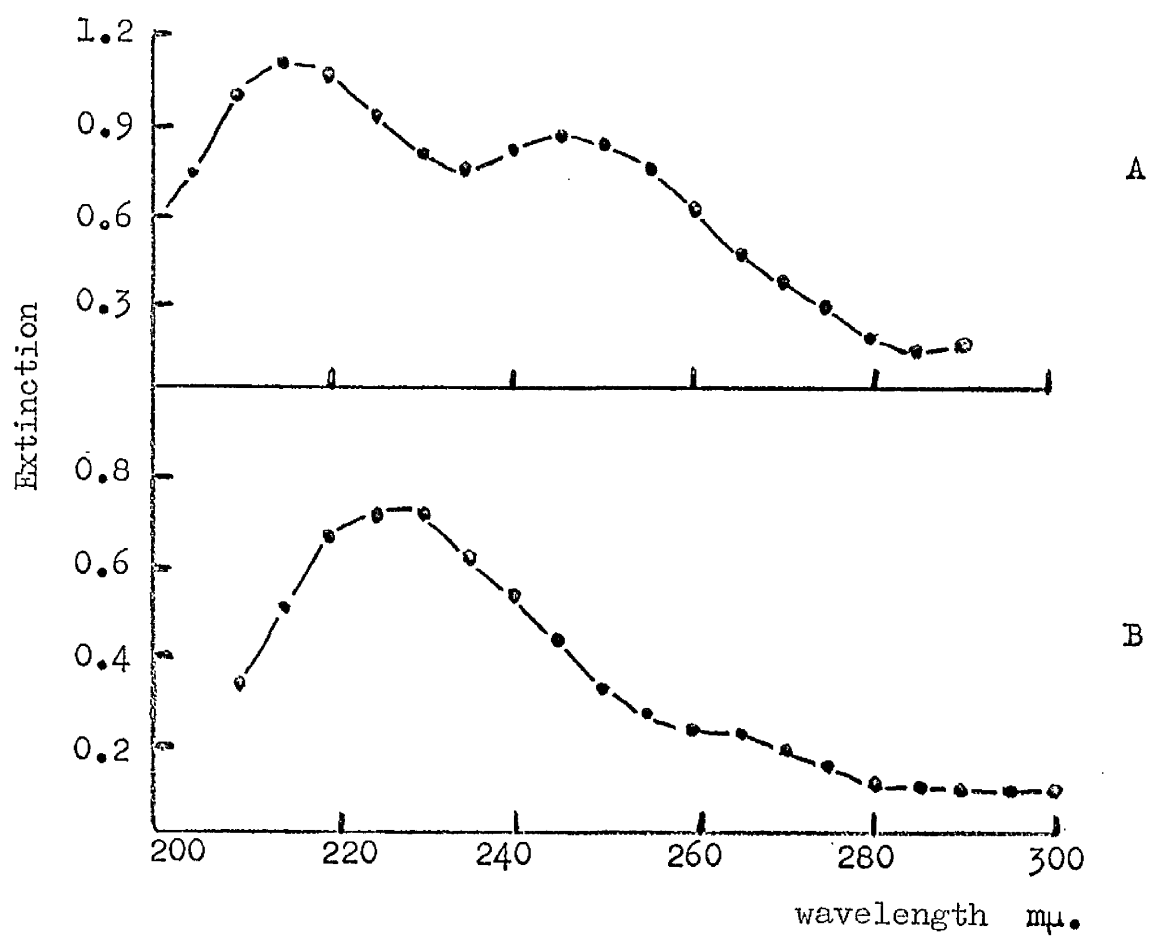


FIGURE 19

- A. The spectrum of 1% calsolene oil in water
- B. The spectrum of 2% cetyl-trimethyl-ammonium bromide in water

FIGURE 19.



merase was very slowly soluble in a 5% solution of this detergent and the solution shows little enzymic activity. A spectrum of the detergent in water at pH 6.5 is shown in Fig. 19. There is a little absorption in the 260/280 mμ region.

Despite its disadvantages sodium deoxycholate was chosen for further investigation.

v. Streptomycin fractionation of deoxycholate treated RNA polymerase

The polymerase in tris/HCl buffer pH 7.5, made 10% with respect to sodium deoxycholate, was treated in the cold with varying volumes of 10% streptomycin sulphate, added slowly dropwise with continuous stirring. The precipitate formed by addition of streptomycin sulphate to the enzyme in 10% DOC was removed by centrifuging the fraction for 5 min. at 600g. This pellet contained no RNA polymerase activity. The supernatant was removed and centrifuged at 161,000g to yield a pellet and a supernatant fraction (SSS). Again the RNA polymerase activity was found in the supernatant fraction.

Fig. 20 shows the effect on incorporation of $[^3\text{H}]$ UMP into RNA by the RNA polymerase preparation after treatment with varying concentrations of streptomycin sulphate. The optimum activity in the SSS fraction was obtained after treatment with 1% streptomycin sulphate. The time of centrifugation was examined (Fig. 21) and enzyme preparations of maximal activity were obtained by centrifugation at 161,000g for 60 min.. This time was routinely used in

FIGURE 20

The effect of increasing concentrations of streptomycin sulphate on the specific activity of the RNA polymerase

The reaction mixture was identical to that in Fig. 1. The crude enzyme preparation was dissolved in 10% DOC and treated with varying concentrations of streptomycin sulphate. The fractions were then centrifuged and the supernatants assayed as shown. The samples were incubated at 37° for 30 min.

FIGURE 20.

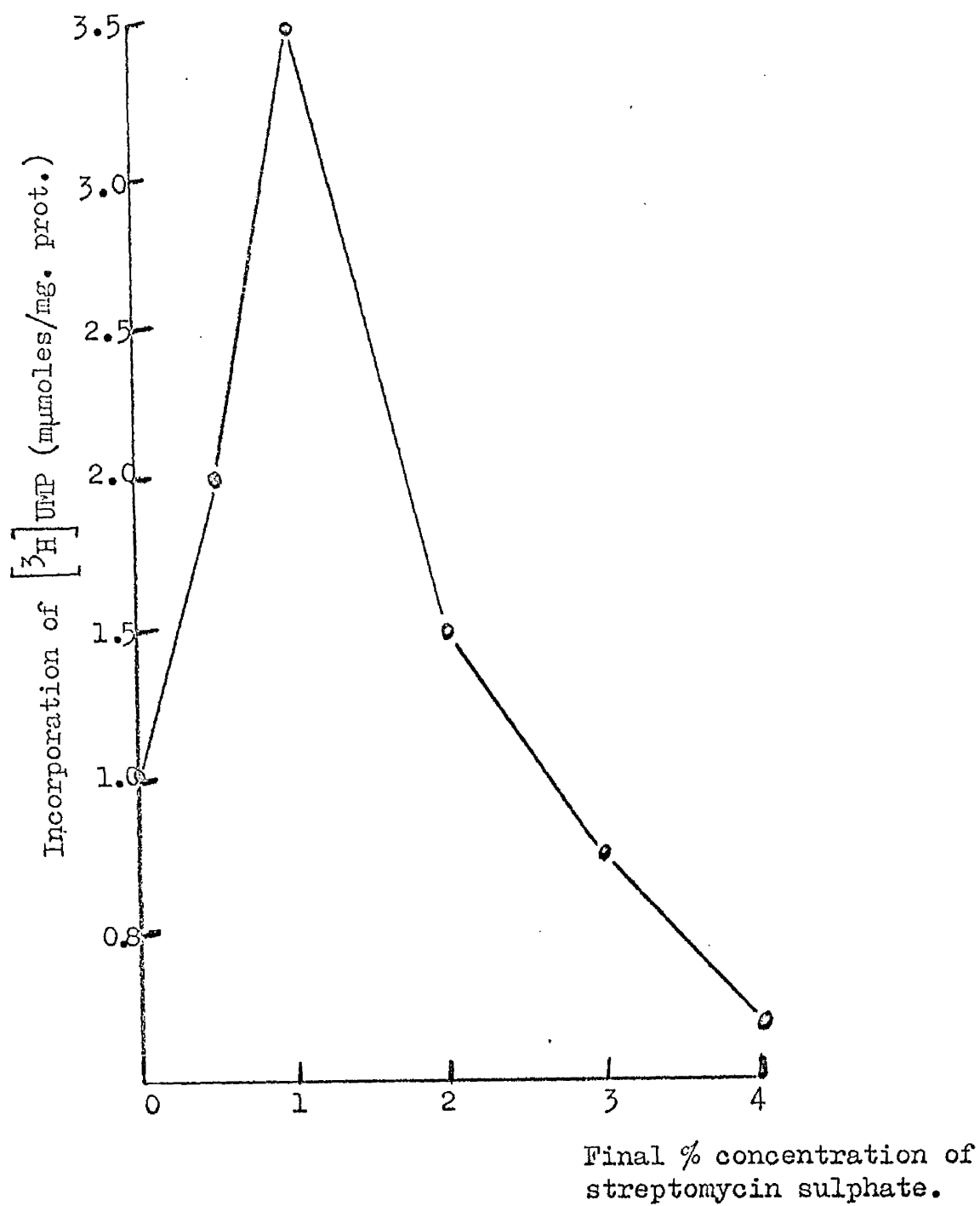
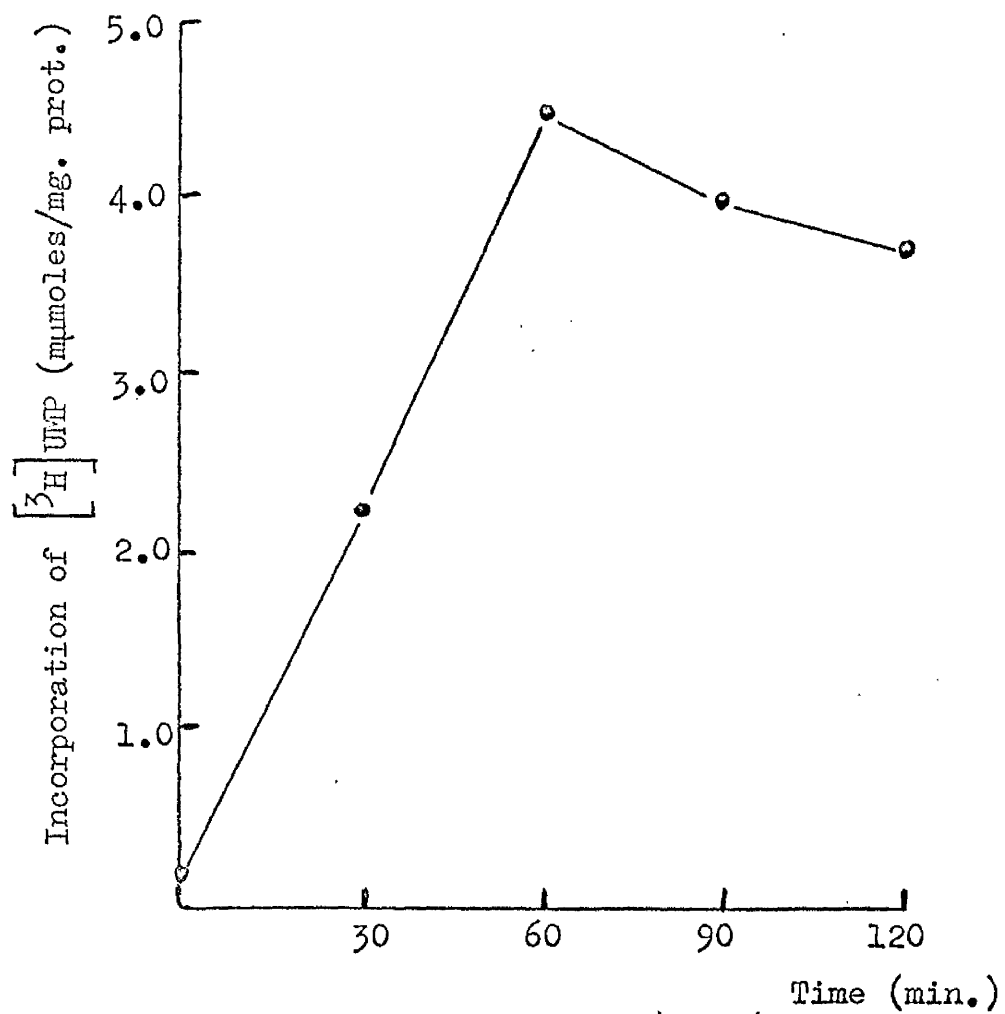


FIGURE 21

The effect of various conditions of centrifugation on the RNA
polymerase activity of the streptomycin sulphate supernatant

The reaction mixture was identical to that of Fig. 1. The enzyme was prepared as described in Fig. 20 and centrifuged in the 50 rotor of a Spinco Model L centrifuge for varying times at 161,000 g. The samples were incubated at 37° for 30 min.

FIGURE 21.



subsequent experiments. The ratio of extinction at 280 m μ to that at 260 m μ , of the DOC extract is 0.68. After treatment with 1% streptomycin sulphate it is 0.84. Table 5 shows the yield of active protein during these procedures. A 51.0% recovery of activity is obtained which corresponds to a purification factor of 4.33 as compared with the crude polymerase fraction.

III. Streptomycin sulphate supernatant fraction (SSS)

i. Assay conditions

Fig. 22 shows the time course of incorporation of [^3H] UMP into RNA by the SSS fraction. It is linear up to 30 min. but falls off rapidly if incubation is continued. Subsequent assays were incubated for 30 min.. The relationship between activity and protein concentration is shown in Fig. 23. After an initial lag up to 75 $\mu\text{g./ml.}$ the rate of incorporation appears linear.

The requirements of the system were investigated with respect to Mn^{2+} and Mg^{2+} ions, in the presence and absence of ammonium sulphate. A summary of the results is shown in Fig. 24. In the absence of ammonium sulphate there was little incorporation, while in its presence the optimal Mn^{2+} concentration was again 1 $\mu\text{mole/}$ assay. On the addition of Mn^{2+} ions much higher levels of enzyme activity were observed than was the case with Mg^{2+} ions. When the effects of varying concentrations of ammonium sulphate on the activity of the preparation (Fig. 25) were measured, keeping the Mn^{2+} level at 1 $\mu\text{mole/assay}$, the graph showed a maximum at 320 $\mu\text{moles/}$

Yields of RNA polymerase after fractionation
with DOC and Streptomycin sulphate

TABLE 5.

| Fraction | Protein mg./ml. | Total Protein mg. | Specific Activity of RNA Polymerase μmoles [³ H] UMP/ mg. prot. | Total RNA Polymerase Activity μmoles [³ H] UMP | % Recovery | Purification Factor |
|---------------------------|--------------------|-------------------------|--|--|------------|------------------------|
| Crude Preparation | 8.9 | 178.0 | 0.9 | 160.2 | 100 | 1 |
| DOC Preparation | 4.5 | 175.0 | 2.4 | 420.0 | 260 | 2.6 |
| Strep.Sulph. Supernat. | 0.88 | 21.4 | 3.9 | 83.5 | 51.0 | 4.33 |

The RNA polymerase activities were assayed as described in

Fig. 1. The protein levels were assayed by the method of Lowry et al. (1951).

FIGURE 22

The time course of the incorporation of $[^3\text{H}]$ UMP into RNA by
the supernatant fraction from 1% streptomycin sulphate

The reaction mixture was identical to that of Fig. 1. The enzyme was prepared as described in Fig. 20. The samples were incubated at 37° for varying times as indicated.

FIGURE 22.

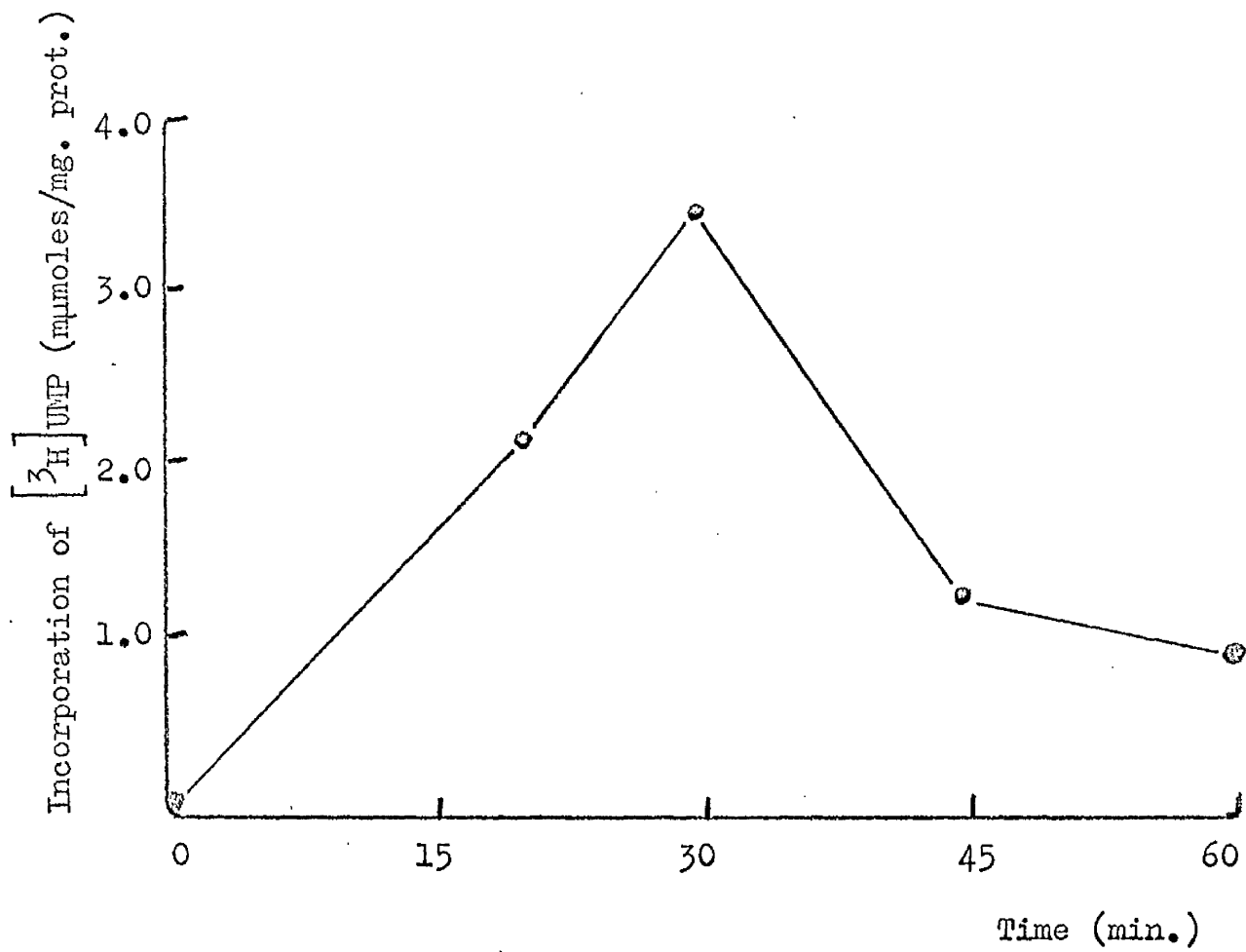


FIGURE 23

The effect of protein concentration on the incorporation
of [3 H]UMP into RNA by the RNA polymerase from the
streptomycin sulphate supernatant fraction

The reaction mixture was identical to that used in Fig. 1, except that the concentration of enzyme protein added, was varied as shown. The samples were incubated at 37° for 30 min.

FIGURE 23.

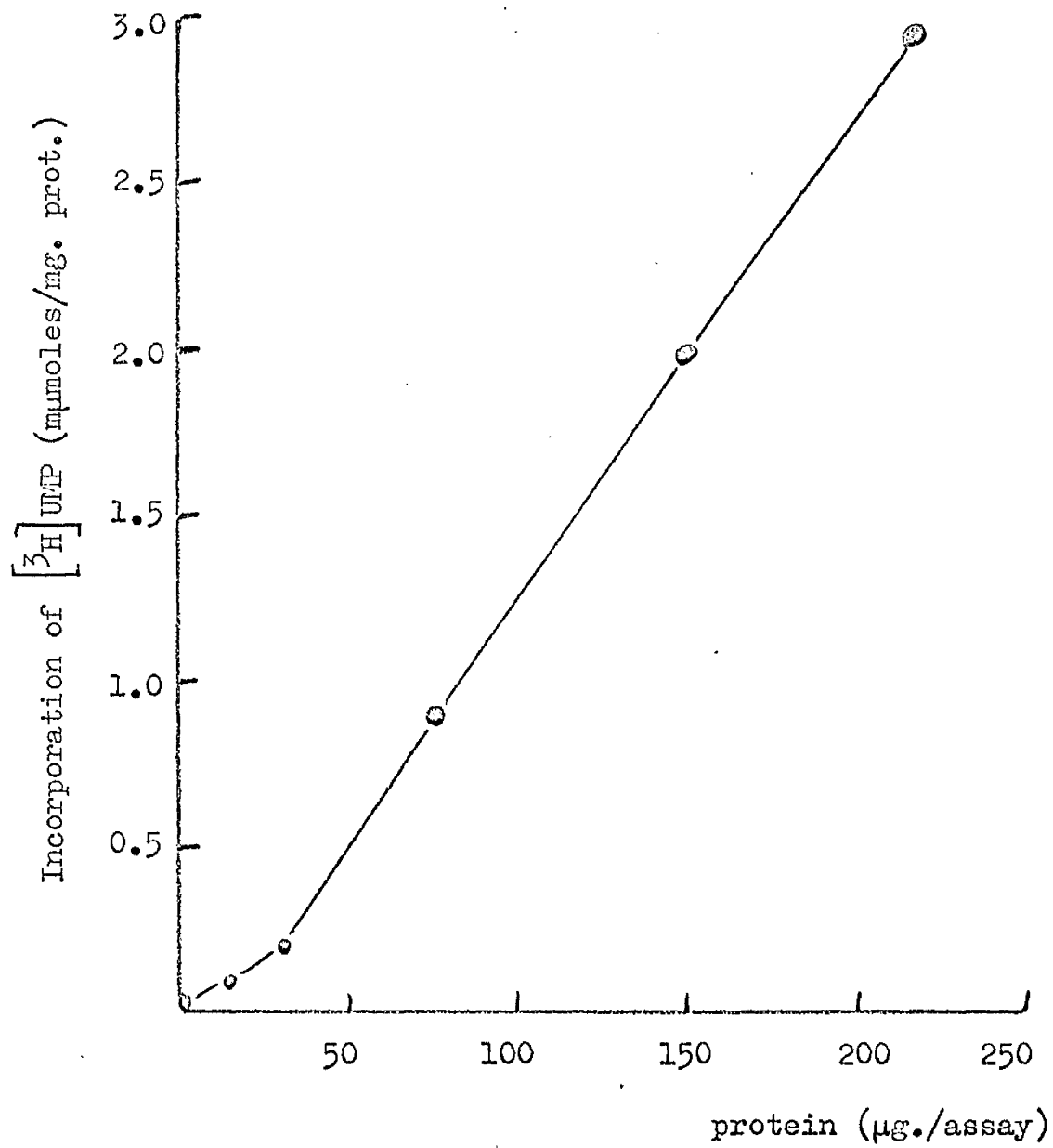


FIGURE 24

The effect of various ions on the
SSS RNA polymerase preparation

- 1) The effect of addition of Mn^{2+} or Mg^{2+} to the assay, in the
presence and absence of ammonium sulphate

The reaction mixtures were those used in Fig. 3.

- represents the presence of Mn^{2+} and $(NH_4)_2SO_4$
—▲— represents the presence of Mg^{2+} and $(NH_4)_2SO_4$
- - ● - - represents the presence of Mn^{2+}
- - ▲ - - represents the presence of Mg^{2+}

The samples were incubated at 37° for 30 min.

FIGURE 24.

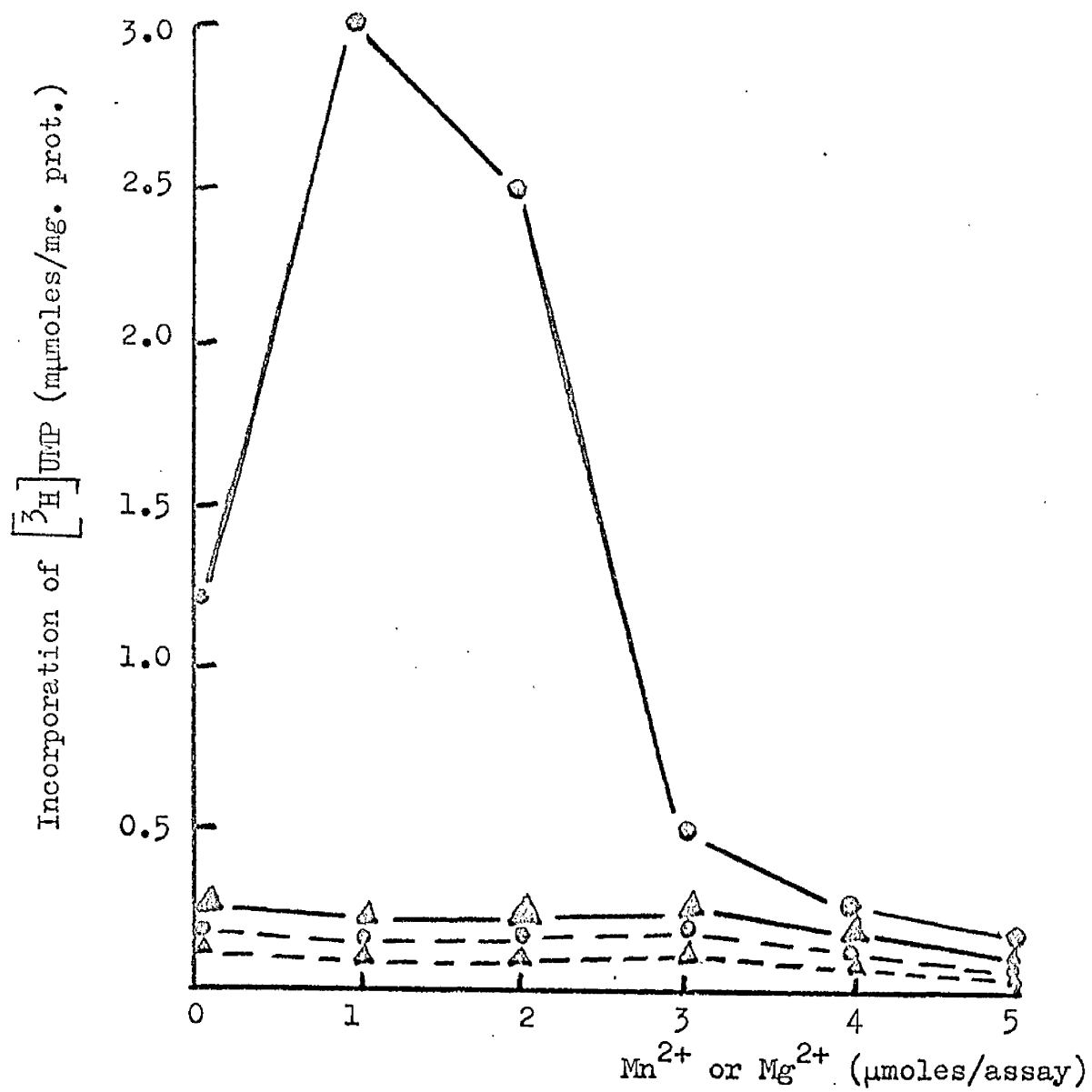


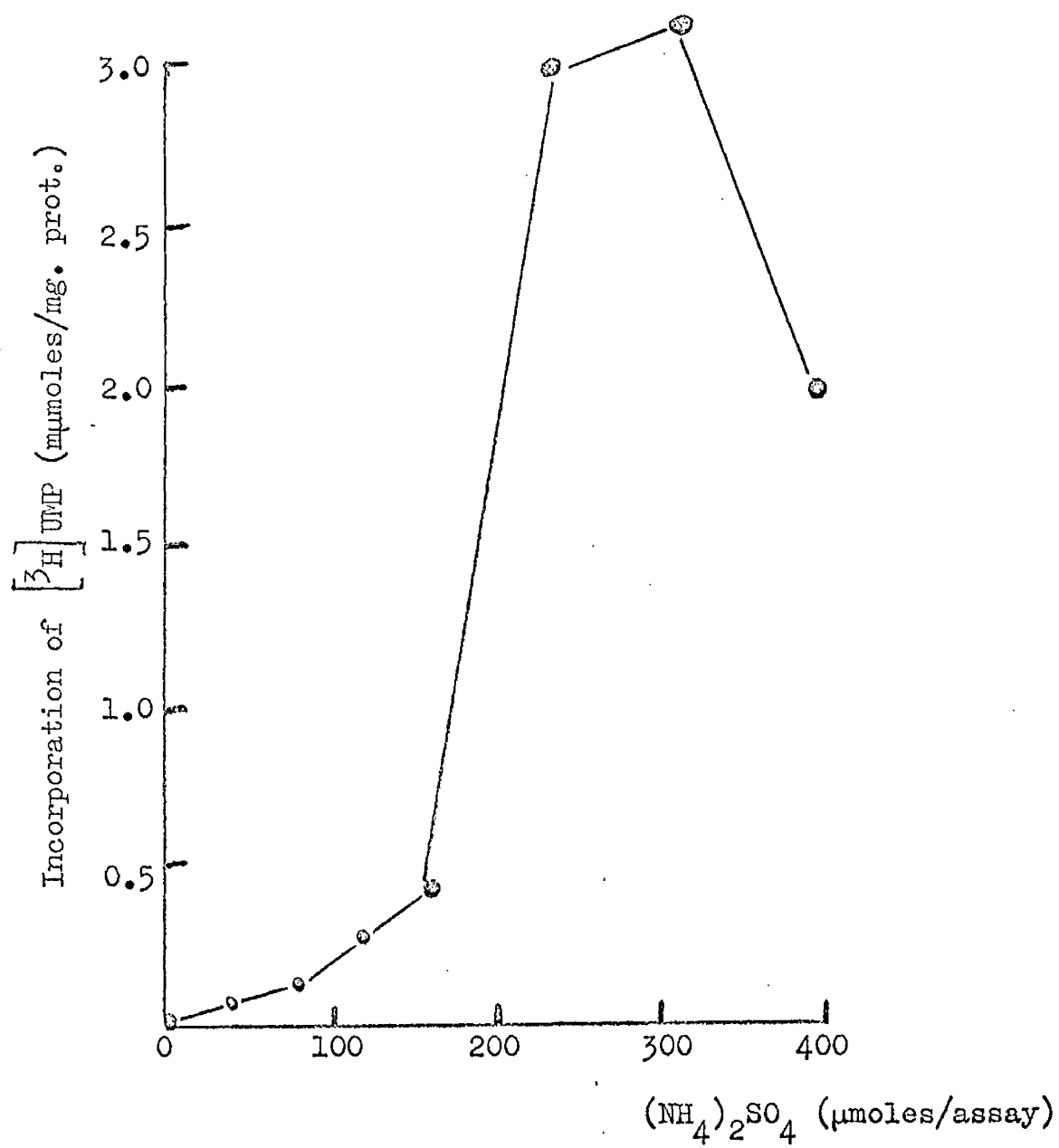
FIGURE 25

The effect of various ions on the incorporation of
 $[^3\text{H}]$ UMP by the SSS RNA polymerase preparation

- 2) The effect of varying concentrations of $(\text{NH}_4)_2\text{SO}_4$ in the
presence of Mn^{2+}

The reaction mixture was identical to that of Fig. 4. The samples were incubated at 37° for 30 min.

FIGURE 25.



assay as compared with 240 μ moles/assay required for crude enzyme.

The effect of varying concentrations of EDTA on the system were seen to be slight (Fig. 26) and in contrast to the crude preparation it was seen that the concentration of -SH groups present in the assay mixture did not appear to affect the incorporation of nucleoside triphosphates greatly (Fig. 27).

It was not possible to test the activity of the SSS preparation in phosphate buffer due to the interaction of DOC with the buffer. The activity of the preparation at pH values between 6.4 and 9.0 was tested using triethanolamine/HCl buffer. The result is shown in Fig. 28. The optimum occurred at pH 7.0 and there is a marked drop of activity on either side.

ii. Enzyme stability

The specific activity of the enzyme was measured after storing the streptomycin sulphate preparation at 4^o, -10^o and -60^o for varying times. Fig. 29 shows the fall in rate of incorporation of ribonucleotides which resulted. It was noted that on removal from the centrifuge the SSS fraction was a clear, non-viscous liquid. After sitting in ice for an hour the liquid became highly viscous and a white precipitate was formed. This made further operations with the fraction technically difficult. Samples left for some time (e.g. over 48 hr.) at 4^o became so highly viscous that they were discarded. The fractions which were frozen immediately on removal from the centrifuge were clear and non-viscous when thawed but they

FIGURE 26

The effect of varying EDTA concentrations on the incorporation
of [^3H]UMP by the SSS RNA polymerase preparation

The reaction mixture was identical to that of Fig. 7. The
samples were incubated at 37° for 30 min.

FIGURE 26.

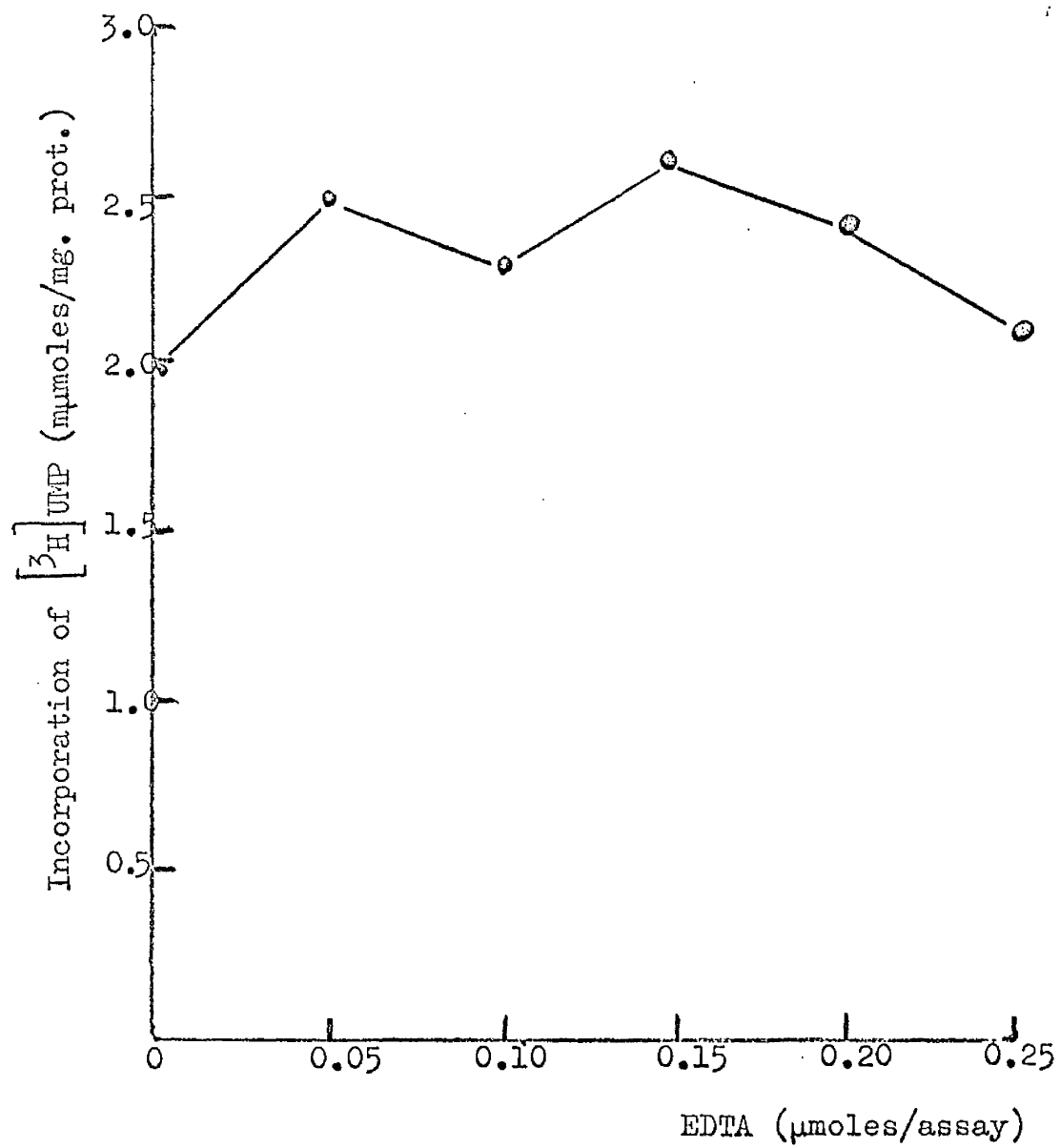


FIGURE 27

The effects of varying the concentration of 2-mercapto-ethanol
present on the activity of the SSS RNA polymerase

The reaction mixture was identical with that described in Fig. 1 except that the concentrations of 2-mercapto-ethanol added were varied as indicated.

The samples were incubated at 37° for 30 min.

FIGURE 27.

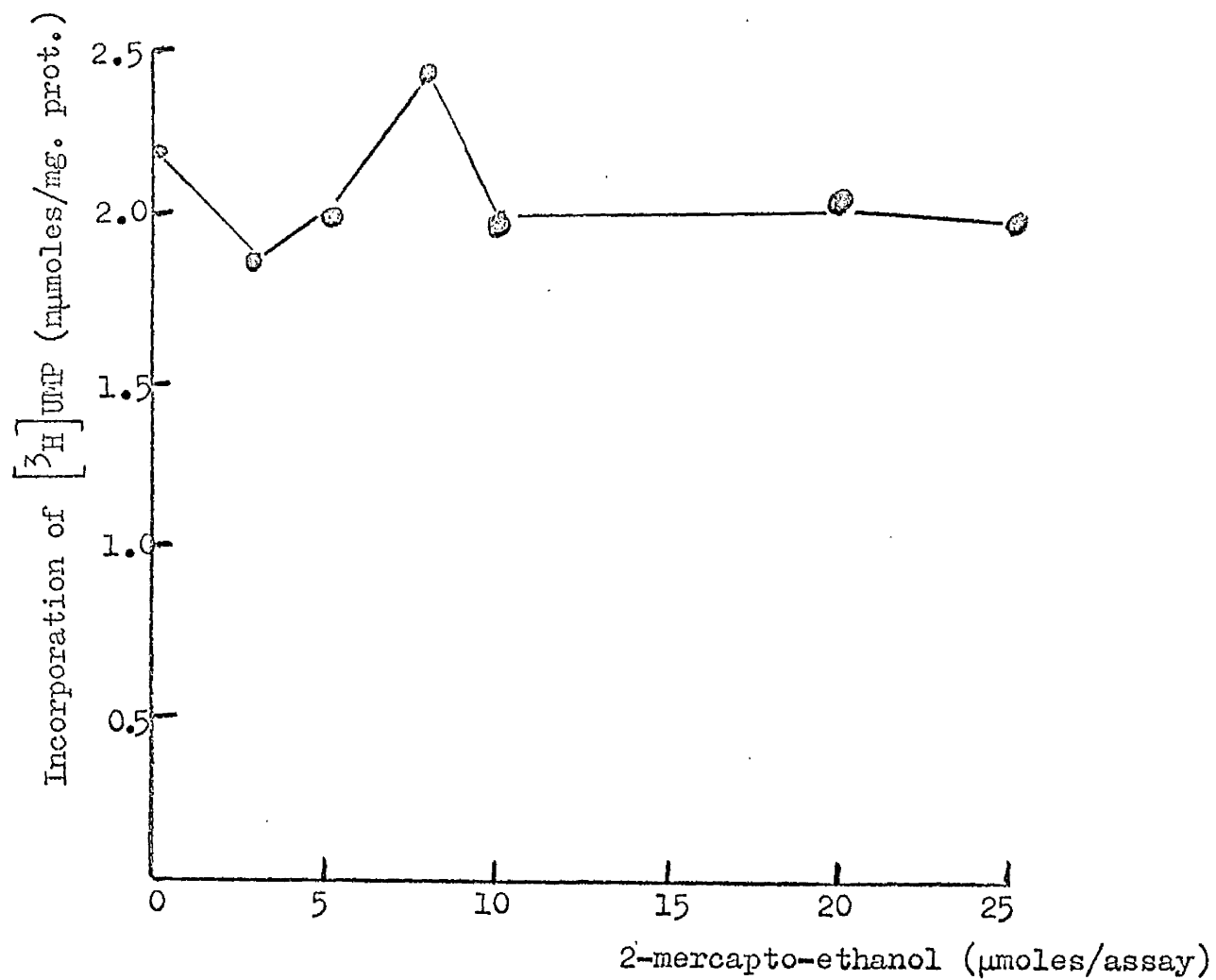


FIGURE 28

The effect of pH on the SSS RNA polymerase activity

The reaction mixture was identical with that described in Fig. 1, except that the pH of the triethanolamine buffer used was varied as indicated.

The samples were incubated at 37° for 30 min.

FIGURE 28.

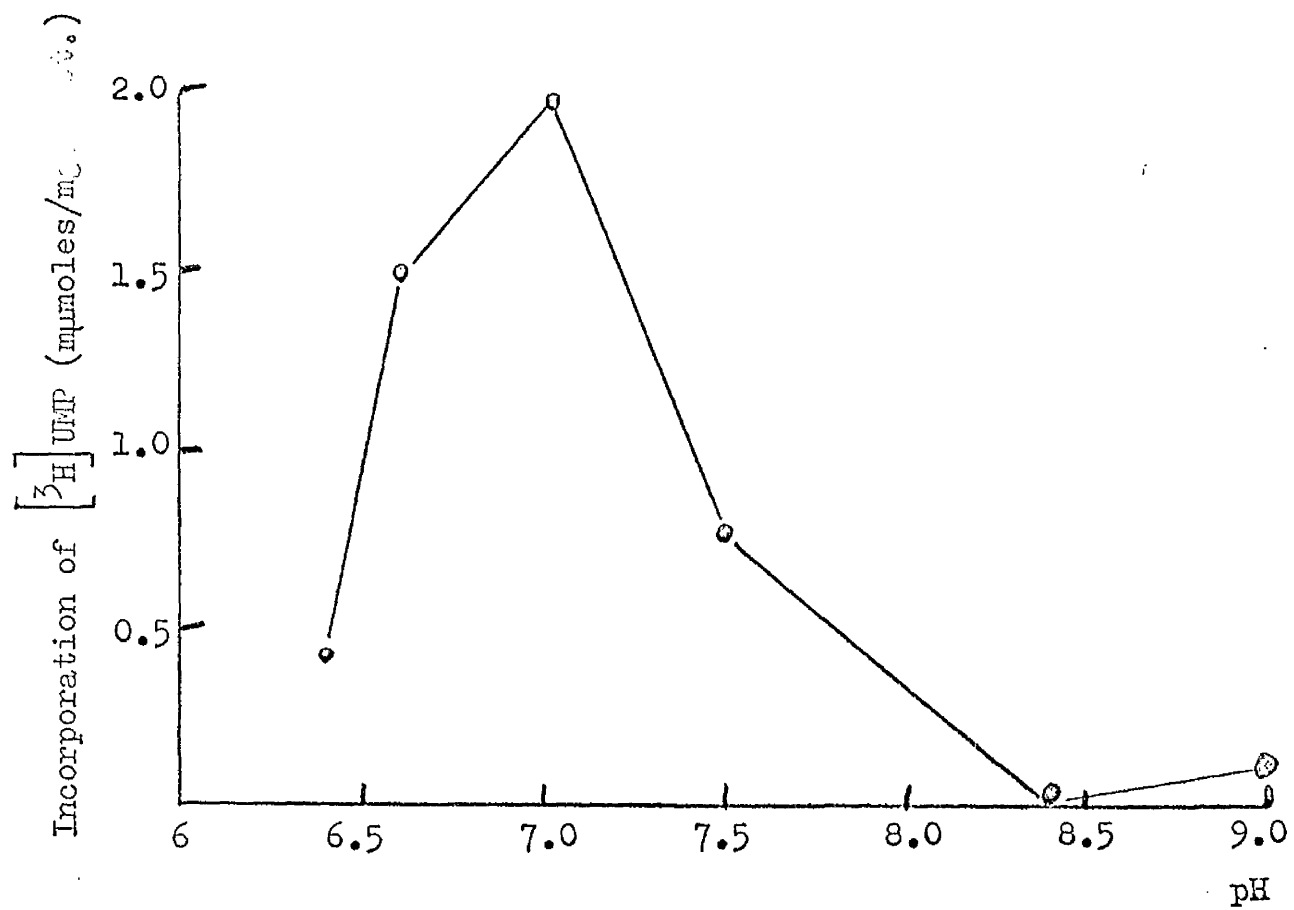


FIGURE 29

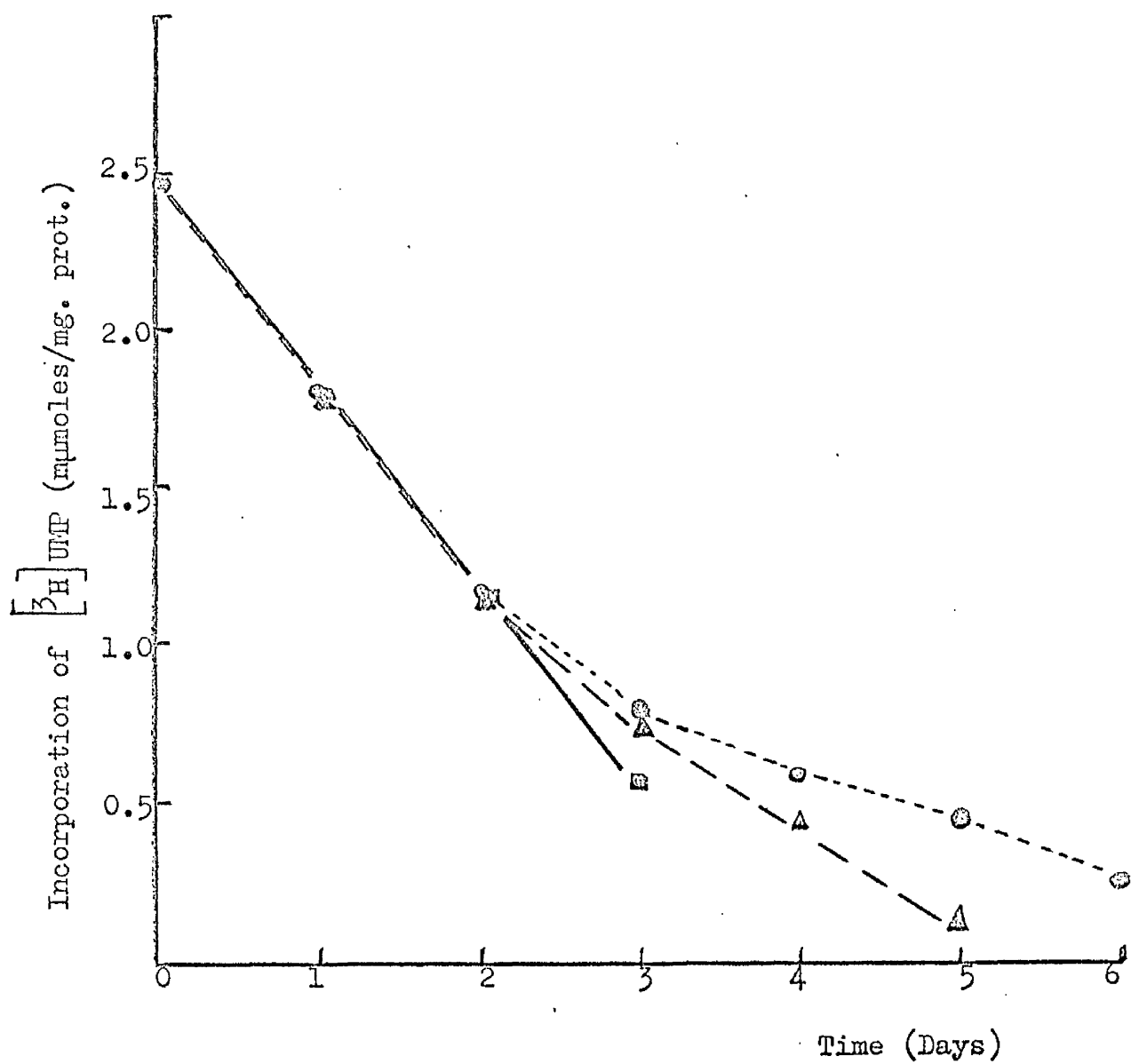
A study of storage conditions of the SSS RNA polymerase

The streptomycin sulphate supernatant fraction was stored at various temperatures prior to assay. The reaction mixture used was identical to that described in Fig. 1.

The samples were incubated at 37° for 30 min.

| | |
|---------|---|
| —■— | represents storage at 4°C (for 3 days only) |
| - -▲- - | represents storage at -10°C |
| ••●•• | represents storage at -60°C |

FIGURE 29.



too became opaque and viscous on standing. When streptomycin sulphate was added to a DOC solution, as had already been noted, a white precipitate formed instantly. If this precipitate was removed by centrifuging at 10,000g for 10 min. and the supernatant decanted and allowed to stand, a further white precipitate slowly formed. This could account for the reaction of the enzyme fraction described above. When the fresh enzyme preparation was made 30% with respect to glycerol, formation of the white precipitate was greatly delayed as also was the subsequent rise in viscosity. Storage at -60° in 30% glycerol was investigated but while an improvement, the specific activity of the enzyme fraction was seen to be still falling. The preparation was judged unsuitable for storage and fresh batches used in subsequent experiments.

IV. Properties of the RNA polymerase preparations

The crude enzyme preparation contained considerable amounts of DNA (0.8 mg./mg. protein) and this minimised the effects of adding DNA or RNA to the system (Table 6). Nevertheless some slight stimulation of incorporation was observed with DNA while addition of RNA seemed to have little or no effect. Pancreatic DNase I (30 μ g.) and pancreatic RNase (30 μ g.) were found to reduce incorporation when added to the assay system.

The SSS preparation still contained some DNA (0.12 mg./ml.) but there was a marked stimulation when DNA was added to the assay system (Table 7). In contrast to the crude polymerase preparation,

TABLE 6.

The effects of added DNA, RNA, DNase I and RNase
on the crude RNA polymerase

| Addition to Assay | Incorporation of $[^3\text{H}]\text{UMP}$ μmoles/mg. protein |
|-------------------|---|
| NIL | 1.325 |
| DNA (80 μg.) | 1.425 |
| RNA (80 μg.) | 1.29 |
| DNase (30 μg.) | 0.41 |
| DNA + DNase | 0.645 |
| RNase (30 μg.) | 0.143 |

The reaction mixture contained 50 μmoles tris-HCl buffer, pH 7.5; 1 μmole MnCl_2 ; 6 μmoles 2-mercapto-ethanol; 100 μmoles EDTA; 240 μmoles $(\text{NH}_4)_2\text{SO}_4$; 100 μmoles each of ATP, GTP, CTP and UTP; $[^3\text{H}]\text{UTP}$ (1×10^7 d.p.m./μmole) and 0.1 ml. of enzyme (3 mg. protein/ml.) in a total volume of 0.6 ml.. Phosphocreatine and phosphocreatine kinase were added as in Fig. 1.

DNA, RNA, DNase I and RNase were added to the reaction mixture as shown.

The samples were incubated at 37° for 30 min.

TABLE 7.

The effects of DNA, RNA, DNase I and RNase on the
streptomycin sulphate supernatant fraction

| Addition to Assay | Incorporation of [³ H] UMP by the purified prep. mμmoles/mg. prot. | Incorporation of [³ H] UMP by the crude preparation mμmoles/mg. prot. |
|-------------------|---|--|
| NIL | 1.56 | 1.325 |
| DNA (80 μg.) | 2.6 | 1.425 |
| RNA (80 μg.) | 2.5 | 1.29 |
| DNase (30 μg.) | 1.25 | 0.41 |
| DNA + DNase | 1.85 | 0.645 |
| RNase (30 μg.) | 0.19 | 0.143 |

The reaction mixture contained 50 μmoles tris-HCl buffer, pH 7.5; 1 μmole MnCl₂; 6 μmoles 2-mercapto-ethanol; 100 mμmoles EDTA; 240 μmoles (NH₄)₂SO₄; 100 mμmoles each of ATP, GTP, CTP and UTP; [³H] UTP (1 x 10⁷ d.p.m./μmole) and 0.1 ml. of enzyme (3 mg. protein/ml.) in a total volume of 0.6 ml.. Phosphocreatine and phosphocreatine kinase were added as in Fig. 1.

DNA, RNA, DNase I and RNase were added to the reaction mixture where indicated.

The samples were incubated at 37° for 30 min.

the addition of RNA caused an increase in the activity of this fraction. The extent of the stimulation with RNA was comparable to that obtained with equimolar amounts of DNA, while pancreatic DNase I (30 µg.) and pancreatic RNase (30 µg.) were found to inhibit the reaction. It should be noted that the inhibition by DNase was much less than that observed with the crude preparation.

Incorporation of each of the ribonucleoside 5'-triphosphates was tested in the presence and absence of the other three. Table 8 shows an increased incorporation of any one labelled ribonucleotide when the other three were also present in the reaction mixture. In the absence of any other nucleotide there is a high level of incorporation of $[^3\text{H}]$ UTP. When this experiment was repeated in the absence of ammonium sulphate, no marked stimulation of incorporation occurred on addition of the other three nucleotides. Thus the reaction observed in the absence of ammonium sulphate may represent merely the formation of homopolymer.

Incorporation of each of the ribonucleoside 5'-triphosphates by the SSS preparation was also tested in the presence and absence of the other three ribonucleotides. Table 9 shows that increased incorporation of any one labelled triphosphate was obtained when the other three ribonucleotides were also present in the reaction mixture. In comparison with the crude preparation there was however a significant rise in incorporation in the presence of only one added ribonucleoside 5'-triphosphate.

TABLE 8.

Ribonucleotide incorporation into RNA
by the crude ascites RNA polymerase

The reaction mixture contained 50 μ moles tris-HCl buffer, pH 7.5; 1 μ mole $MnCl_2$; 6 μ moles 2-mercapto-ethanol; 100 $m\mu$ moles EDTA; 80 μ g. native Landschutz DNA; 1.4 μ moles phosphocreatine and 70 μ g. phosphocreatine kinase and 0.1 ml. of enzyme (3 mg. protein/ml.) in a total volume of 0.6 ml..

100 $m\mu$ moles (1×10^7 d.p.m./ μ mole) of labelled ribonucleoside 5'-triphosphate were added as indicated. Additionally where shown 100 $m\mu$ moles of unlabelled ribonucleoside 5'-triphosphates were added.

In table (i) 240 μ moles $(NH_4)_2SO_4$ added.

In table (ii) no $(NH_4)_2SO_4$ added.

The reaction mixtures were incubated at 37° for 30 min.

TABLE 8.

Ribonucleotide incorporation into RNA
by the crude ascites RNA polymerase

| Labelled ribonucleotide | Additions | (i) Incorporation of ribonucleotide μmoles/mg. prot. | (ii) Incorporation of ribonucleotide μmoles/mg. prot. |
|----------------------------|-------------------|---|--|
| $[^3\text{H}]$ ATP | None | 0.28 | 0.45 |
| $[^3\text{H}]$ ATP | UTP GTP CTP | 1.67 | 0.45 |
| $[^3\text{H}]$ GTP | None | 0.23 | 0.05 |
| $[^3\text{H}]$ GTP | ATP UTP CTP | 1.73 | 0.21 |
| $[^3\text{H}]$ CTP | None | 0.58 | 0.48 |
| $[^3\text{H}]$ CTP | ATP GTP UTP | 1.5 | 0.52 |
| $[^3\text{H}]$ UTP | None | 0.70 | 0.40 |
| $[^3\text{H}]$ UTP | ATP GTP CTP | 1.22 | 0.44 |

TABLE 9.

Ribonucleotide incorporation into RNA by the
streptomycin sulphate supernatant fraction

The reaction mixture contained 50 μ moles tris-HCl buffer, pH 7.5; 1 μ mole MnCl_2 ; 6 μ moles 2-mercapto-ethanol; 100 μ moles EDTA; 80 μ g. native Landschutz DNA; 240 μ moles $(\text{NH}_4)_2\text{SO}_4$; 1.4 μ moles phosphocreatine and 70 μ g. phosphocreatine kinase and 0.1 ml. enzyme (3 mg. protein/ml.) in a total volume of 0.6 ml..

100 μ moles (1×10^7 d.p.m./ μ mole) of labelled ribonucleoside 5'-triphosphate were added as indicated. Additionally where shown 100 μ moles of unlabelled ribonucleoside 5'-triphosphate were added.

The samples were incubated at 37° for 30 min.

TABLE 9.

Ribonucleotide incorporation into RNA by the
streptomycin sulphate supernatant fraction

| Labelled ribonucleotide | Additions | Incorporation of ribonucleotide by SSS μmoles/mg. prot. | Incorporation of ribonucleotide by crude preparation μmoles/mg. prot. |
|----------------------------|-------------------|---|--|
| $[^3\text{H}]$ ATP | None | 2.20 | 0.28 |
| $[^3\text{H}]$ ATP | UTP GTP CTP | 2.80 | 1.67 |
| $[^3\text{H}]$ GTP | None | 1.4 | 0.23 |
| $[^3\text{H}]$ GTP | ATP UTP CTP | 3.12 | 1.73 |
| $[^3\text{H}]$ CTP | None | 1.96 | 0.58 |
| $[^3\text{H}]$ CTP | ATP GTP UTP | 2.56 | 1.5 |
| $[^3\text{H}]$ UTP | None | 2.02 | 0.70 |
| $[^3\text{H}]$ UTP | ATP GTP CTP | 2.05 | 1.22 |

A series of experiments was carried out with varying concentrations of actinomycin D (Fig. 30). Initially these were performed using $[^3\text{H}]$ UTP. In the absence of ammonium sulphate only limited inhibition (40%) was observed, but in the presence of ammonium sulphate 80% inhibition occurred at an actinomycin D concentration of 5 $\mu\text{g.}/\text{assay}$. Since it seemed likely that in the absence of ammonium sulphate much of the product was polyuridylylate, the experiments with labelled UTP were repeated with $[^3\text{H}]$ GTP. It was found that in the presence of ammonium sulphate 75% inhibition was observed at an actinomycin D concentration of 5 $\mu\text{g.}/\text{assay}$ while in the absence of ammonium sulphate only 20% inhibition occurred.

A similar series of experiments was carried out to determine the effect of actinomycin D on the SSS preparation (Fig. 31). Here again the initial experiments were performed using $[^3\text{H}]$ UTP. In the presence of ammonium sulphate 20% inhibition occurred at an actinomycin D concentration of 5 $\mu\text{g.}/\text{assay}$. The experiments were repeated using $[^3\text{H}]$ GTP but the same level of inhibition was obtained.

The drop in actinomycin D inhibition which occurs with the crude enzyme in the absence of ammonium sulphate is in agreement with the suggestion already mentioned that under these conditions ribonucleotide incorporation is largely into homopolymers. It would therefore seem probable that the 20% inhibition which occurs

FIGURE 30

The effects of addition of varying concentrations of actinomycin D on the activity of the crude RNA polymerase

The reaction mixture (0.6 ml.) contained 50 μ moles tris/HCl buffer, pH 7.5; 1 μ mole MnCl_2 ; 6 μ moles 2-mercapto-ethanol; 100 μ moles EDTA; 80 μ g. native Landschutz ascites tumour DNA; 100 μ moles each of ATP, GTP, CTP and UTP; 1.4 μ moles phosphocreatine and 70 μ g. phosphocreatine kinase and 0.1 ml. of enzyme (3 mg. protein/ml.)

- A. contained in addition $\left[{}^3\text{H}\right]\text{UTP}$ (1×10^7 d.p.m./ μ mole)
B. contained in addition $\left[{}^3\text{H}\right]\text{GTP}$ (1×10^7 d.p.m./ μ mole)

Various concentrations of actinomycin D were added as indicated.

The samples were incubated at 37° for 30 min.

- represents 240 μ moles $(\text{NH}_4)_2\text{SO}_4$ present
—▲— no $(\text{NH}_4)_2\text{SO}_4$ present

FIGURE 30.

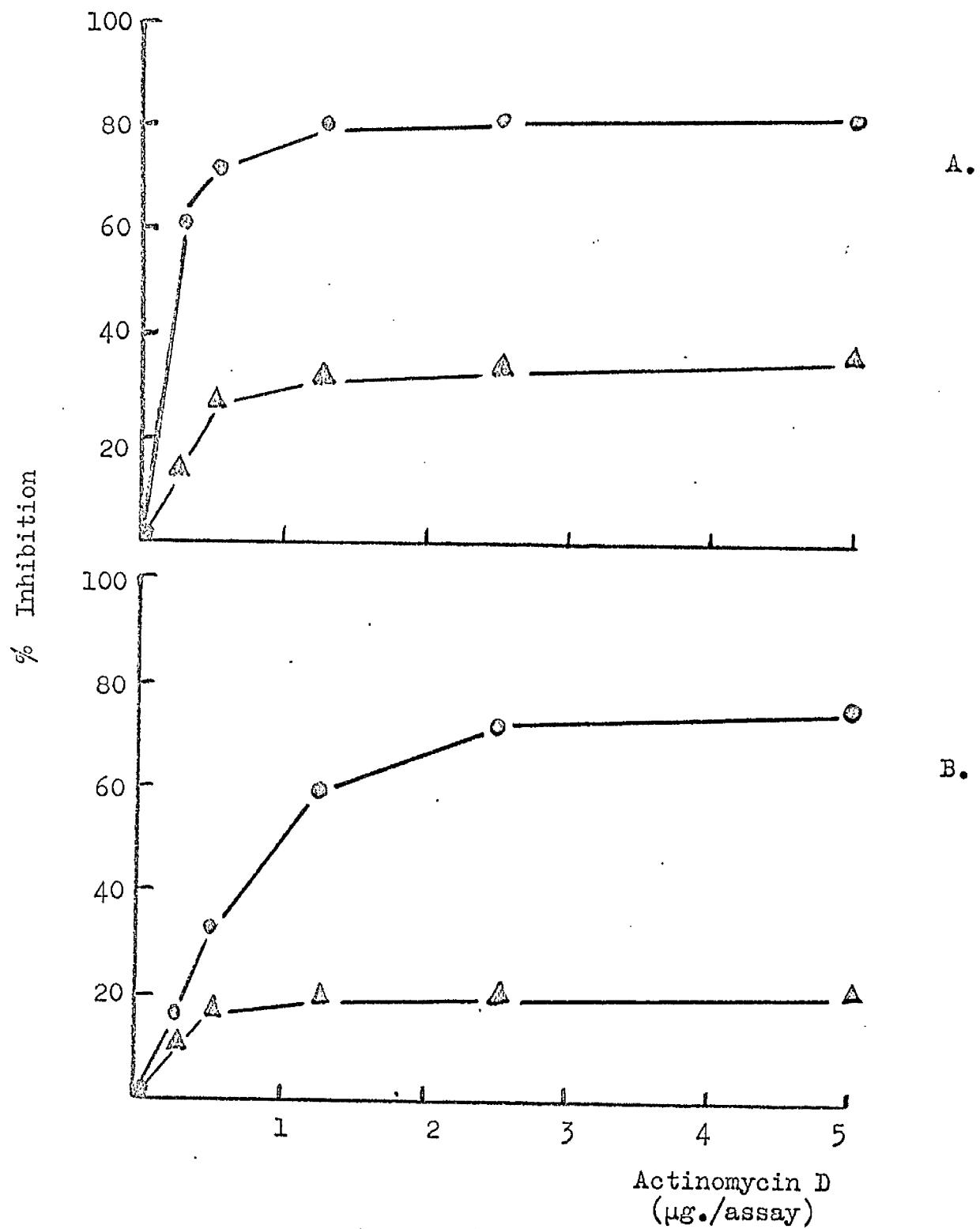


FIGURE 31

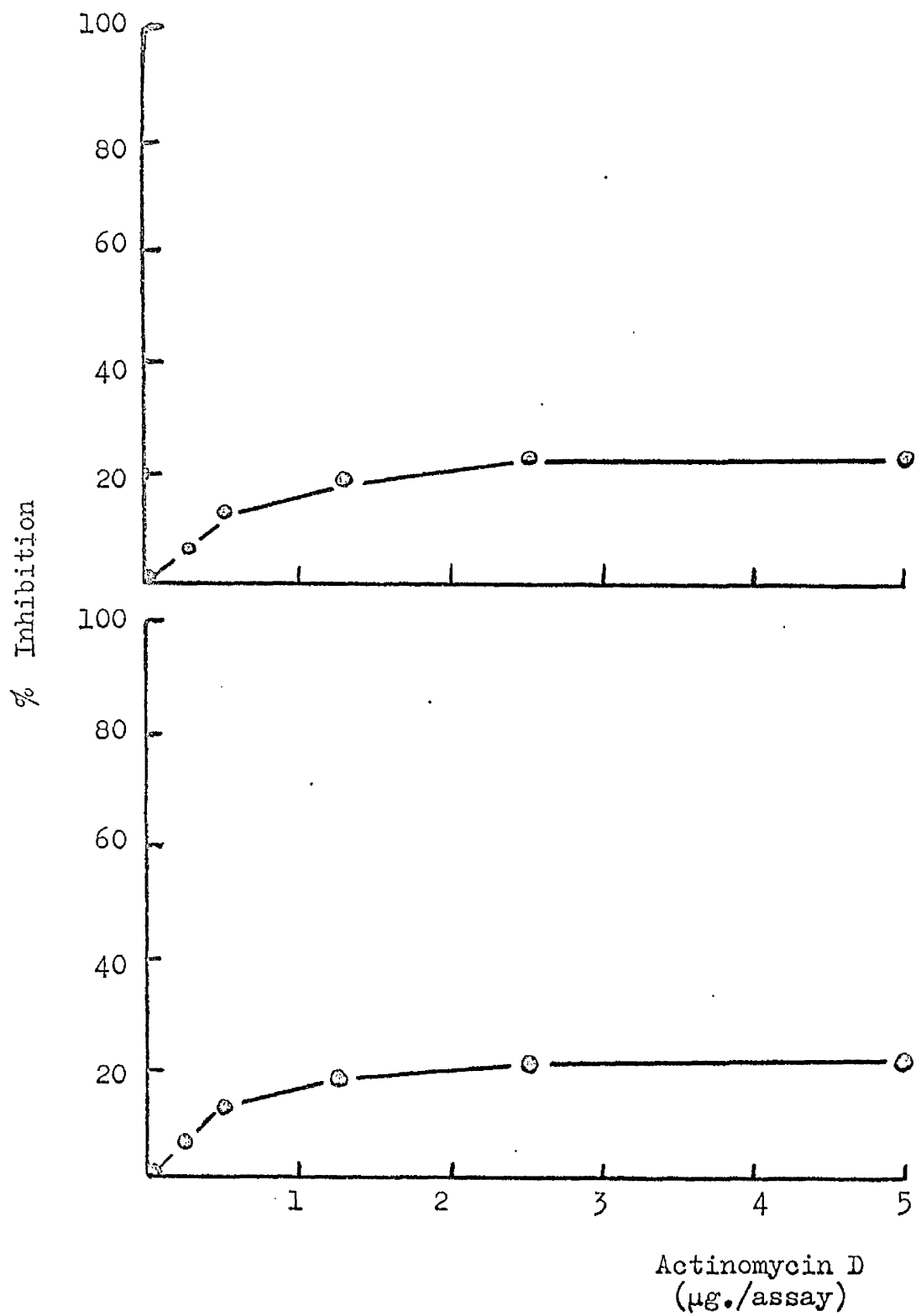
The effects of addition of varying concentrations of
actinomycin D on the incorporation of $[^3\text{H}]$ UMP
into RNA, by the SSS RNA polymerase

The reaction mixture was identical to that of Fig. 30 including 240 μmoles of $(\text{NH}_4)_2\text{SO}_4$.

- A. again contained $[^3\text{H}]$ UTP (1×10^7 d.p.m./ μmole)
- B. again contained $[^3\text{H}]$ GTP (1×10^7 d.p.m./ μmole)

Varying concentrations of actinomycin D were added as indicated and the samples were incubated at 37° for 30 min.

FIGURE 31.



with the SSS fraction in the presence of actinomycin D also reflects an increased homopolymer formation.

The presence of other enzymes in the polymerase preparation was to be expected and the material was assayed for DNA polymerase, DNase, RNase and alkaline phosphatase. Table 10 shows that in the crude preparation there was considerable DNA polymerase activity present. DNase, RNase and alkaline phosphatase were assayed in ionic and pH conditions appropriate to these enzymes. Since these conditions were somewhat different from those employed in the assay of RNA polymerase it seems likely that the levels of activity in Table 10 are higher than would be expected to occur under the routine conditions of assay of RNA polymerase. When the activities of DNase, RNase and alkaline phosphatase were measured in the purified RNA polymerase preparation, (Table 11) the specific activities of these enzymes were about 70% lower than in the crude RNA polymerase fraction. The specific activity of DNA polymerase in contrast, was seen to be apparently unaffected. This may suggest that the DNA polymerase is bound to a fraction of the DNA which is purified along with the RNA polymerase especially as the addition of exogenous denatured DNA caused little stimulation in deoxyribonucleotide incorporation.

The nature of the polyribonucleotide product formed by the crude and purified RNA polymerase fractions was investigated by extracting labelled RNA from the reaction mixtures and submitting

TABLE 10.

A summary of the activities of enzymes
found in the crude ascites preparation

| Enzyme | Activity |
|----------------------|---|
| RNA polymerase | 1.34 μ moles UMP incorporated/mg. protein |
| DNA polymerase | 408 μ moles dTMP incorporated/mg. protein |
| DNase | 50 μ moles deoxyribonucleotide liberated/mg. protein |
| RNase | 50 μ moles ribonucleotide liberated/mg. protein |
| Alkaline phosphatase | 150 μ moles phosphate liberated/mg. protein |

RNA polymerase:

The reaction mixture was as described in Fig. 1.

DNA polymerase:

The reaction mixture was as described under 'Methods' p.39.

DNase:

The reaction mixture was as described under 'Methods' p.39.

RNase:

The reaction mixture was as described under 'Methods' p.40.

Alkaline phosphatase:

The reaction mixture was as described under 'Methods' p.40.

The samples were incubated at 37° for 30 min.

TABLE 11.

A summary of the activities of enzymes found in the
streptomycin sulphate supernatant fraction

RNA polymerase:

The reaction mixture was as described in Fig. 1.

DNA polymerase:

The reaction mixture was as described under 'Methods' p.39.

DNase:

The reaction mixture was as described under 'Methods' p.39.

RNase:

The reaction mixture was as described under 'Methods' p.40.

Alkaline phosphatase:

The reaction mixture was as described under 'Methods' p.40.

The samples were incubated at 37° for 30 min.

TABLE 11.

A summary of the activities of enzymes found in the
streptomycin sulphate supernatant fraction

| Enzyme | Activity of SSS | Activity of Crude Preparation |
|----------------------|---|---|
| RNA Polymerase | 2.53 μ moles UMP incorp./ mg. prot. | 1.34 μ moles UMP incorp./ mg. prot. |
| DNA Polymerase | 400 μ moles dTMP incorp./ mg. prot. | 408 μ moles dTMP incorp./ mg. prot. |
| DNase | 11.6 μ moles deoxyribonucleotide liberated/mg. protein | 50 μ moles deoxyribonucleotide liberated/mg. protein |
| RNase | 16 μ moles ribonucleotide liberated/mg. protein | 50 μ moles ribonucleotide liberated/mg. protein |
| Alkaline Phosphatase | 40.3 μ moles Pi liberated/ mg. protein | 150 μ moles Pi liberated/ mg. protein |

it to analysis on sucrose density gradients. Figure 32 shows the result of such an experiment. The RNA polymerase reaction was carried out in the presence of ammonium sulphate by the crude polymerase fraction using $[^3\text{H}]\text{GTP}$. The labelled polyribonucleotide was run in the gradient with unlabelled Landschutz ascites tumour whole-cell RNA. The bulk of the radioactivity was found in a position corresponding to approximately 15s. A similar experiment was carried out with RNA formed using $[^3\text{H}]\text{UTP}$. Figure 33 shows that there was no alteration to the pattern of radioactivity obtained. In both of these experiments about 80% of total ribonucleotide incorporation is represented on the gradient. Since it had already been shown that in the absence of ammonium sulphate there was little incorporation of $[^3\text{H}]\text{GTP}$ (Table 8), $[^3\text{H}]\text{UTP}$ was employed when investigating the product of the crude polymerase in the absence of $(\text{NH}_4)_2\text{SO}_4$. Figure 34 shows that this RNA is much smaller, and here only 30% of the estimated RNA produced can be detected on the gradient. The polyribonucleotide product, formed by the SSS RNA polymerase in the presence of ammonium sulphate, using $[^3\text{H}]\text{GTP}$, (Figure 35) sediments in a position similar to that obtained with the crude polymerase in the presence of ammonium sulphate. Once again about 75% of the calculated ribonucleotide incorporation is represented on the gradient.

FIGURE 32

Sedimentation analysis in a sucrose density gradient of $[^3\text{H}]\text{GMP}$
labelled RNA formed by the crude RNA polymerase fraction,
in the presence of $(\text{NH}_4)_2\text{SO}_4$

A 5% to 20% linear sucrose gradient was formed. A mixture of unlabelled Landschutz ascites tumour whole-cell RNA and labelled polyribonucleotide was layered on top and the gradient centrifuged at 150,000g for 3 hr.. The gradient was then fractionated by the dropwise procedure. Each sample was diluted with 0.5 ml. H_2O , and the extinction measured at 256 m μ . The radioactivity contained in each fraction was then assayed using a dioxane based scintillator.

—●—●— whole-cell RNA (E_{256})
—▲—▲— labelled polyribonucleotide (d.p.m.)

FIGURE 32.

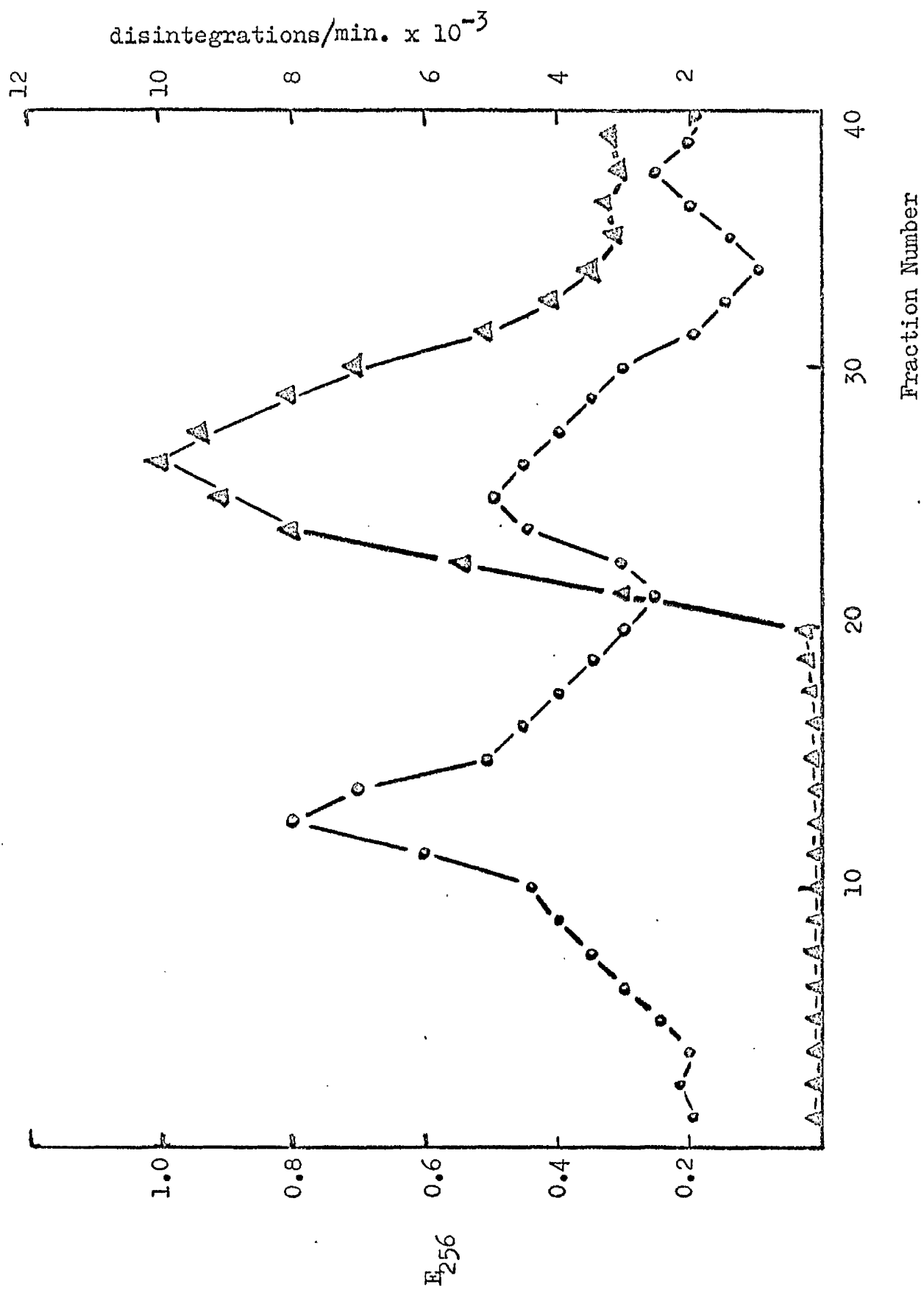


FIGURE 33

Sedimentation analysis in a sucrose density gradient of $[^3\text{H}]$ UMP
labelled RNA formed by the crude RNA polymerase fraction,
in the presence of $(\text{NH}_4)_2\text{SO}_4$

The gradients were prepared and fractionated as described in
Figure 32.

—●—●— whole cell RNA (E_{256})
—▲—▲— labelled polyribonucleotide (d.p.m.)

FIGURE 33.

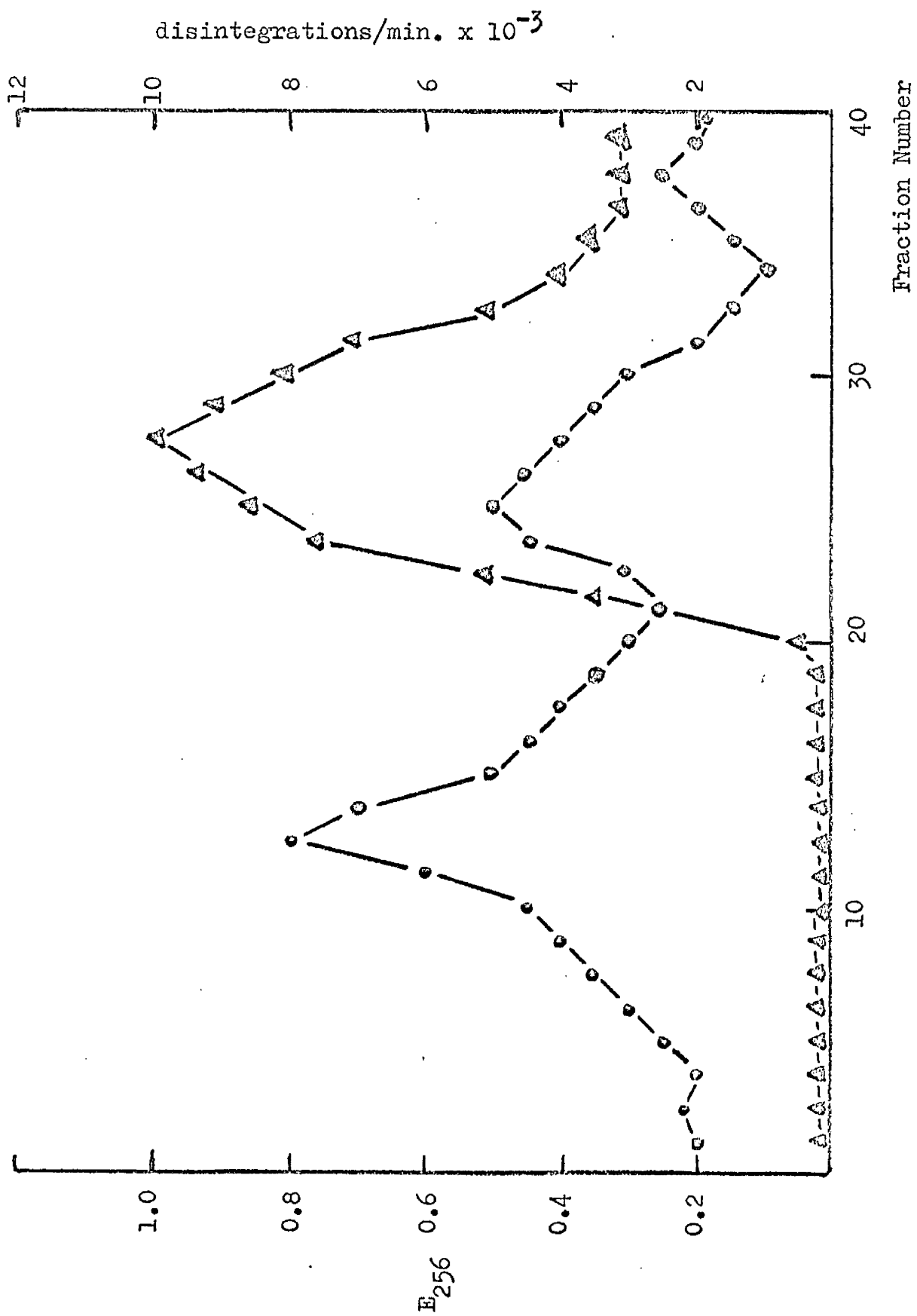


FIGURE 34

Sedimentation analysis in a sucrose density gradient of $[^3\text{H}]\text{UMP}$
labelled RNA formed by the crude RNA polymerase fraction,
in the absence of $(\text{NH}_4)_2\text{SO}_4$

A 5% to 20% linear sucrose gradient was formed. A mixture of unlabelled Landschutz ascites tumour whole-cell RNA and labelled polyribonucleotide was layered on top and the gradient centrifuged at 150,000g for 3 hr.. The gradient was then fractionated by the dropwise procedure. Each sample was diluted with 0.5 ml. H_2O , and the extinction measured at 256 m μ . The radioactivity contained in each fraction was then assayed using a dioxane based scintillator.

—●—●—

whole-cell RNA (E_{256})

—▲—▲—

labelled polyribonucleotide (d.p.m.)

FIGURE 34.

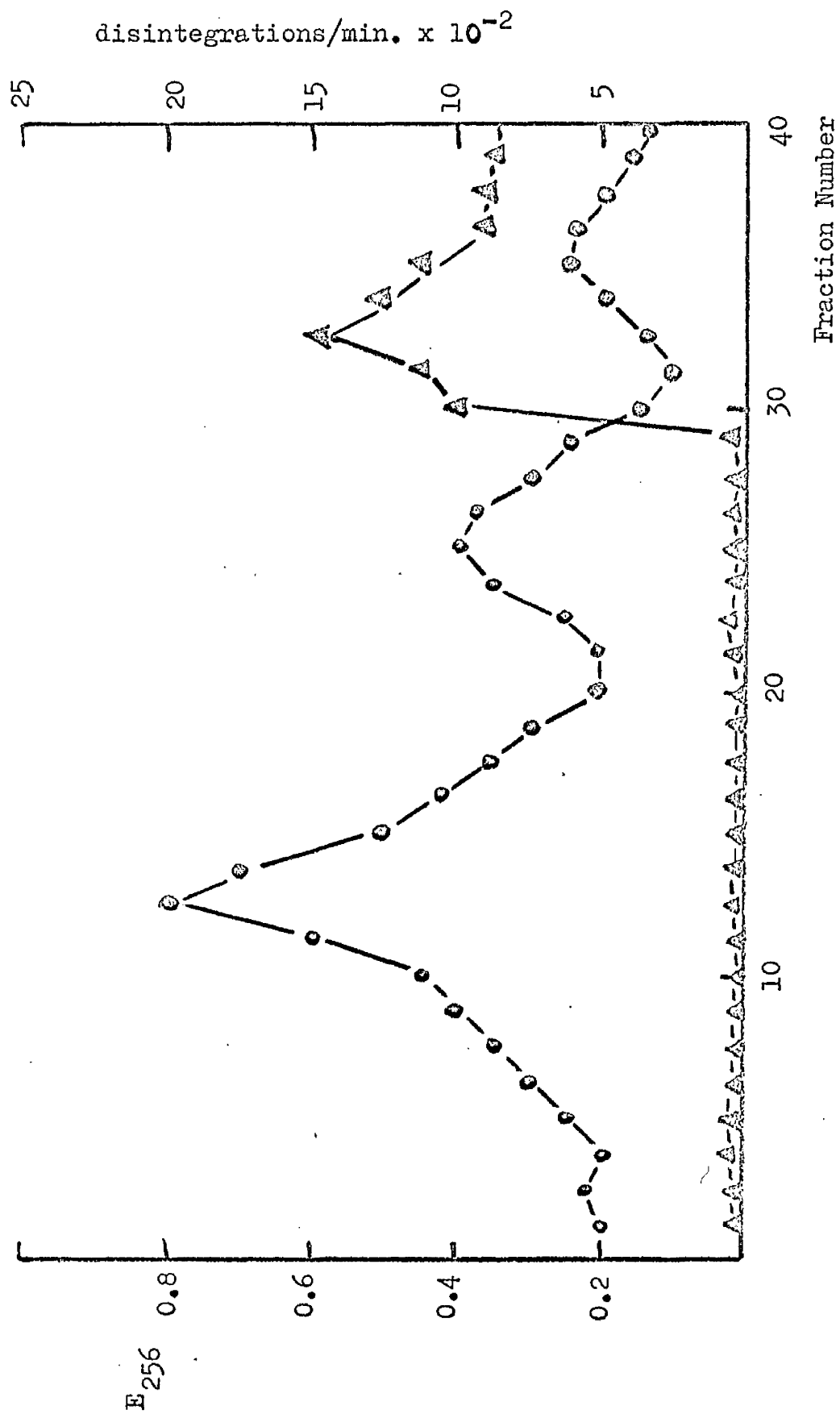


FIGURE 35

Sedimentation analysis in a sucrose density gradient of $[^3\text{H}]$ GTP

labelled RNA formed by the SSS RNA polymerase fraction,

in the presence of $(\text{NH}_4)_2\text{SO}_4$

A 5% to 20% linear sucrose gradient was formed. A mixture of Landschutz ascites tumour whole-cell RNA and labelled polyribonucleotide was layered on top and the gradient centrifuged at 150,000g for 3 hr.. The gradient was then fractionated by the dropwise procedure. Each sample was diluted with 0.5 ml. H_2O , and the extinction measured at 256 m μ . The radioactivity contained in each fraction was then assayed in a dioxane based scintillator.

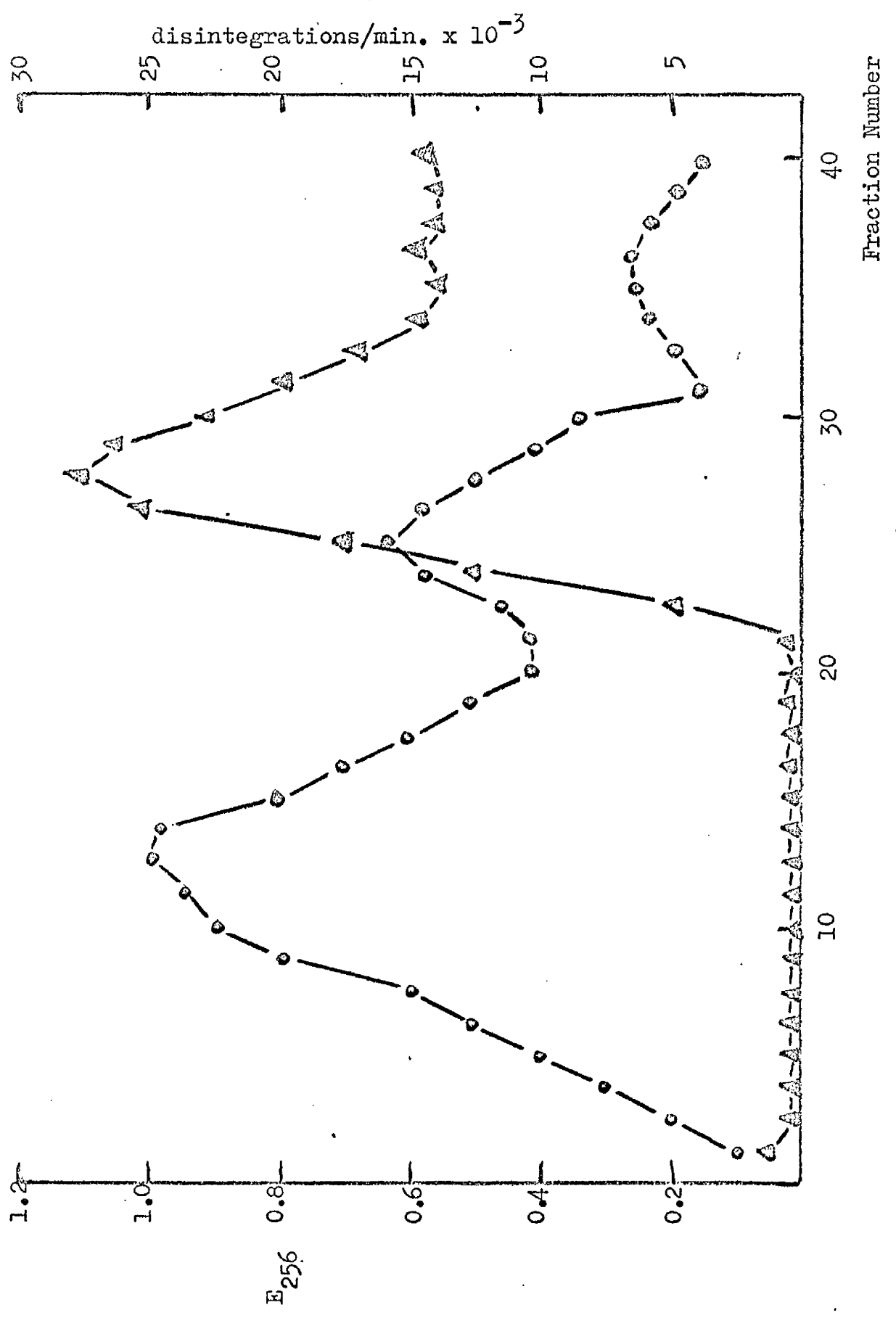
—●—●—

whole-cell RNA (E_{256})

—▲—▲—

labelled polyribonucleotide (d.p.m.)

FIGURE 35.



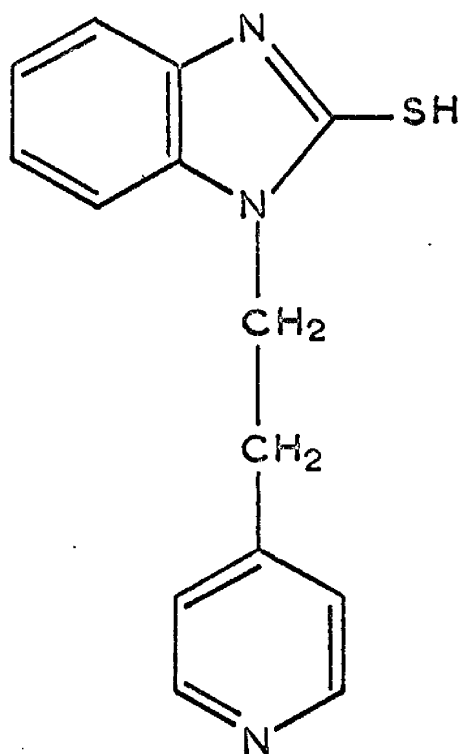
V. Experiments involving the drug 2-mercapto-1(β -4-pyridethyl) benzimidazole (MPB)

MPB was originally examined as an inhibitor of viral replication. It appears to act in a similar way to actinomycin D in that it inhibits those viruses which require DNA-directed RNA synthesis (Walters, Burke and Skehel (1967), Bucknall (1967)). It is however unlikely that the two inhibitors act in exactly the same way since their structures are so different. Bucknall (1967) has shown that MPB inhibits nucleic acid synthesis and virus multiplication in vivo, but MPB can also be shown to inhibit nucleoside phosphorylation (Skehel, Hay, Burke and Cartwright (1967)) which means that its effect on the incorporation of uridine and thymidine into RNA and DNA observed by Bucknall could be due to inhibition of nucleoside phosphorylation, rather than to inhibition of nucleic acid synthesis. It was therefore decided to investigate the compound in an in vitro system.

The effects of MPB on purified RNA polymerase from M. lysodeikticus have been tested (Keir, personal communication) but no inhibition was observed. It seemed possible however that inhibition would only occur in the presence of some component other than the active enzyme or that the form of the bacterial enzyme was altered in some way during the purification process. This suggested that the crude enzyme from Landschutz ascites tumour was a suitable system for investigation with MPB. The compound MPB is

FIGURE 36.

M.P.B.



highly insoluble at 37° in water at concentrations above 25 $\mu\text{g.}/\text{ml.}$. It was however soluble in dimethyl sulphoxide and for subsequent studies was routinely dissolved in this.

(a) Inhibition of RNA polymerase

MPB was found to cause a higher degree of inhibition of RNA polymerase if the enzyme was incubated at 37° for 15 min. prior to assay. The effect of such an incubation of the enzyme at 37° has already been shown in Fig. 9. In further studies with MPB the normal assay constituents were incubated in the presence of DMS but in the absence of the four ribonucleoside triphosphates which were added at the beginning of the second period of incubation. A time course of the subsequent incorporation is shown in Fig. 37. Comparison of Figs. 9 and 37 shows that in this system there is little inhibition due to DMS.

Fig. 38 shows the effect on the activity of the enzyme of a prior incubation for 15 min. at 37° in the presence of 60 $\mu\text{g.}$ MPB. While the initial rate of the reaction seems unchanged, the rate of incorporation is reduced by MPB at times longer than 15 min.. Various levels of MPB concentration were examined under the same conditions. Fig. 39 shows that there is no further decrease in activity at concentrations of MPB above 100 $\mu\text{g.}/\text{ml.}$. While this could be due to the poor solubility of MPB in the reaction mixture it is to be noted that in Fig. 43 at low protein concentrations lower amounts of MPB (25 $\mu\text{g.}$ and 50 $\mu\text{g.}$) produce a similar effect.

FIGURE 37

The effect of a prior incubation of the crude RNA polymerase
preparation in the presence of dimethyl sulphoxide

FIGURE 37.

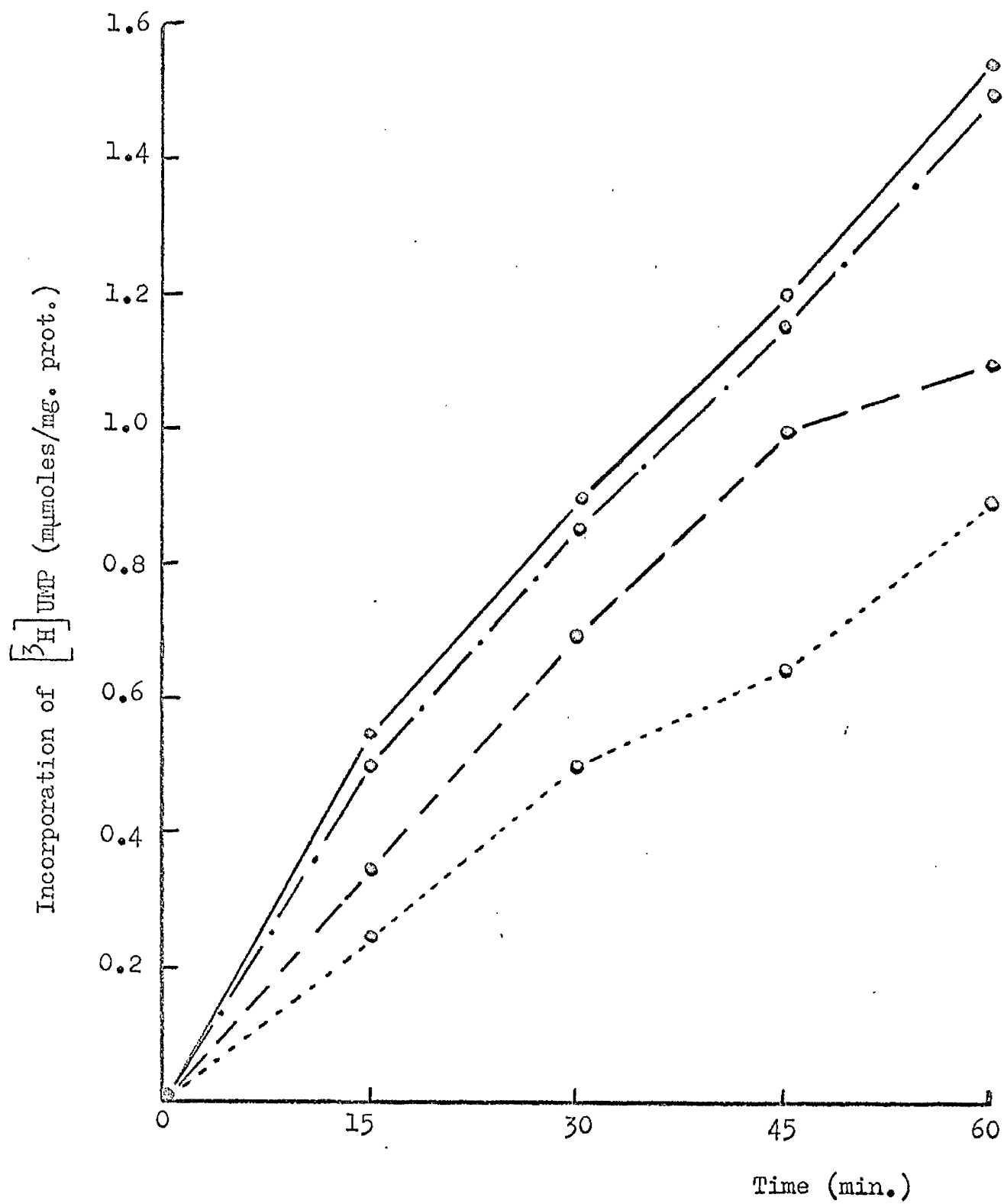


FIGURE 38

The effect of a prior incubation of the crude RNA polymerase
preparation in the presence of MPB

The reaction mixture contained 50 μ moles tris-HCl buffer, pH 7.5; 1 μ mole MnCl_2 ; 6 μ moles 2-mercapto-ethanol; 240 μ moles $(\text{NH}_4)_2\text{SO}_4$; 100 μ moles each of ATP, GTP, CTP and UTP; $[\text{}^3\text{H}]$ UTP (1×10^7 d.p.m./ μ mole); and 0.1 ml. of enzyme (3 mg. protein/ml.) in a total volume of 0.6 ml.

In addition,

- - ① - - represents 0.04 ml. H_2O added.
- ① ——— represents 0.04 ml. DMS added.
- . . — ① — . . — represents 60 μ g. MPB added in 0.04 ml. DMS.

The samples were given a prior incubation at 37° for 15 min.. The ribonucleoside triphosphates were then added and the time course of the subsequent incorporation at 37° followed.

FIGURE 38.

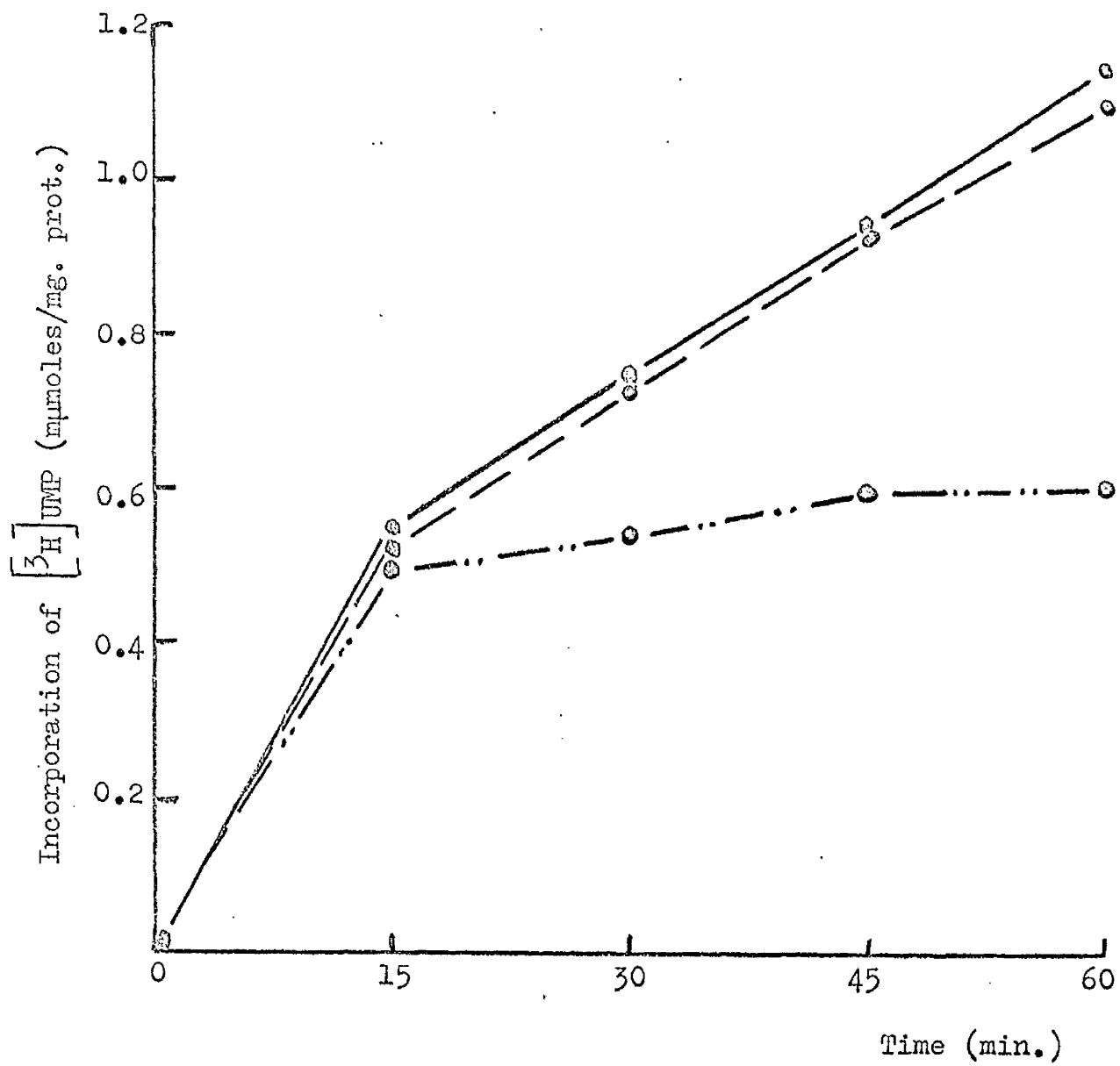


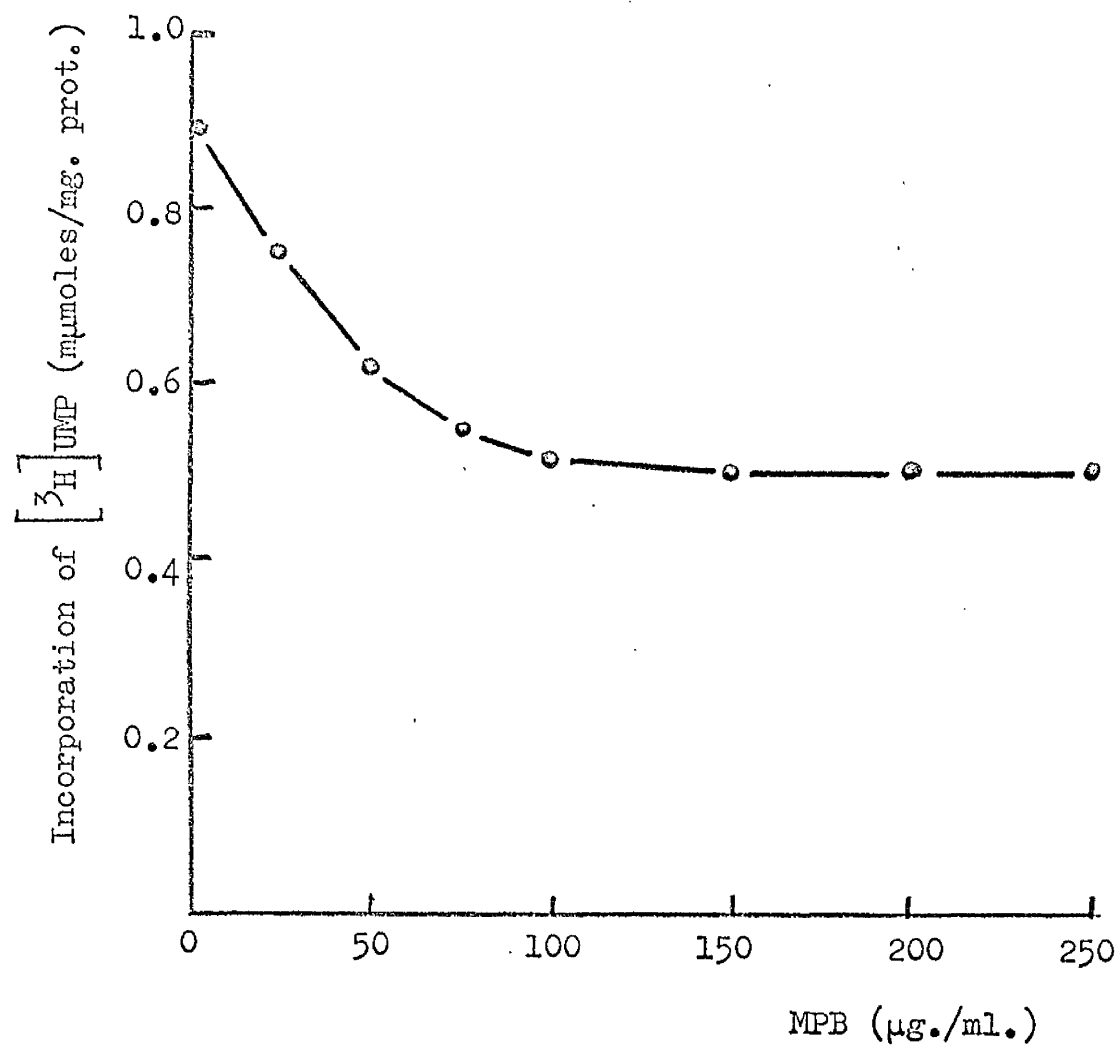
FIGURE 39

The effect of increasing concentrations of MPB
on the activity of the crude RNA polymerase

The reaction mixture was identical to that described in Fig. 38. In addition, varying concentrations of MPB were added in 0.04 ml. of DMS.

The samples were given a prior incubation at 37° for 15 min. and as before the ribonucleotides added and incubation at 37° continued for a further 30 min.

FIGURE 39.



Since the effects of MPB might have been on some component of the assay system other than the RNA polymerase, experiments were carried out using a simplified system containing only ammonium sulphate, manganese ions, ribonucleoside triphosphates, tris/HCl and enzyme. In the absence of ammonium sulphate no inhibition was noted with MPB Fig. 40, while in its presence up to 50% inhibition may occur with the drug. Similar results were obtained with both $[^3\text{H}]$ UTP and $[^3\text{H}]$ GTP.

Attempts were made to investigate the mode of action of MPB. Varying amounts of ammonium sulphate were tested as is shown in Fig. 41 but the degree of inhibition caused by the drug (100 $\mu\text{g.}/\text{ml.}$) remained fairly constant.

The effects of varying the concentration of manganese chloride, above 0.4 $\mu\text{moles}/\text{assay}$ were observed. Fig. 42 shows there is no change in the percentage inhibition in the presence of MPB (100 $\mu\text{g.}/\text{ml.}$).

While maintaining the MPB concentration at 15 $\mu\text{g.}$ the volume of crude enzyme preparation added was altered. Maximal inhibition (Fig. 43) was reached when the protein/MPB ratio was 4:1 i.e. at 60 $\mu\text{g.}$ protein. When the level of MPB was raised to 30 $\mu\text{g.}$ using the same enzyme preparation, maximal inhibition occurred with 120 $\mu\text{g.}$ protein. At 300 $\mu\text{g.}$ protein it is interesting to note that the inhibition due to the lower concentration of MPB is reduced but not that due to the higher. This suggests that a component of the

FIGURE 40

The effect of the presence or absence of $(\text{NH}_4)_2\text{SO}_4$
on MPB inhibition of the crude RNA polymerase

The reaction mixture contained 50 μmoles tris-HCl buffer, pH 7.5; 1 μmole MnCl_2 ; 6 μmoles 2-mercapto-ethanol; 100 μmoles each of ATP, GTP, CTP and UTP; $[\text{}^3\text{H}]\text{UTP}$ or $[\text{}^3\text{H}]\text{GTP}$ (1×10^7 d.p.m./ μmole); and 0.1 ml. of enzyme (3 mg. protein/ml.) in a total volume of 0.6 ml.

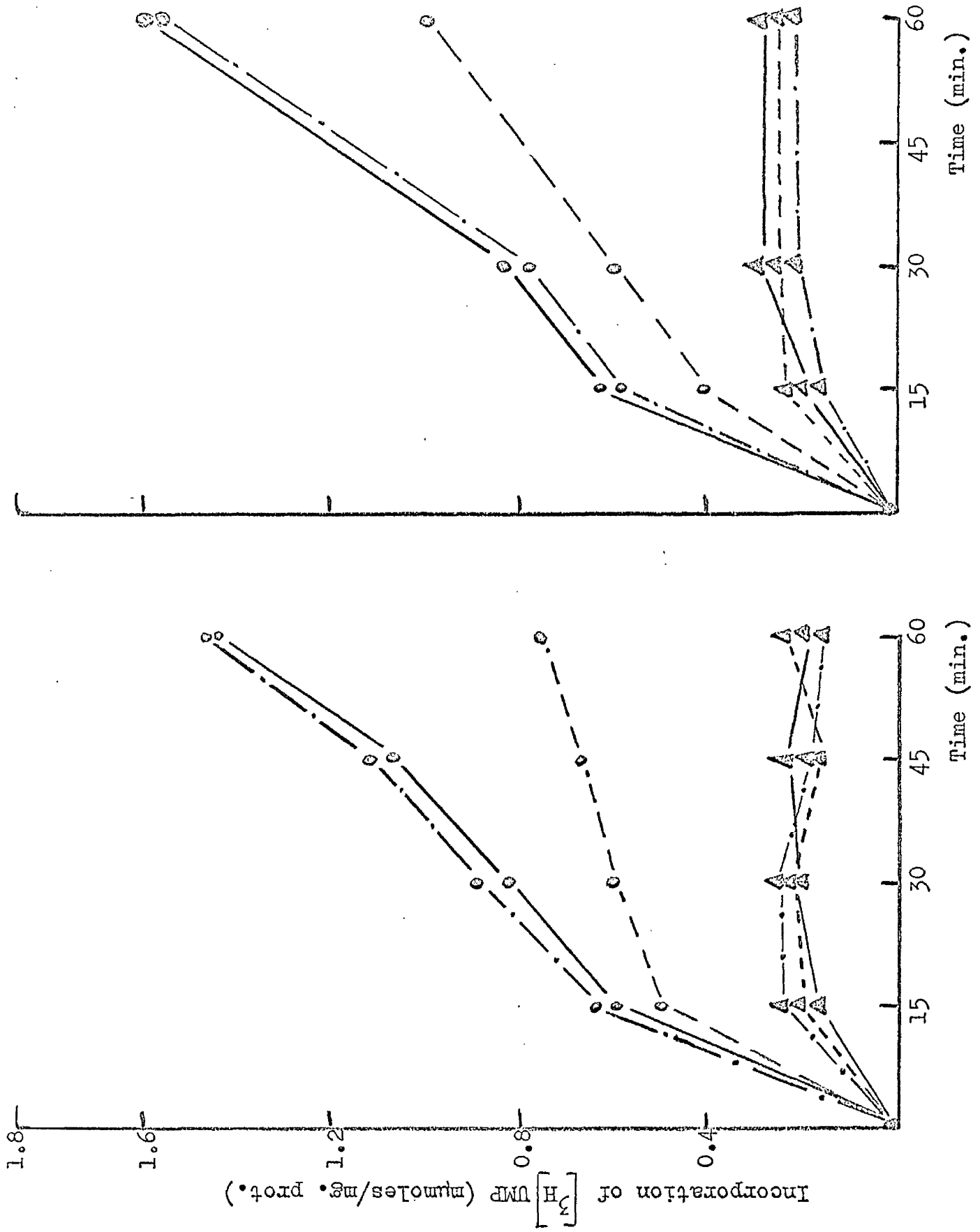
In addition,

- represents 0.04 ml. H_2O and 240 μmoles $(\text{NH}_4)_2\text{SO}_4$ added.
- represents 0.04 ml. DMS and 240 μmoles $(\text{NH}_4)_2\text{SO}_4$ added.
- represents 0.04 ml. DMS containing 60 $\mu\text{g.}$ MPB and 240 μmoles $(\text{NH}_4)_2\text{SO}_4$ added.
- represents 0.04 ml. H_2O added.
- represents 0.04 ml. DMS added.
- represents 0.04 ml. DMS containing 60 $\mu\text{g.}$ MPB added.

The samples were given a prior incubation at 37° for 15 min.. The ribonucleotides were added as before and the time course of the subsequent incorporation at 37° followed.

- A. contained $[\text{}^3\text{H}]\text{UTP}$
- B. contained $[\text{}^3\text{H}]\text{GTP}$

FIGURE 40.



A.

B.

FIGURE 41

The effect of various concentrations of $(\text{NH}_4)_2\text{SO}_4$ on
the MPB inhibition of the crude RNA polymerase

The reaction mixture contained 50 μ moles tris-HCl buffer, pH 7.5; 1 μ mole MnCl_2 , 6 μ moles 2-mercapto-ethanol; 100 μ moles each of ATP, GTP, CTP and UTP; $[^3\text{H}]$ UTP (1×10^7 d.p.m./ μ mole); and 0.1 ml. of enzyme (3 mg. protein/ml.) in a total volume of 0.6 ml.

In addition,

—●—

represents the presence of 0.04 ml. DMS and varying concentrations of $(\text{NH}_4)_2\text{SO}_4$ as shown.

—▲—

represents the presence of 0.04 ml. DMS containing 60 μ g. MPB, and varying concentrations of $(\text{NH}_4)_2\text{SO}_4$ as indicated.

The samples were given a prior incubation at 37° for 15 min.. The ribonucleotides were added as before and the time course of the subsequent incorporation at 37° followed.

FIGURE 41.

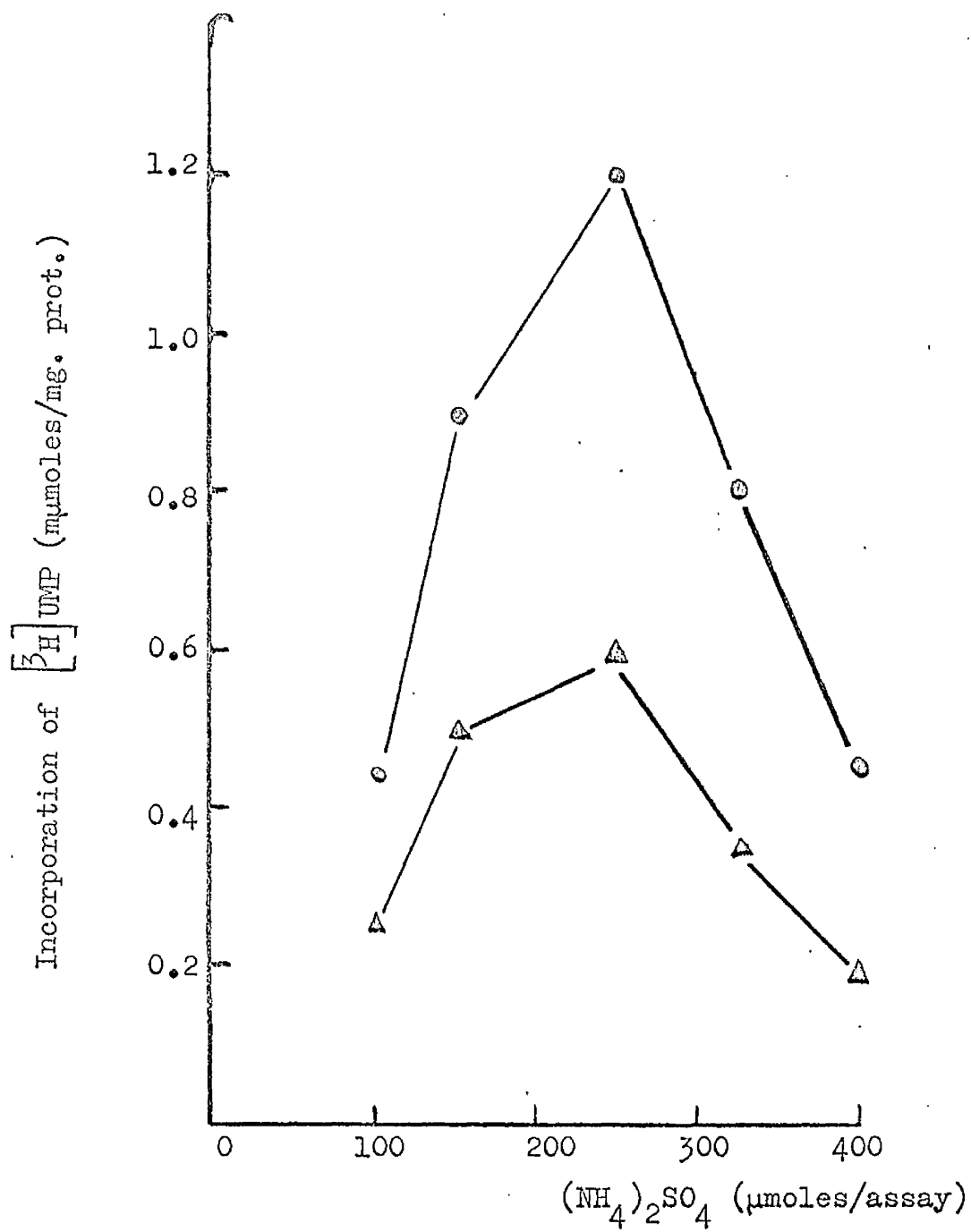


FIGURE 42

The effect of various concentrations of Mn^{2+} on the MPB
inhibition of the crude RNA polymerase

The reaction mixture contained 50 μ moles tris-HCl buffer, pH 7.5; 240 μ moles $(NH_4)_2SO_4$; 6 μ moles 2-mercapto-ethanol; 100 μ moles each of ATP, GTP, CTP and UTP; $[^3H]UTP$ (1×10^7 d.p.m./ μ mole); and 0.1 ml. of enzyme (3 mg. protein/ml.) in a total volume of 0.6 ml.

In addition,

—●— represents the presence of 0.04 ml. DMS and varying concentrations of Mn^{2+} as indicated.

—▲— represents the presence of 0.04 ml. DMS containing 60 μ g. MPB, and varying concentrations of Mn^{2+} as indicated.

The samples were given a prior incubation at 37° for 15 min.. The ribonucleotides were added as before, and the time course of the subsequent incorporation at 37° followed.

FIGURE 42.

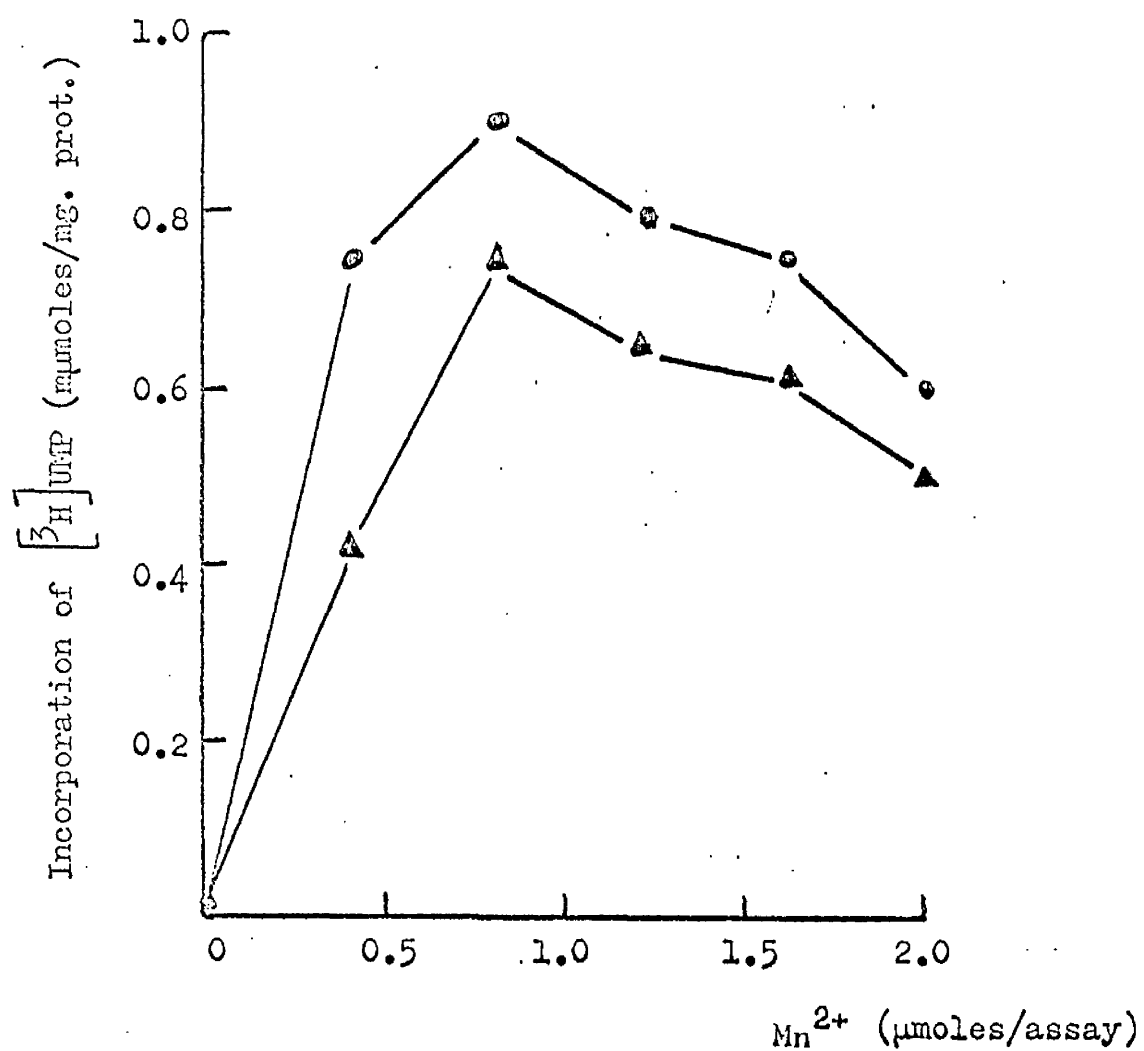


FIGURE 43

The effect of various protein concentrations on the MPB
inhibition of the crude RNA polymerase

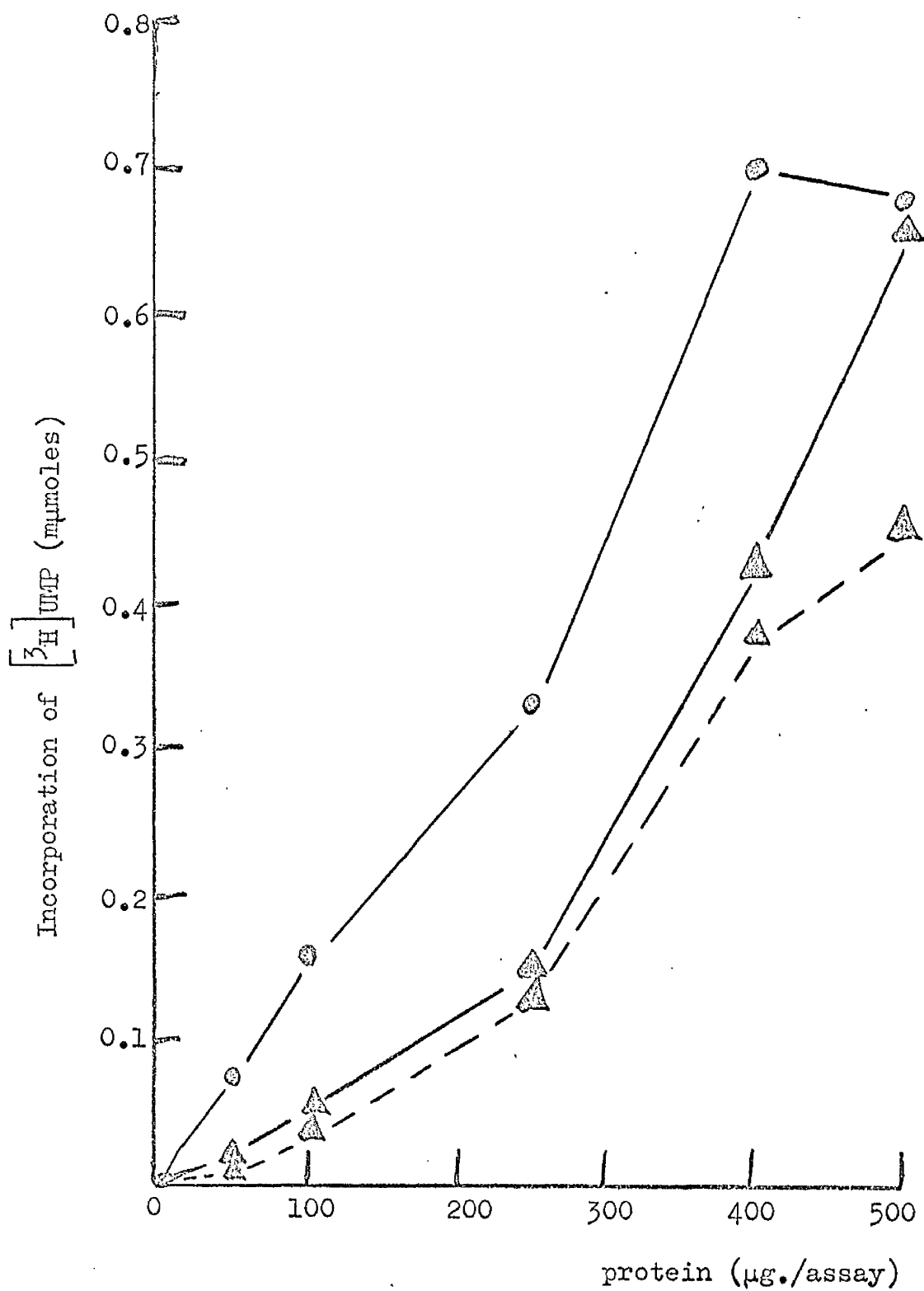
The reaction mixture contained 50 μ moles tris-HCl buffer, pH 7.5; 1 μ mole MnCl_2 , 240 μ moles $(\text{NH}_4)_2\text{SO}_4$; 6 μ moles 2-mercapto-ethanol; 100 μ moles each of ATP, GTP, CTP and UTP; $[\text{}^3\text{H}]\text{UTP}$ (1×10^7 d.p.m./ μ mole); and various amounts of enzyme as indicated, in a total volume of 0.6 ml.

In addition,

- represents 0.04 ml. DMS added.
- ▲— represents 0.04 ml. DMS containing 15 μ g. MPB added.
- - -▲- - - represents 0.04 ml. DMS containing 30 μ g. MPB added.

The samples were given a prior incubation at 37° for 15 min.. The ribonucleotides were added as before, and the time course of the subsequent incorporation at 37° followed.

FIGURE 43.



crude enzyme preparation is reacting with the MPB and that the addition of excess of this component can partially reverse the inhibition due to MPB.

Since it seemed possible that MPB formed complexes with -SH groups on the protein present, varying amounts of 2-mercapto-ethanol were added (Fig. 44). Above 3 μ moles/assay increasing concentrations of 2-mercapto-ethanol do not reduce the effect of MPB. In the presence of 20 μ moles/assay however the activity of the enzyme drops, in the absence of MPB but the presence of the drug minimises any further decrease in incorporation.

Various levels of the ribonucleoside triphosphates were similarly examined (Fig. 45) but once again there was no apparent reversal of inhibition.

The effect of increasing concentrations of MPB on the SSS polymerase fraction were also tested. Fig. 46 shows that the maximum level of inhibition was 30% which is lower than the inhibition observed with the crude enzyme. A similar level of inhibition was obtained with both labelled UTP and GTP.

(b) The interaction between MPB and nucleic acid derivatives

Since MPB may interact with the DNA component of the enzyme preparation experiments were performed to determine whether any such complex could be detected spectrophotometrically. Spectra were plotted using the Unicam SP 800. The spectrum of MPB in water and DMS is shown in Fig. 47. There is an additional absorption

FIGURE 44

The effect of increasing concentrations of 2-mercapto-ethanol
on the MPB inhibition of the crude RNA polymerase

The reaction mixture contained 50 μ moles tris-HCl buffer, pH 7.5; 1 μ mole MnCl_2 ; 240 μ moles $(\text{NH}_4)_2\text{SO}_4$; 100 μ moles each of ATP, GTP, CTP and UTP; $[\text{}^3\text{H}]\text{UTP}$ (1×10^7 d.p.m./ μ mole); 0.1 ml. of enzyme (3 mg. protein/ml.) and various concentrations of 2-mercapto-ethanol in a total volume of 0.6 ml.

In addition,

—●—

represents 0.04 ml. DMS added.

—▲—

represents 0.04 ml. DMS containing 30 μ g. MPB added.

-▲--

represents 0.04 ml. DMS containing 120 μ g. MPB added.

The samples were given a prior incubation at 37° for 15 min.. The ribonucleotides were added as before, and the time course of the subsequent incorporation at 37° followed.

FIGURE 44.

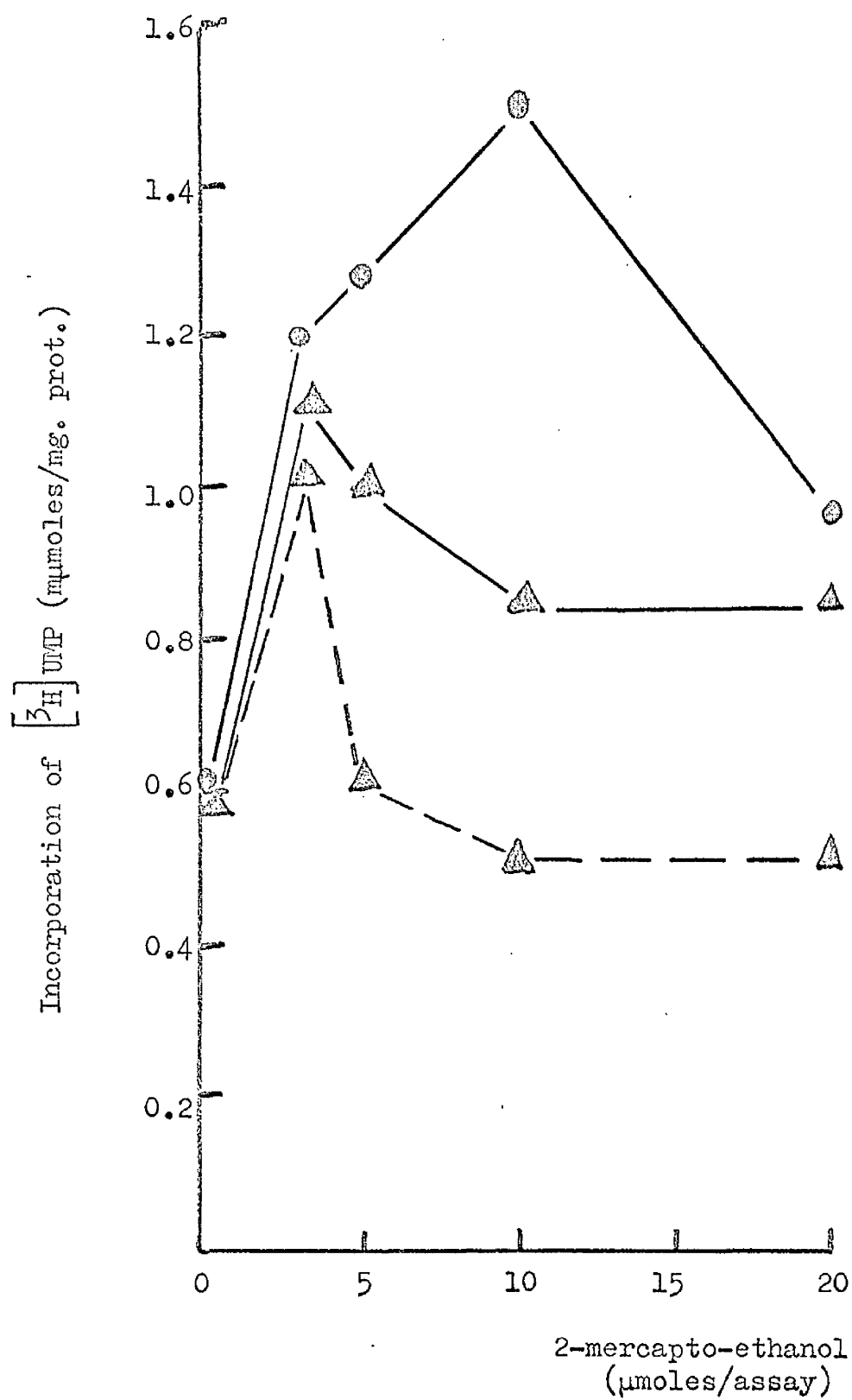


FIGURE 45

The effect of increasing concentrations of ribonucleotides
on the MPB inhibition of the crude RNA polymerase

The reaction mixture contained 50 μ mole tris-HCl buffer, pH 7.5; 1 μ mole MnCl_2 ; 240 μ moles $(\text{NH}_4)_2\text{SO}_4$; 6 μ moles 2-mercapto-ethanol; 0.1 ml. of enzyme (3 mg. protein/ml.), various concentrations of all four ribonucleotides as indicated and $\left[\begin{smallmatrix} 3 \\ \text{H} \end{smallmatrix} \right] \text{UTP}$ (1×10^7 d.p.m./ μ mole) in a total volume of 0.6 ml.

In addition,

— 0 —

represents 0.04 ml. DMS added.

— Δ —

represents 0.04 ml. DMS containing 60 μ g. MPB added.

The samples were given a prior incubation at 37° for 15 min.. The ribonucleotides were added as before, and the time course of the subsequent incorporation at 37° followed.

FIGURE 45.

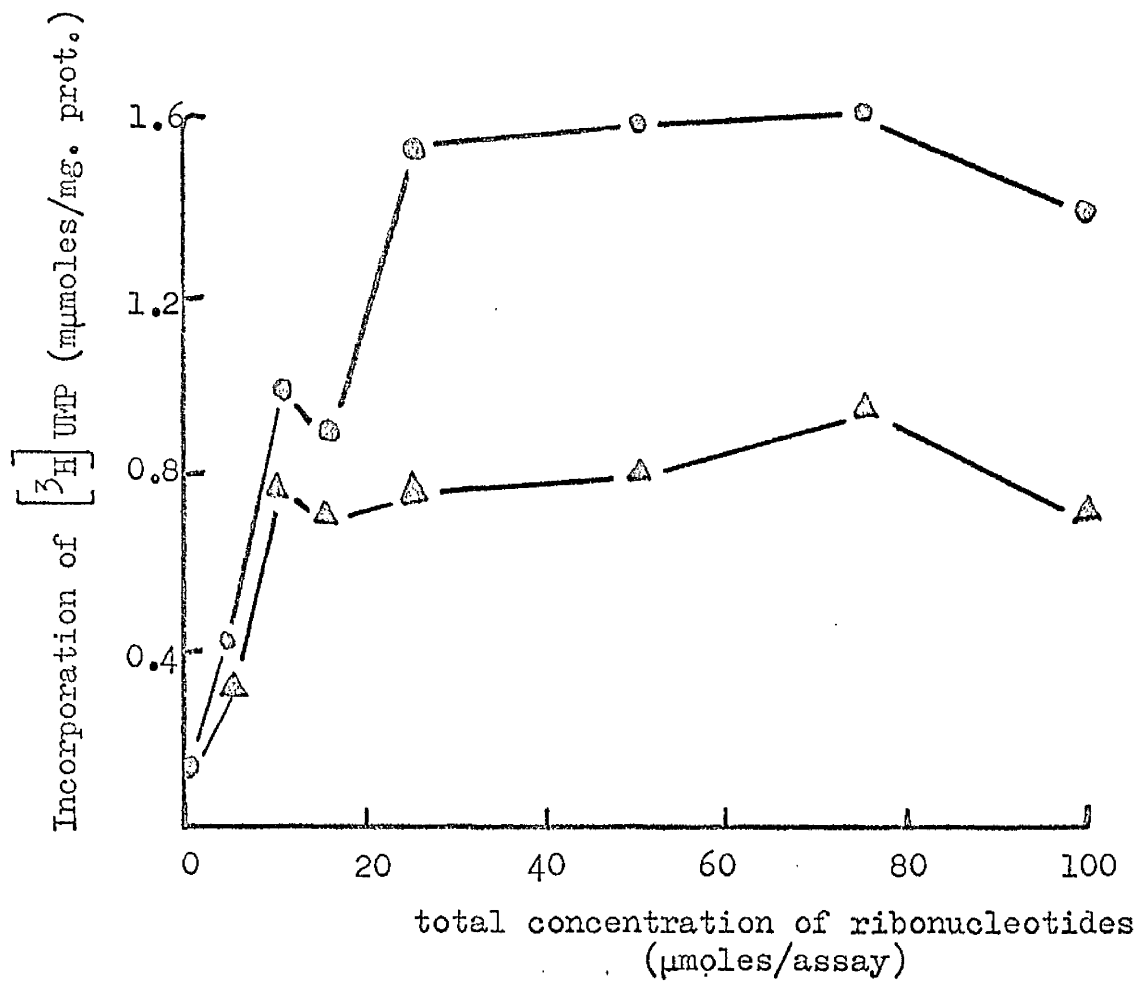


FIGURE 46

The effect of increasing concentrations of MPB
on the incorporation of $[^3\text{H}]$ UMP into RNA,
by the SSS RNA polymerase

The reaction mixture was identical to that of Fig. 38. In addition, varying concentrations of MPB were added in 0.04 ml. DMS. The samples were incubated at 37° for 30 min.

- A. contained $[^3\text{H}]$ UTP.
- B. contained $[^3\text{H}]$ GTP.

FIGURE 46.

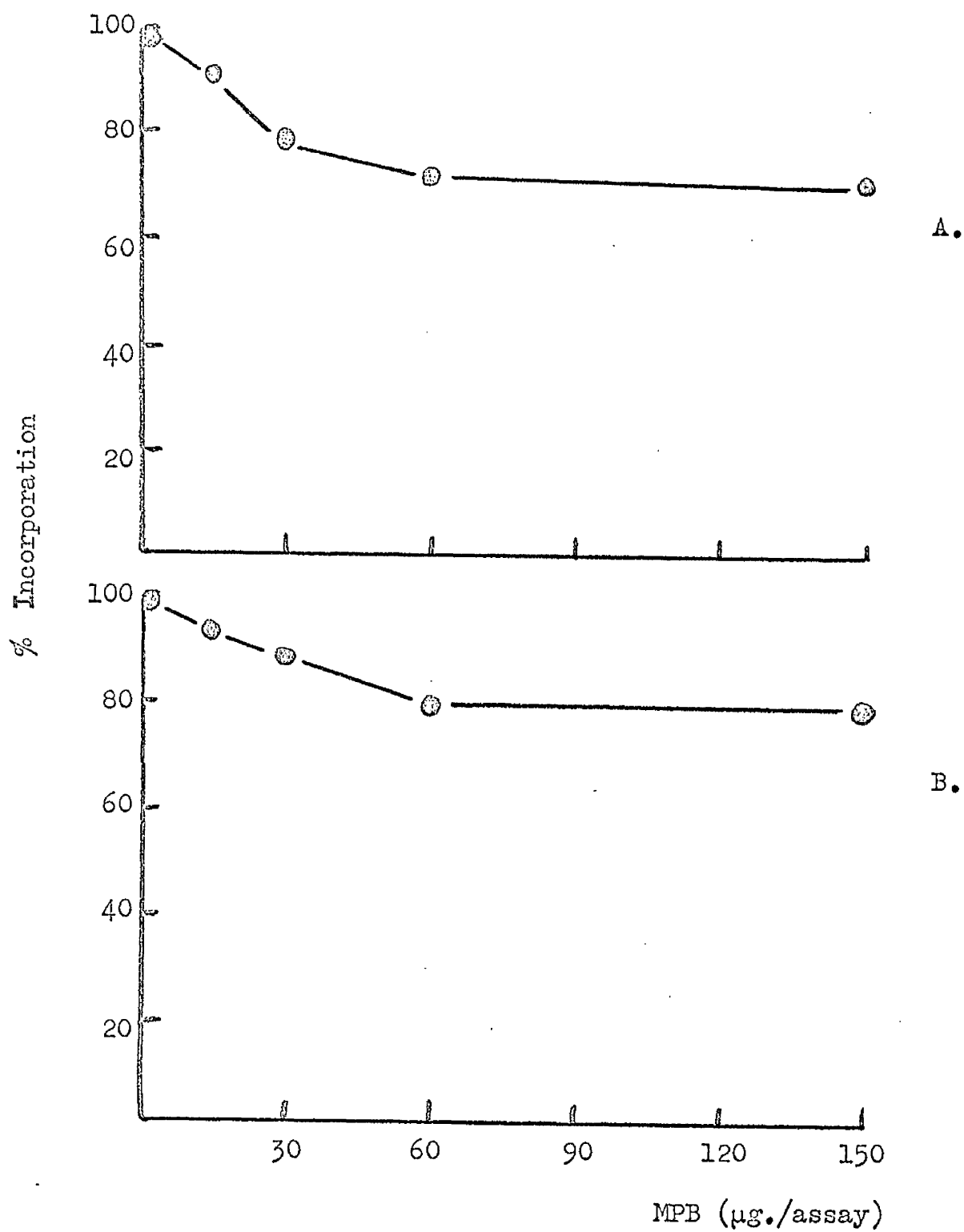


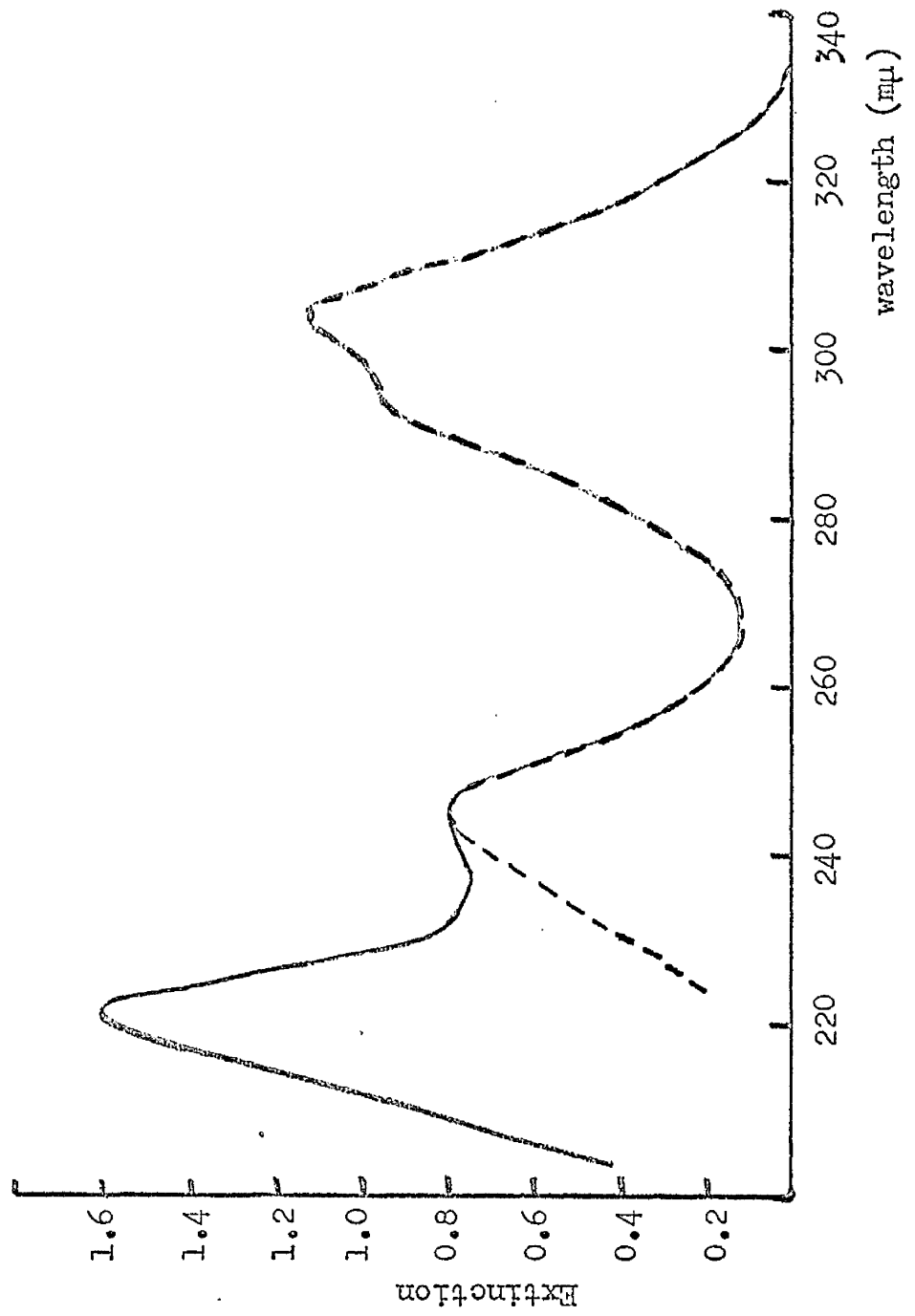
FIGURE 47

The ultraviolet absorption spectrum of 2-mercapto-1-
(3-4 pyridethyl) benzimidazole (MPB)

— represents MPB dissolved in water (20 $\mu\text{g.}/\text{ml.}$)
using a water blank.

-- represents MPB dissolved in DMS (20 $\mu\text{g.}/\text{ml.}$)
using a DMS blank.

FIGURE 47.



peak at 222 mμ when MPB is dissolved in water. This may be due to DMS stabilising tautomerism within the substituted benzimidazole molecule.

Little effect was noted when MPB was added to solutions of the bases, nucleosides, nucleotides, RNA or DNA but in the reverse condition i.e. observing changes in the spectrum of MPB, there was a consistent alteration to the absorbance peak at 246 mμ. These samples were run with 10% DMS and 0.01M-tris/HCl pH 7.5 in both test and control. Later the nucleotides, RNA and DNA were checked in the absence of DMS but with the same effect. There was no alteration to any other part of the spectrum. The results are summarised on Figs. 48 to 52. In the presence of 0.4M-ammonium sulphate a decreased effect was noted. This was most clearly observed with RNA and DNA (Fig. 53). From this it seemed unlikely that the effect was relevant to the inhibition of RNA polymerase by MPB as this had already been noted to be $(\text{NH}_4)_2\text{SO}_4$ -dependent (Fig. 40). An attempt was made to isolate the complex formed between the bases and MPB by chromatography at pH 7.0 in water on Whatman No. 1. MPB was seen to remain at the origin. A mixture of $[^{14}\text{C}]$ adenosine and MPB did not produce any detectable complex after chromatography, all the radioactivity being found in a position corresponding to free adenosine.

The melting temperature of the calf thymus DNA being used with MPB was examined under various conditions. Spectrophotometric

FIGURE 48

The effect of various concentrations of deoxyadenosine on
the ultraviolet absorption spectrum of MPB

The spectrum of MPB was observed under the following
conditions :-

| | Test Cell | Reference Cell |
|--------------------|--|--|
| A — | 0.5 μ moles of MPB 10% (v/v) DMS 0.01M-tris/HCl pH 7.5 to 5 ml. | 10% (v/v) DMS 0.01M-tris/HCl pH 7.5 to 5 ml. |
| B - - - | 0.5 μ moles of MPB 0.5 μ moles deoxyadenosine 10% (v/v) DMS 0.01M-tris/HCl pH 7.5 to 5 ml. | 0.5 μ moles deoxyadenosine 10% (v/v) DMS 0.01M-tris/HCl pH 7.5 to 5 ml. |
| C - . - . - | 0.5 μ moles of MPB 1.0 μ mole deoxyadenosine 10% (v/v) DMS 0.01M-tris/HCl pH 7.5 to 5 ml. | 1.0 μ mole deoxyadenosine 10% (v/v) DMS 0.01M-tris/HCl pH 7.5 to 5 ml. |
| D - . . - . . - | 0.5 μ moles of MPB 2.5 μ moles deoxyadenosine 10% (v/v) DMS 0.01M-tris/HCl pH 7.5 to 5 ml. | 2.5 μ moles deoxyadenosine 10% (v/v) DMS 0.01M-tris/HCl pH 7.5 to 5 ml. |

FIGURE 48.

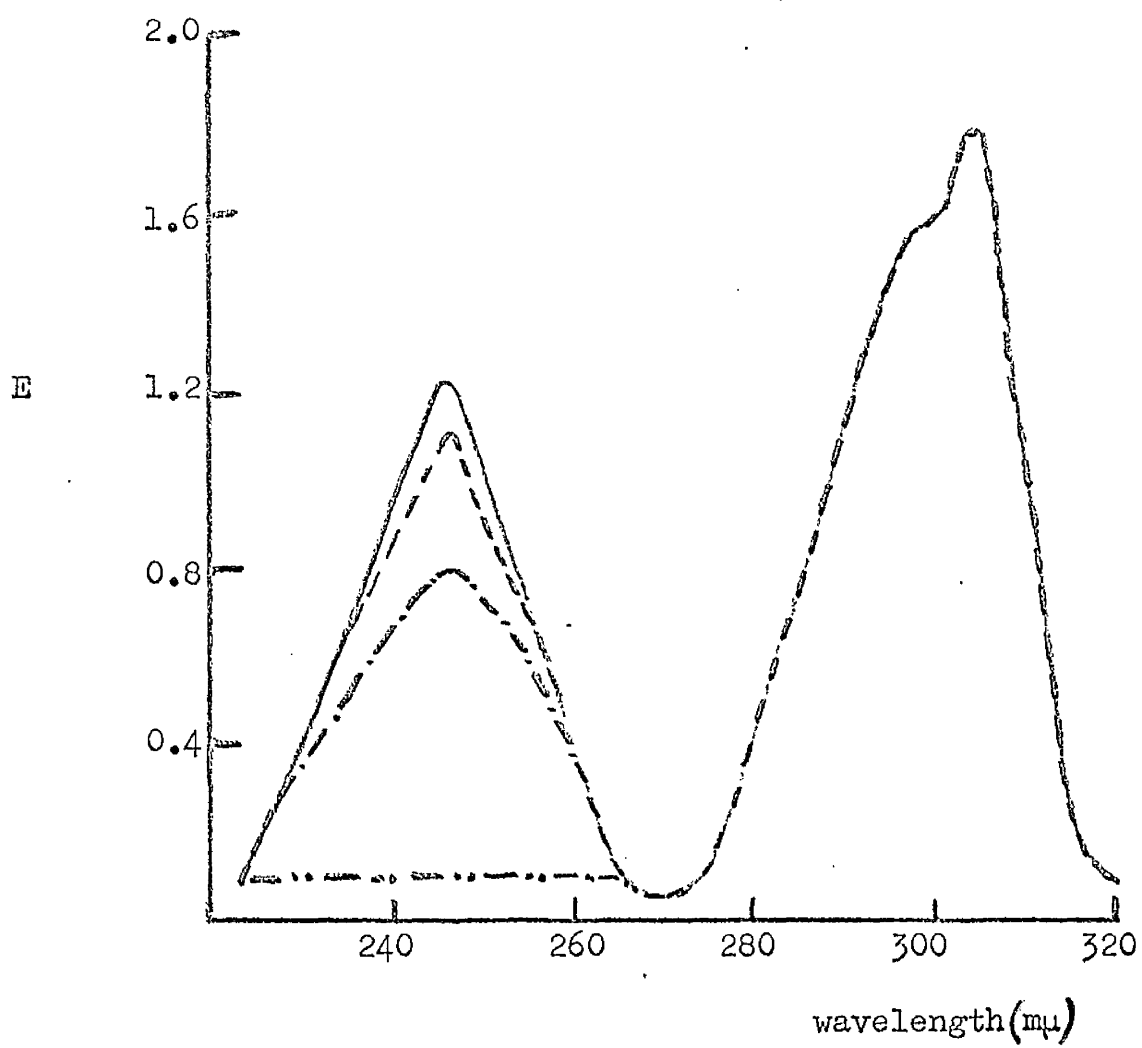


FIGURE 49

The decrease in E_{246} of MPB due to the presence of varying

concentrations of adenine and guanine derivatives

As in Figure 48, test and control samples differed only in MPB content. The spectra of MPB obtained resembled those shown in Fig. 48. The drop in absorption at 246 m μ was measured and plotted ($-\Delta E_{246}$) against purine concentration.





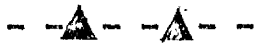

- A.  increasing adenine concentration
  increasing adenosine concentration
  increasing AMP concentration
- B.  increasing guanine concentration
  increasing guanosine concentration
  increasing GMP concentration

FIGURE 49.

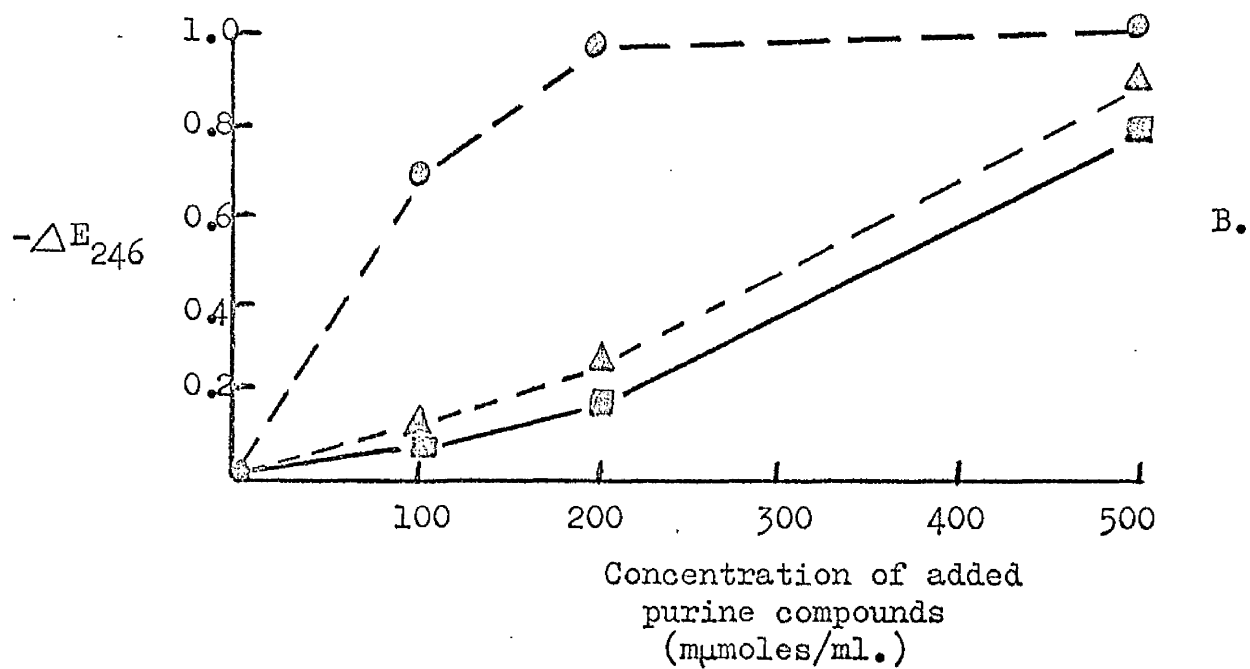
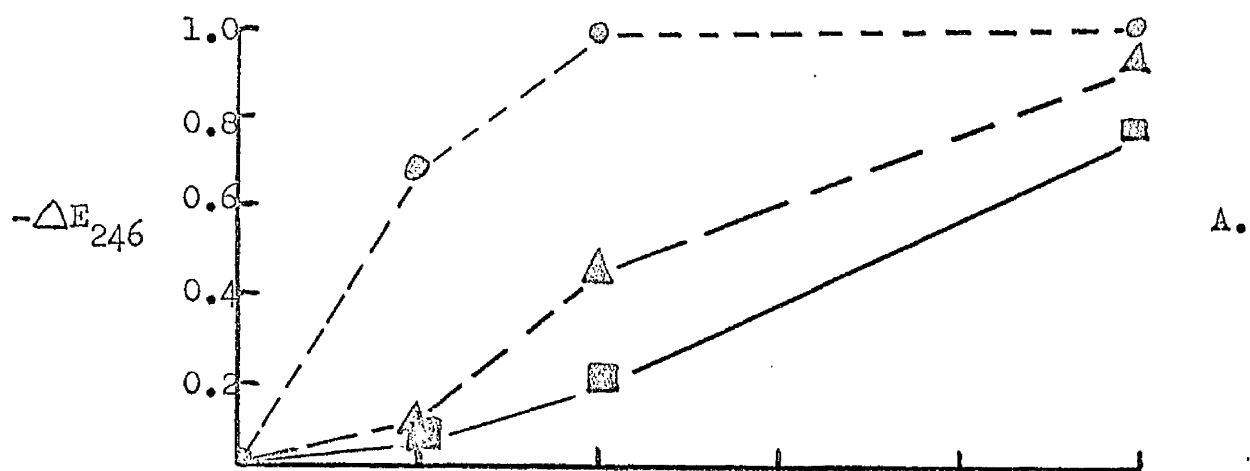


FIGURE 50

The decrease in E_{246} of MPB due to the presence of varying concentrations of adenine and guanine derivatives

As in Figure 48, test and control samples differed only in MPB content. The spectra of MPB obtained resembled those shown in Figure 48. The drop in absorption at 246 m μ was measured and plotted ($-\Delta E_{246}$) against purine concentration.


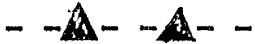
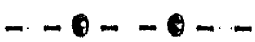
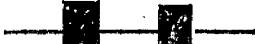
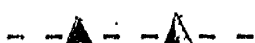

- A.
- | | |
|--|---|
|  | increasing adenine concentration |
|  | increasing deoxyadenosine concentration |
|  | increasing dAMP concentration |
- B.
- | | |
|---|---|
|  | increasing guanine concentration |
|  | increasing deoxyguanosine concentration |
|  | increasing dGMP concentration |

FIGURE 50.

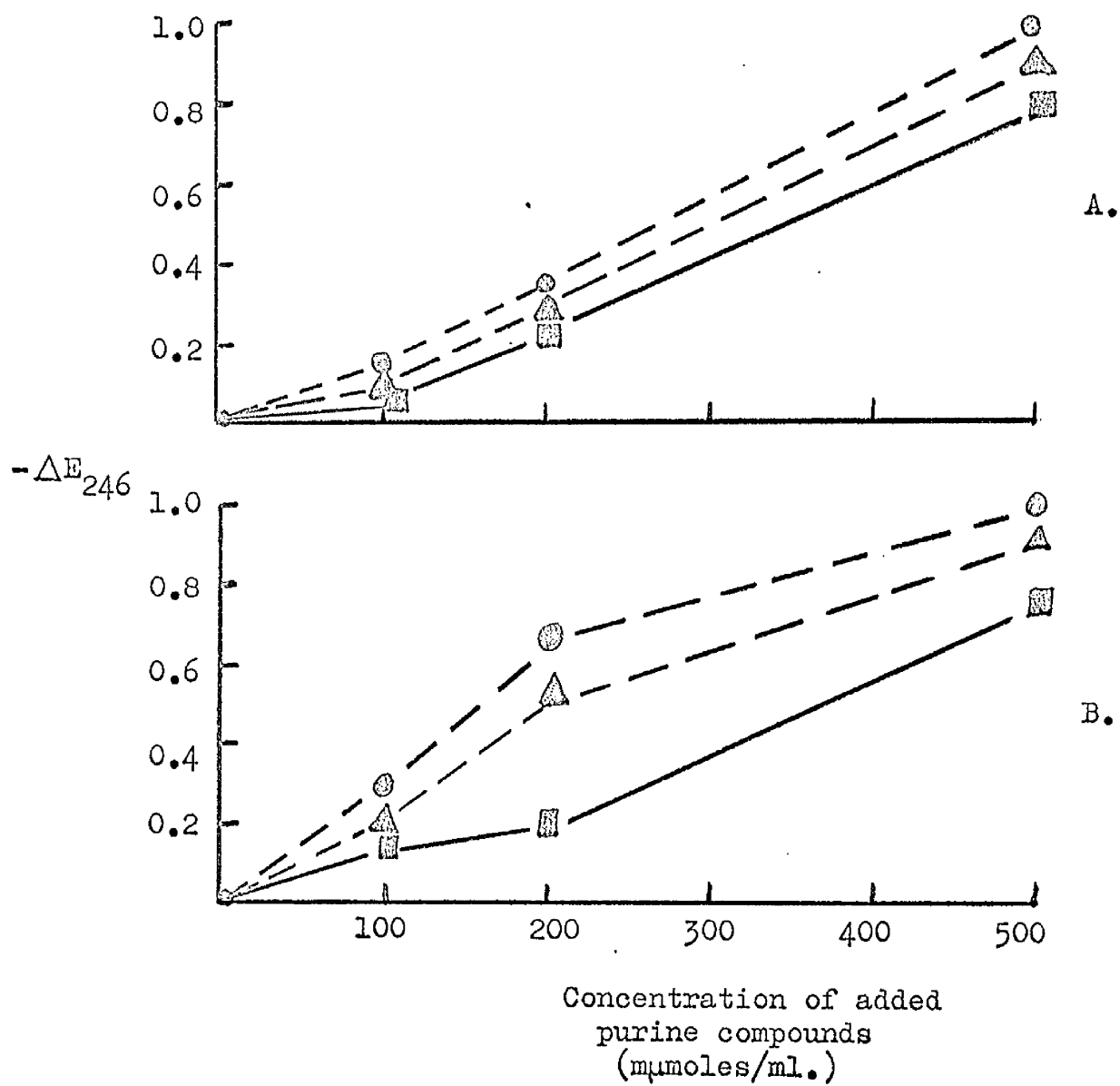


FIGURE 51

The decrease in E_{246} of MPB due to the presence of varying concentrations of cytosine and uracil derivatives

As in Figure 48, test and control samples differed only in MPB content. The spectra of MPB obtained resembled those shown in Figure 48. The drop in absorption at 246 m μ was measured and plotted ($-\Delta E_{246}$) against pyrimidine concentration.

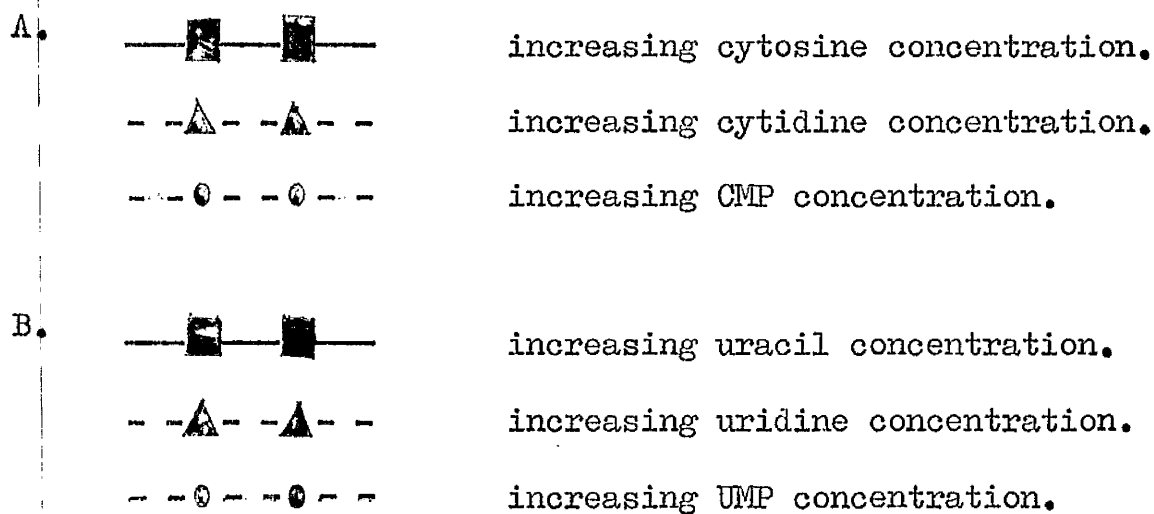


FIGURE 51.

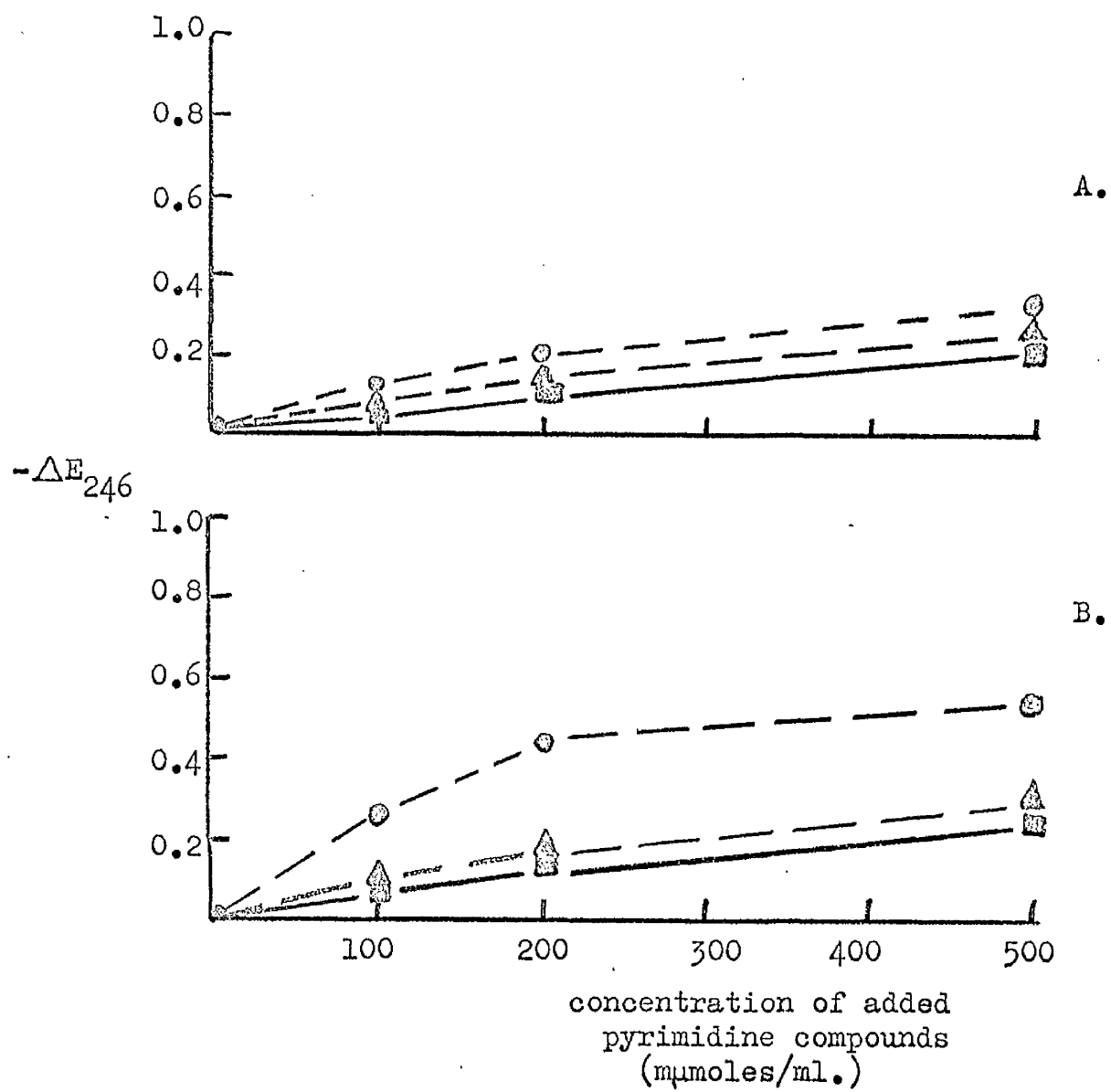


FIGURE 52

The decrease in E_{246} of MPB due to the presence of varying concentrations of cytosine and thymine derivatives

As in Figure 48, test and control samples differed only in MPB content. The spectra of MPB obtained resembled those shown in Figure 48. The drop in absorption at 246 m μ was measured and plotted ($-\Delta E_{246}$) against pyrimidine concentration.

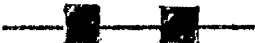

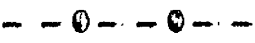


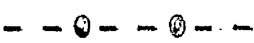
- A.
- | | |
|---|---|
|  | increasing cytosine concentration. |
|  | increasing deoxycytidine concentration. |
|  | increasing dCMP concentration. |
- B.
- | | |
|---|-------------------------------------|
|  | increasing thymine concentration. |
|  | increasing thymidine concentration. |
|  | increasing TMP concentration. |

FIGURE 52.

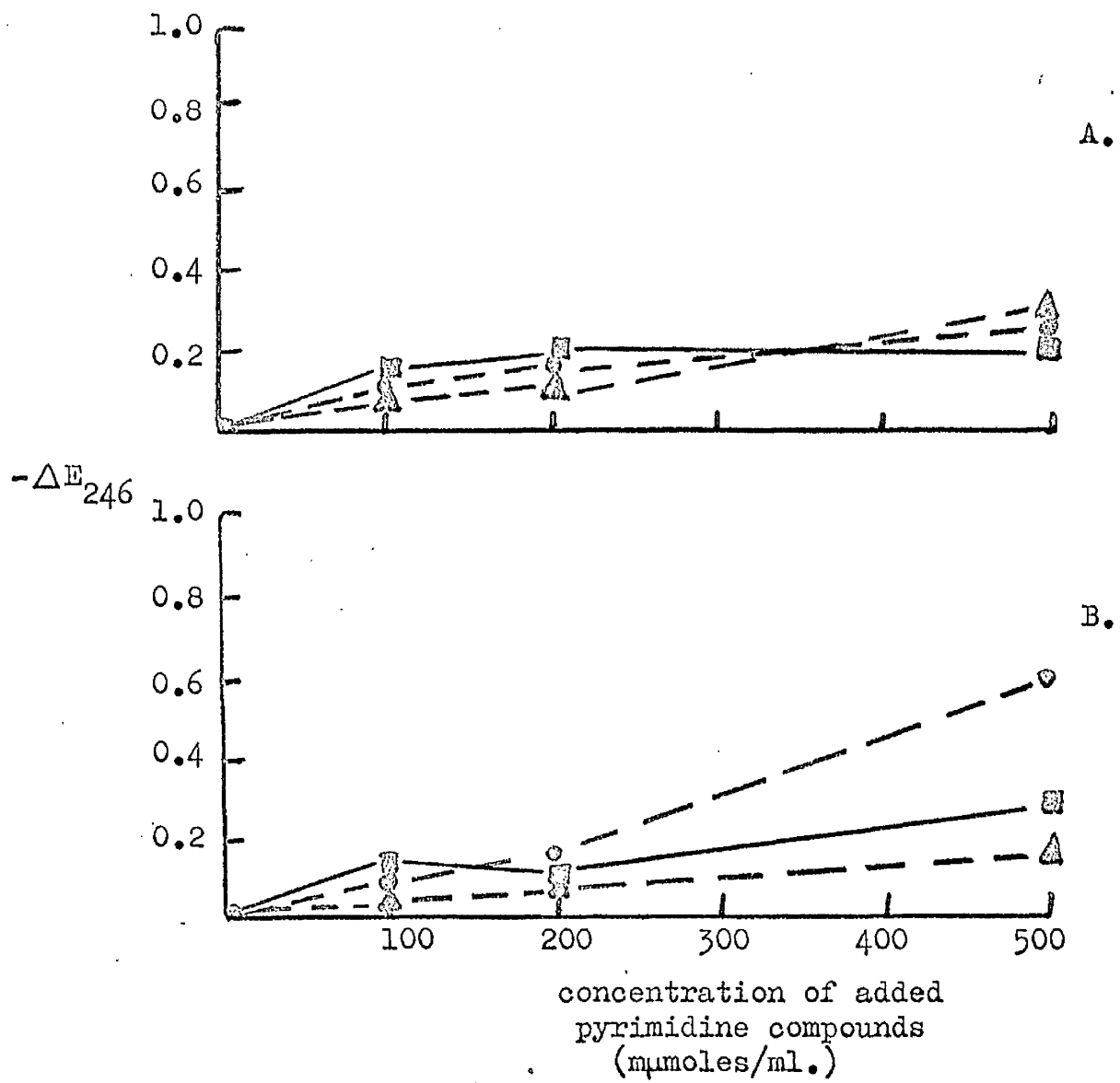


FIGURE 53

The decrease in E_{246} of MPB due to the presence of varying concentrations of the nucleic acids and of adenine derivatives

As in Figure 48, test and control samples differed only in MPB content. The spectra of MPB obtained resembled those shown in Figure 48. The drop in absorption at 246 mμ was measured and plotted ($-\Delta E_{246}$) against nucleic acid concentration or purine concentration.

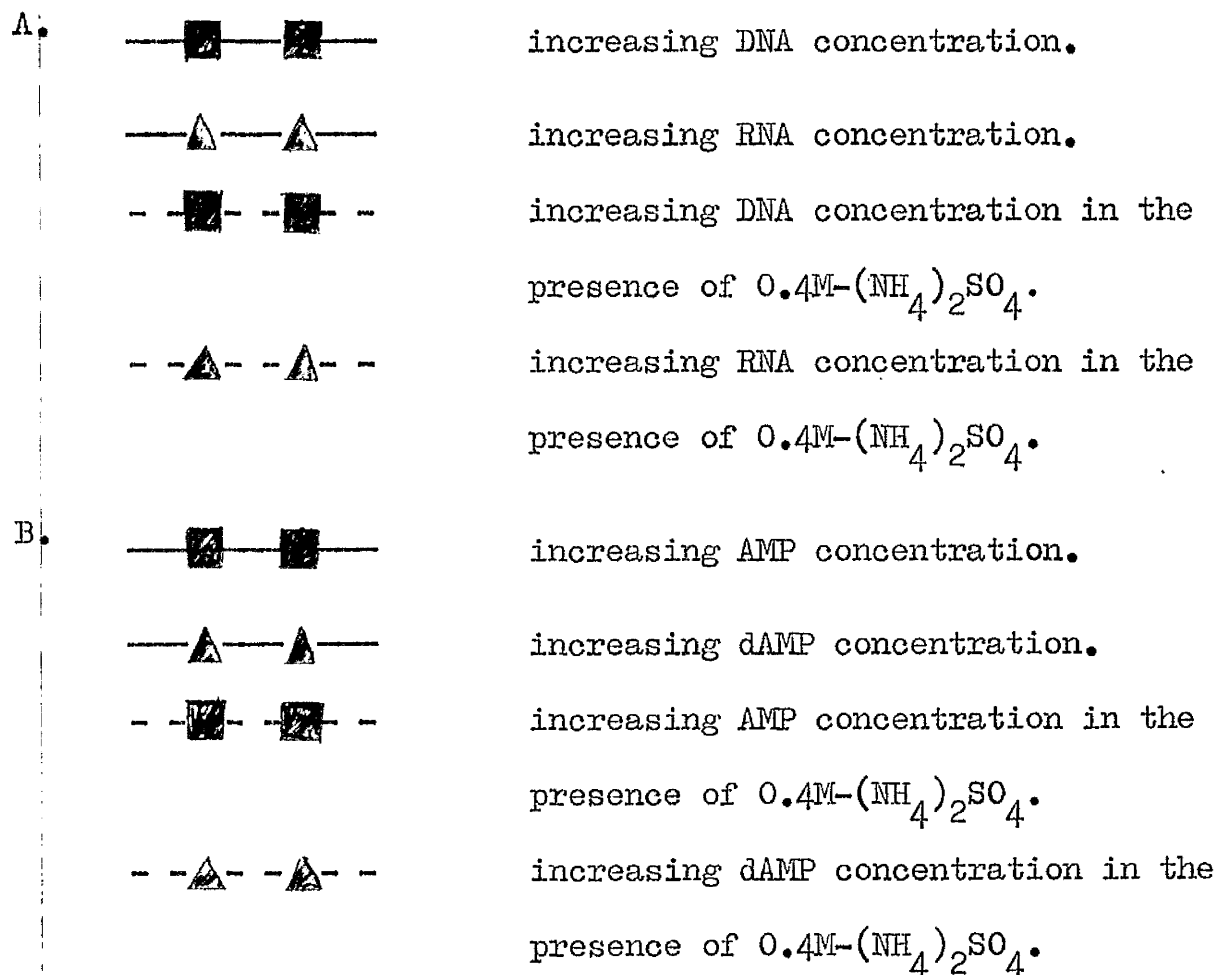
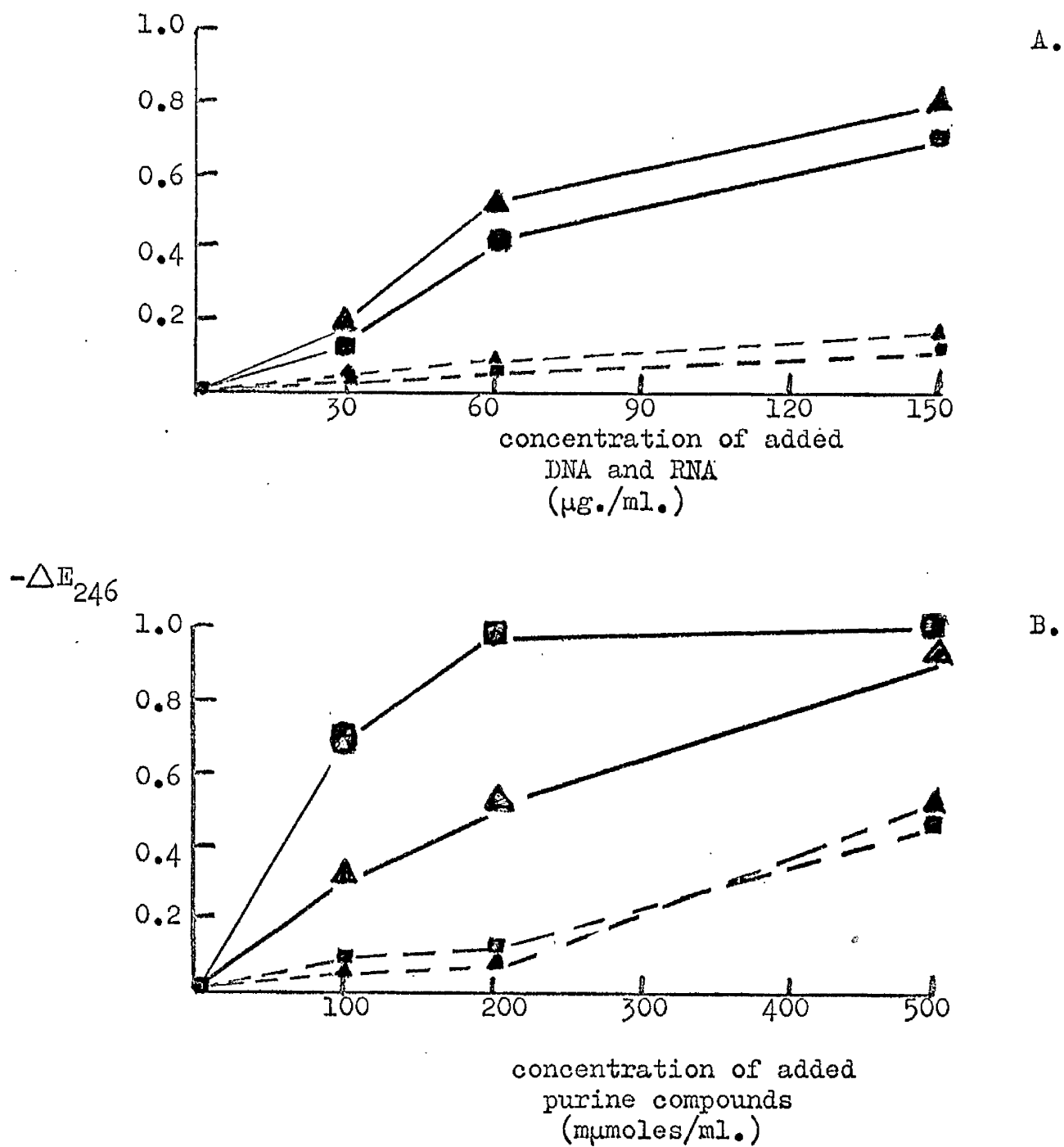


FIGURE 53.



measurements on the denaturation of DNA by heat are shown in Fig. 54. It can be seen that 10% DMS lowers the degree of hyperchromicity obtained on heating. MPB lowers this even more and the rise in absorbance is much less steep. When the DNA was treated with pronase (1 mg./ml.) by dialysing against water for 4 hr. at 60°, it was seen that 10% DMS and also MPB had no marked effect on the optical density readings. These results are consistent with the MPB reacting with some protein component of the system, causing the DNA strands to be less readily separated.

FIGURE 54

The melting temperature of Landschutz ascites tumour DNA
under various conditions

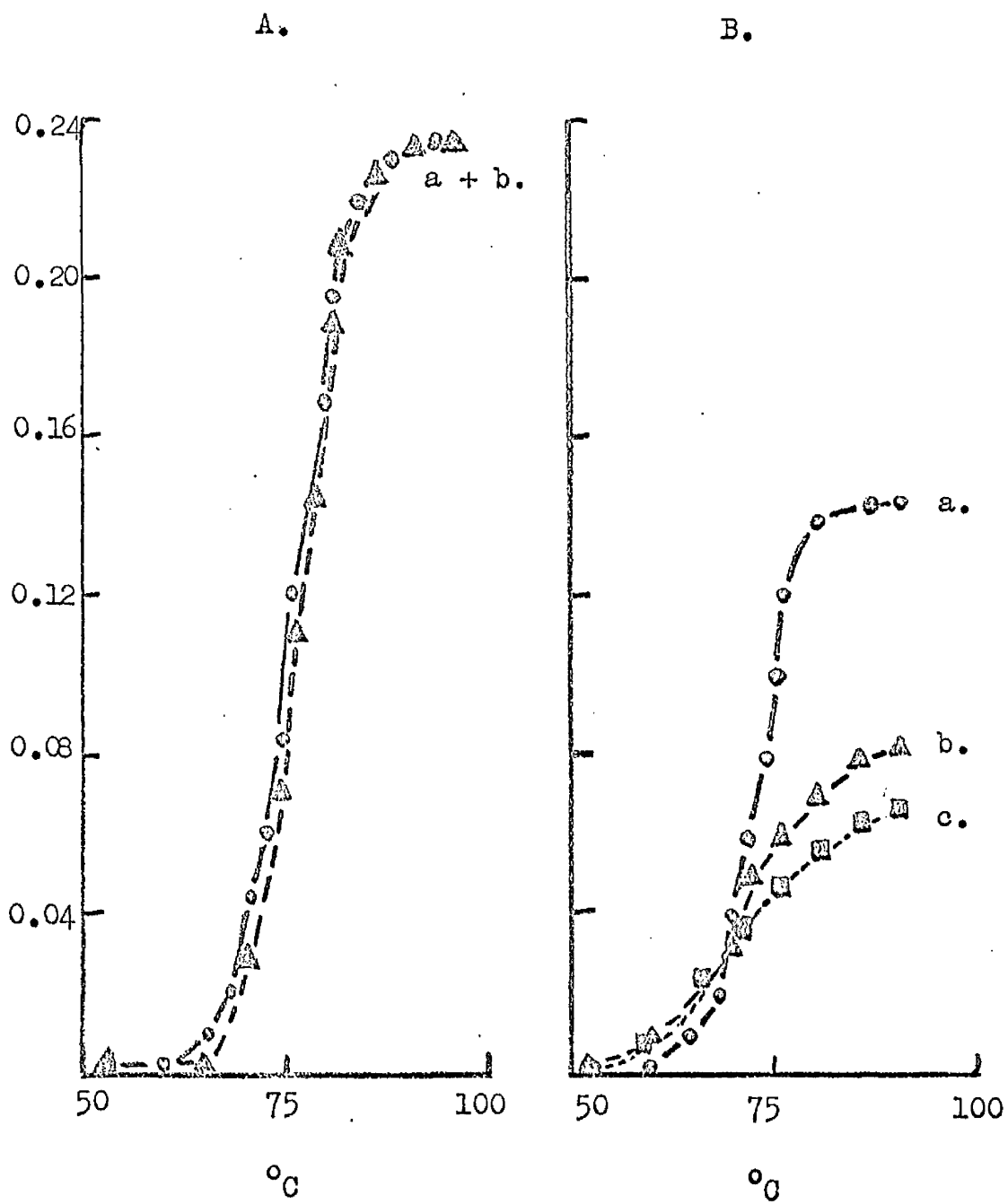
- A. The Landschutz ascites tumour DNA used, was treated with pronase (1 mg./ml.) by dialysing the mixture at 60°, for 4 hr. against water.

| | Test Cell | Reference Cell |
|------------|---|---|
| a —●— | DNA (30 µg./ml.) in 0.01M-tris/HCl pH 7.5 | 0.01M-tris/HCl, pH 7.5 |
| b --▲-- | MPB (30 µg./ml.) DMS (10%, v/v) DNA (30 µg./ml.) in 0.01M-tris/HCl pH 7.5 | MPB (30 µg./ml.) DMS (10%, v/v) in 0.01M-tris/HCl pH 7.5 |

- B. Landschutz ascites tumour DNA was used.

| | Test Cell | Reference Cell |
|------------|---|---|
| a —●— | DNA (30 µg./ml.) in 0.01M-tris/HCl pH 7.5 | 0.01M-tris/HCl pH 7.5 |
| b --▲-- | DMS (10%, v/v) DNA (30 µg./ml.) in 0.01M-tris/HCl pH 7.5 | DMS (10%, v/v) in 0.01M-tris/HCl pH 7.5 |
| c --■-- | MPB (30 µg./ml.) DMS (10%, v/v) DNA (30 µg./ml.) in 0.01M-tris/HCl pH 7.5 | MPB (30 µg./ml.) DMS (10%, v/v) in 0.01M-tris/HCl pH 7.5 |

FIGURE 54.



D I S C U S S I O N

DISCUSSION

I. A comparison of crude and purified RNA polymerase preparations

a. Characteristics of the enzyme

The ionic requirements of the purified enzyme preparation are quite similar to those of the crude enzyme. The optimal incorporation of ribonucleoside 5'-triphosphates in both systems was obtained in the presence of 1 $\mu\text{mole/assay}$ of Mn^{2+} (Figs. 3 and 24). Ammonium sulphate was found to stimulate both preparations but a comparison of Figs. 4 and 25 shows the level causing the maximal incorporation to have narrowed from 160-320 $\mu\text{moles/assay}$ to 240-320 $\mu\text{moles/assay}$, while in the absence of ammonium sulphate increased concentrations of Mn^{2+} ions caused no increase in ribonucleoside triphosphate incorporation beyond the level which would have been found with the crude preparation. When the SSS fraction was assayed using Mg^{2+} ions instead of Mn^{2+} , the results resembled those obtained with the crude preparation, no increase in specific activity being noted in the presence of 240 $\mu\text{moles/assay}$ of ammonium sulphate (Fig. 24). This may point to the existence of two RNA polymerases in the crude preparation as is suggested by Widnell and Tata (1966) from their work on rat liver nuclei, although in the ascites system there is no evidence of a preference for Mg^{2+} . A marked preference for ammonium sulphate was shown by the Mn^{2+} -dependent enzyme (Figure 5) suggesting that the increased incorporation is not due merely to a rise in ionic strength. This

is also in accordance with the results of Widnell and Tata (1966).

The pH range giving the highest level of activity is slightly lower (6.4-7.6, Fig. 28) with the purified enzyme than with the crude preparation (6.5-8.5, Fig. 6), while the requirement for 2-mercapto-ethanol becomes much less important (Figs. 8 and 27). These observations may relate to some conformational changes in the enzyme protein.

The increase in activity observed on adding DNA to the reaction mixture is considerably greater with the purified enzyme preparation (Table 7), although the crude enzyme appears more sensitive to the addition of DNase. The observation of DNase sensitivity however confirms that some DNA remains in the purified preparations. In the crude system, the addition of RNA neither stimulates nor inhibits the polymerase, but it is significant that with the purified preparation RNA is almost as good a primer as DNA (Table 7).

The utilisation, by crude RNA polymerase preparations, of one ribonucleoside 5'-triphosphate in the absence of the other three may be taken as some measure of the extent of homopolymer formation. It is evident (Table 8) that even in the presence of ammonium sulphate the maximum amount of homopolymer formation is about 50% of the total activity. While marked incorporation of uracil nucleotides occurs in the absence of ATP, GTP and CTP, the quantity of guanine nucleotide incorporated under similar conditions

is quite small. On addition of all four ribonucleotides, a significant stimulation of incorporation is noted. On the other hand, in the absence of ammonium sulphate there is no increase in ribonucleotide incorporation when all four are present. The level of enzyme activity observed in the absence of ammonium sulphate when one or all four ribonucleotides are present, closely resembles the level of activity noted in the presence of ammonium sulphate when only one ribonucleotide is added. This would strongly suggest that in the absence of ammonium sulphate most of the product is homopolymer.

It is of interest that the guanine nucleotides behave differently from those of adenine, cytosine and uracil (Table 8). It is known however that guanine nucleotides show a marked tendency to associate within themselves and with one another (Michelson et al. (1967)). This can in some circumstances be minimised e.g. by the addition of high concentrations of urea, and it is not impossible that in the presence of $0.4M-(NH_4)_2SO_4$ a similar phenomenon occurs. This however seems a less likely explanation in view of the finding that $[^3H]GTP$ incorporation is considerably enhanced by the addition of ATP, UTP and CTP (Table 8).

With the purified enzyme, up to 90% of the total polymerising activity may be obtained in the presence of only one ribonucleotide (Table 9). As will be discussed later, purified preparations of RNA polymerase from E. coli may also catalyse homopolymer formation

so that an increase in this capacity may not represent the purification of a separate enzyme at the expense of the original DNA-dependent, RNA polymerase, but rather a modification of its specificity. An analysis of the product was not carried out, but the incorporation of each ribonucleoside 5'-triphosphate was assayed in the presence of the other three unlabelled ribonucleotides. A comparison of the proportions of adenine, uracil, guanine and cytosine incorporated showed these to be unchanged in the purified enzyme, despite the loss of a large percentage of DNA.

The product of the crude RNA polymerase assay obtained in the presence of $(\text{NH}_4)_2\text{SO}_4$ was isolated and its profile on a sucrose density gradient was examined (Figs. 32 and 33). About 80% of the total ribonucleotide incorporated was present in the RNA extracted from the assay mixture. The 20% not extracted, was probably in the form of oligonucleotides. The bulk of the RNA synthesised in this case would appear, on comparison with whole-cell RNA from Landschutz ascites tumour, to be around 15s. This experiment was run using $[^3\text{H}]\text{UTP}$ or $[^3\text{H}]\text{GTP}$ as the labelled ribonucleotide (Figs. 32 and 33 respectively). There was little alteration in the pattern of radioactivity obtained.

The RNA formed by the crude polymerase in the absence of ammonium sulphate was also extracted and examined (Fig. 34). As has already been discussed there is little $[^3\text{H}]\text{GMP}$ incorporation in the absence of ammonium sulphate and so $[^3\text{H}]\text{UTP}$ was employed in

this experiment. Here only 30% of the calculated product was obtained after extraction, suggesting that a much larger proportion of oligonucleotides was formed in the absence of ammonium sulphate. Here too, sucrose gradient analysis of the RNA shows that the maximum sedimentation coefficient is 6s.

The material produced by the purified RNA polymerase in the presence of ammonium sulphate was extracted and examined in a similar fashion (Figure 35). About 70% of the total ribonucleotide incorporation is represented in the RNA layered on the gradient. This constitutes a drop of 10% when considered beside the results obtained from the crude polymerase fraction, and is perhaps indicative of a larger fraction of homopolymer formation (assuming oligonucleotides to be predominantly homopolymers). Nevertheless the proportion of labelled nucleotide in the extracted RNA is still considerably greater than was observed with the crude enzyme in the absence of ammonium sulphate. Furthermore the product appears to be more closely related in size to that formed by the crude enzyme in the presence of ammonium sulphate.

b. The effect of MPB and actinomycin D on the two RNA polymerase preparations

The effects of MPB on incorporation of ribonucleoside triphosphates by the crude and purified RNA polymerase preparations were tested. In initial experiments with the crude preparation a maximum inhibition was obtained when the ratio of protein:MPB was

4:1. The addition of a further amount of crude enzyme reduced the level of inhibition obtained. It was therefore thought that there was an interaction between the drug and one or more components of the crude enzyme preparation.

From consideration of the chemistry of the substituted benzimidazole compound it seemed likely that inhibition of the polymerase could be caused by -SH interaction with the enzyme protein (A. Porte, Personal communication). Figure 44 shows however that increasing concentrations of 2-mercapto-ethanol do not minimise the effect of MPB. Another interesting feature of the inhibition is the effect of ammonium sulphate. Figure 40 shows that in the complete absence of ammonium sulphate no inhibition occurs, while Figure 41 shows a maximal inhibition using 240 μ moles of ammonium sulphate/assay which is consistent with the suggestion that ammonium sulphate causes dissociation of deoxynucleoprotein allowing greater access of the MPB to the DNA molecule. This will be discussed further in conjunction with the results obtained with actinomycin D.

Attempts were made to study the possible interactions of MPB with bases, nucleosides and nucleotides. Figure 45 shows that increasing the concentration of ribonucleoside 5'-triphosphates present in the assay mixture does not decrease the inhibitory effect of the drug. Figure 48 however shows a typical effect on the spectrum of MPB in DMS when a nucleoside is added in increasing

amounts.

The subsequent figures (Figs. 49 - 52) summarise the effects observed and demonstrate a maximal decrease in ultraviolet absorption at 246 m μ with adenine and guanine nucleotides. Addition of RNA or DNA to the solution of MPB in DMS gives rise to a similar effect, thus suggesting some form of interaction between the purines and MPB. MPB is slightly soluble in water (up to about 20 μ g./ml.), so the spectrum of MPB was re-examined in the presence of the various purine compounds, RNA or DNA. No difference in E_{246} was noted although when water is used as solvent a new ultraviolet absorption peak at 218 m μ appears. This extra peak may be due to stabilisation of the MPB molecule in either the enol- or keto-form.

Addition of 0.4M-ammonium sulphate to both test and reference cells causes the E_{246} effect to be greatly reduced (Fig. 53). As no inhibition is obtained in vitro unless $(\text{NH}_4)_2\text{SO}_4$ is present it would seem unlikely that this purine-MPB interaction is the sole explanation of inhibition of RNA polymerase by MPB. In interpreting the results from Figures 48-53 it must be remembered that the traces obtained are those from difference spectra and the alteration in ultraviolet absorption at 246 m μ may only represent a small fraction of the total absorption at this wavelength. There is however no marked alteration in absorption at any other wavelength. As is reported in the results section (p.71) no trace of a chemical MPB-

adenosine complex is found.

Studies on the melting temperature of DNA (Fig. 54) show that the treatment of a partially purified preparation of ascites tumour DNA with DMS causes a drop in the degree of hyperchromicity obtained and also a more gradual separation of the DNA strands. When MPB is present, these effects are more pronounced. However if the DNA is treated with pronase before addition of DMS, or DMS and MPB these effects on the melting temperature are lost, which shows that in the presence of protein a more stable complex is formed between MPB and DNA.

The purified enzyme is less susceptible to inhibition by MPB (Fig. 46). This may be due to failure to form the correct protein-MPB-DNA complex or to the ability of the preparation to incorporate ribonucleoside 5'-triphosphates in the absence of a primer.

The effect of actinomycin D on the incorporation of ribonucleotides by the crude RNA polymerase fraction was examined using first $[^3\text{H}]$ UTP and later $[^3\text{H}]$ GTP. Figure 30 describes the results obtained. In the presence of ammonium sulphate and actinomycin D (5 $\mu\text{g./ml.}$) 80% and 75% inhibition was observed with $[^3\text{H}]$ UTP and $[^3\text{H}]$ GTP respectively. Similarly in the absence of the salt 40% and 20% inhibition was noted, again with $[^3\text{H}]$ UTP and $[^3\text{H}]$ GTP respectively. This compares with 54% inhibition of RNA polymerase obtained by Eason and Smellie (1964) in a Krebs ascites tumour system, in the presence of ammonium sulphate and an equivalent

concentration of actinomycin D. Actinomycin D is believed to inhibit RNA polymerase by binding to deoxyguanosine residues in the DNA helix. Thus actinomycin D inhibition is most noticeable when the RNA polymerase is dependent on a DNA primer. This would suggest that in the absence of ammonium sulphate, the crude polymerase is not greatly dependent on such a primer. This would again support the suggestion that in such circumstances much of the ribonucleotide is incorporated into a homopolymer fraction.

It was noted that in the presence of ammonium sulphate and added uridine triphosphate there was a higher level of incorporation than in the presence of any one other nucleotide. This means that it is possible that even in the presence of all four ribonucleotides and ammonium sulphate a large proportion of the UMP is incorporated into a homopolymer fraction. Thus it would suggest that using $[^3\text{H}]\text{UTP}$ as the labelled ribonucleotide an artificially high level of incorporation would be observed. No such discrepancy is however seen in Table 8. By following $[^3\text{H}]\text{GTP}$ incorporation this possibility is removed.

In the experiments with actinomycin D, 80% inhibition of UMP and 75% inhibition of GMP incorporation was observed when $(\text{NH}_4)_2\text{SO}_4$ was included in the reaction mixture, whereas the comparable figures for UMP and GMP in the absence of $(\text{NH}_4)_2\text{SO}_4$ were 40% and 10% respectively. These results suggest that in the absence of $(\text{NH}_4)_2\text{SO}_4$ as much as 40% of the observed incorporation of UMP

may represent heteropolymer formation, while not more than 20% of the GMP uptake can be ascribed to this type of reaction. This therefore presents the paradoxical situation that 80% of the GMP incorporation appears to contribute to the synthesis of homopolymers while it is known that virtually no GMP is incorporated in the absence of ATP, CTP and UTP. It should however be remembered that in the absence of ammonium sulphate, $[^3\text{H}]$ GMP incorporation is very low and the margin of error is thus increased when calculating the percentage inhibition obtained on the addition of actinomycin D.

The effect of actinomycin D on the purified polymerase system was also examined. Here the level of inhibition observed was lower than that found with the crude enzyme in the presence of comparable concentrations of actinomycin D. The experiment was performed using both $[^3\text{H}]$ UTP and $[^3\text{H}]$ GTP as the labelled ribonucleotide. Identical levels of inhibition were obtained.

This purified preparation is more dependent on exogenous DNA than the crude enzyme and at first this result seems anomalous. However in the absence of exogenous DNA and in the presence of DNase, ribonucleotide incorporation continues. RNA has already been shown to act as a primer for the purified enzyme. Thus actinomycin D which inhibits only DNA primed synthesis, may allow the purified enzyme to incorporate ribonucleotides, without demonstrating a requirement for DNA.

Parallel experiments were performed to investigate the effect

of MPB on the crude and purified enzyme preparations. Using the crude polymerase fraction the drug was shown to cause inhibition of ribonucleotide incorporation in the presence of ammonium sulphate but not in its absence (Fig. 40). Again the results of this experiment were followed using $[^3\text{H}]\text{UTP}$ and $[^3\text{H}]\text{GTP}$ as the labelled ribonucleoside triphosphate. In the absence of ammonium sulphate, similar levels of incorporation of GMP and UMP were observed. In the presence of the salt however, inhibition by MPB fell from 50% to 40%, on changing from $[^3\text{H}]\text{UTP}$ to $[^3\text{H}]\text{GTP}$. Again this is the reverse of the result expected if there were a drop in the extent of homopolymer being formed. The action of MPB within the purified polymerase system was also investigated using both forms of labelled ribonucleotide, in the presence of ammonium sulphate. The inhibition obtained dropped from about 30% to about 20% when $[^3\text{H}]\text{UTP}$ was replaced with $[^3\text{H}]\text{GTP}$.

From the above results it would seem that the fraction of total RNA synthesis corresponding to homopolymer formation is reduced on the addition of the other three ribonucleotides to the reaction mixture.

The data obtained on homopolymer formation in the presence and absence of ammonium sulphate by the crude RNA polymerase requires some interpretation. It would appear that in the presence of ammonium sulphate not only is there a higher degree of ribonucleotide incorporation, but the product is largely heteropolymer. In

the absence of the salt the enzyme largely supports homopolymer formation.

Actinomycin D has been shown to be bound more firmly to DNA when the native helical structure is preserved (Kahan, Kahan and Hurwitz (1963)) and it would thus seem unlikely that ammonium sulphate is causing the strands of the DNA molecule to separate. Addition of denatured DNA to the crude polymerase assay system has no marked effect on the level of ribonucleotide incorporation obtained. This is due to the high level of DNA already present in the crude enzyme preparation.

Breuer and Florini (1966) have suggested that ammonium sulphate may lead to the dissociation of proteins from the deoxy-ribonucleoprotein, while Fuchs et al. (1967) have studied the effect of ionic strength on a purified RNA polymerase preparation from E. coli in some detail. They found that free enzyme and enzyme bound to DNA but not synthesising RNA are inhibited above an ionic strength of about 0.15. However the rate of RNA synthesis by those enzyme molecules which were already taking part in the formation of RNA chains was stimulated by increasing salt concentration up to an optimum ionic strength of 0.36. Thus under the salt concentrations used with the ascites tumour enzyme there are two possible effects contributing to the formation of heteropolymers rather than homopolymers i.e. the partial removal of some inhibitory material from the DNA complex and also the stimulation of the poly-

merase already bound to the DNA.

Widnell and Tata (1966) have shown evidence for an enzyme in rat liver nuclei which is activated by Mg^{2+} in the absence of ammonium sulphate and which is more susceptible to inhibition by actinomycin D than is the Mn^{2+} /ammonium sulphate-activated enzyme. No evidence of this was obtained with the present system.

c. The presence of other enzymes in the two RNA polymerase preparations

Table 11 shows that the purified preparation contained about a third of the DNase, RNase or alkaline phosphatase activity of the original preparation.

The presence of a DNA polymerase attached to the chromatin of mammalian cells has been reported by Patel, Howk and Wang (1967). The specific activity of this enzyme in the ascites preparations remains unchanged despite the streptomycin sulphate fractionation, although the concentration of DNA in the SSS fraction has been reduced to 10% of the level of the crude preparation which would suggest that DNA polymerase is firmly bound to a fraction of the DNA which is extracted along with the RNA polymerase.

II. A comparison with other RNA polymerase systems from various sources

a. Methods of preparation of RNA polymerase

In bacterial systems, RNA polymerase appears to be largely in a soluble form, consequently considerable purification may be obtained by an initial phase of differential centrifugation. This

step is frequently followed by fractionation with various concentrations of streptomycin sulphate and/or protamine sulphate to remove contaminating DNA and protein. The enzyme can then be concentrated by precipitation with ammonium sulphate and further purified by fractionation with calcium phosphate gel or chromatography on columns of DEAE cellulose and sephadex G100 (Chamberlin and Berg (1962), Stevens (1961), Weiss and Nakamoto (1961b), Krakow and Ochoa (1963) and Maitra and Hurwitz (1967)).

Fuchs et al. (1968) have described a rather different purification of RNA polymerase. The ruptured bacterial cells are centrifuged as before and the resulting pellet suspended in a complex buffer of tris-Mg-acetate pH 7.3. The suspension is then adsorbed to DEAE cellulose and the cellulose packed into a column. The proteins present are eluted with a KCl gradient in the above buffer. The active portion, corresponding to 0.2M-KCl is then precipitated with 50% saturated ammonium sulphate and further purified by centrifugation in sucrose/glycerol gradients. Such preparations have a sedimentation coefficient of 20s, whereas preparations obtained using the procedures previously described yielded RNA polymerase with a sedimentation coefficient of at most 15s. It is concluded therefore that protamine sulphate and streptomycin sulphate may destroy the natural structure of the enzyme during purification.

RNA polymerase from both plant and mammalian sources can be isolated in both aggregate and soluble forms. Huang and Bonner

(1962) described an aggregate preparation from pea nuclei, while Mans and Novelli (1964) demonstrated a soluble RNA polymerase in maize seedlings. Little work has however been done to purify RNA polymerase from plant sources.

It is possible to detect RNA polymerase activity in the nuclei of most mammalian cells but the levels of activity found differ widely (Hopper, Ho and Furth (1966)). This may be partly an artefact of isolation but it may also be a reflection of the differing rates of growth found in mammalian cells e.g. the increase in RNA polymerase activity following partial hepatectomy in rats (Tsukado and Lieberman (1965)).

It has already been mentioned that the enzyme from animal tissues may occur in a soluble or in an aggregate form. The soluble form has been partially purified by several groups of workers. Hopper, Ho and Furth (1966) have described soluble preparations from various bovine and guinea pig tissues. Fractions with higher specific activities have been prepared by Ishihama (1967) from HeLa cells and Ballard and Williams-Ashman (1965) from rat testes. The methods used by these groups are variants of the procedures described by Chamberlin and Berg (1962). A French pressure cell is used by Ishihama to disrupt the cells which are then centrifuged at 105,000g. The high speed supernatant fraction so obtained, is treated with protamine sulphate, the precipitate resuspended and fractionated on a column of DEAE cellulose. The

active fraction can then be precipitated with 40% saturated ammonium sulphate. Williams-Ashman and his collaborators homogenise rat testes in dilute tris/HCl buffer pH 7.5 to rupture the cells. The active fraction is then precipitated with ammonium sulphate, resuspended in a tris/HCl buffer pH 7.5 made 30% with respect to glycerol and passed through a DEAE cellulose column, also 30% with respect to glycerol. The active fraction is eluted by means of an ammonium sulphate gradient in the same glycerol buffer and the enzyme is concentrated by precipitation with 55% saturated ammonium sulphate.

Widnell and Tata (1966) among others have attempted to purify the aggregate form of RNA polymerase but no notable success has resulted. Goldberg in 1961 noted the stimulatory effect of sodium deoxycholate on an aggregate RNA polymerase preparation from HeLa cells. As this detergent had earlier been noted for its solubilising effects on the aggregate enzyme from Landschutz ascites cells it seemed a suitable method of obtaining a soluble preparation. In the present study, attempts were made to treat the preparation with protamine sulphate, ammonium sulphate and DEAE cellulose. These were not altogether successful owing to interaction of the reagents with DOC, but a soluble purified preparation was obtained using streptomycin sulphate fractionation.

b. Chemical properties of RNA polymerase

In comparing the results obtained from various RNA polymerase

systems the degree of purity of the enzyme must be considered. Variations may thus be due to other contaminating elements in the assay mixture or to genuine characteristics of the enzyme.

Widnell and Tata (1966) describe two aggregate RNA polymerase activities from rat liver nuclei. The first is stimulated by the presence of ammonium sulphate and shows a higher activity in the presence of Mn^{2+} than in Mg^{2+} . The second shows no stimulation by ammonium sulphate and yields a higher rate of nucleotide incorporation in the presence of Mg^{2+} than in Mn^{2+} . Both the mammalian enzymes of Furth and Ho (1965) and Ballard and Williams-Ashman (1965) seem to be stimulated by ammonium sulphate and to prefer Mn^{2+} . However Ramuz et al. (1965) have demonstrated a soluble polymerase from rat liver nuclei which is inhibited at high ionic strength but which shows higher levels of activity in the presence of Mn^{2+} than in Mg^{2+} . The aggregate enzyme studied here appears to show a higher rate of incorporation in the presence of low concentrations of Mn^{2+} but to be stimulated by the addition of ammonium sulphate. Whereas the bulk of the evidence from mammalian sources shows a preference for Mn^{2+} ions, the polymerase obtained from E. coli would appear to be more highly active in the presence of Mg^{2+} (Stevens and Henry (1963); Fuchs et al. (1967)). As has already been discussed, the ascites RNA polymerase gives a maximum ribonucleotide incorporation in the presence of Mn^{2+} ions and 0.4M-
(NH_4)₂SO₄.

Ammonium sulphate has been used extensively both in the preparation of purified systems and in assaying RNA polymerase, but the influence of various other ions has also been tested on this system. Goldberg (1961) working with HeLa and L cells found the same level of increased incorporation could be obtained in the presence of equimolar amounts of KCl or NH_4Cl . The Mn^{2+} activated enzyme prepared by Widnell and Tata (1966) shows a preference for ammonium sulphate which cannot be replaced by equimolar amounts of other ammonium or sulphate salts. This effect is similar to that observed with the system described in this thesis (Fig. 5).

DNA-dependent RNA polymerase might almost be said by definition to catalyse the incorporation of all four ribonucleoside 5'-triphosphates into RNA, in a sequence directed by that of the template DNA present. It has also been shown by Furth and Loh (1964) that in bovine lymphosarcoma the RNA polymerase is more highly active in the presence of native rather than denatured DNA. However in the presence of suitable primers and nucleotides, highly purified preparations of the enzyme will catalyse the synthesis of various artificial polynucleotides.

E. coli RNA polymerase was shown by Chamberlin and Berg (1964) to catalyse a DNA-dependent synthesis of long chain poly A when ATP was the only ribonucleoside triphosphate added. The homopolymer synthesis was inhibited on addition of one, two or three of the other ribonucleoside triphosphates and was noted to require the

addition of denatured DNA. This type of incorporation is thought to be due to reiterative copying of short sequences of thymidylic acid residues in the DNA template rather than the existence of a separate polymerase. The inhibition of homopolymer formation caused by addition of the other ribonucleoside triphosphates could be due to the enzyme being attached to the template at one base complementary to the base of the entering triphosphate and so preventing further copying of thymidylic acid sequences.

Roy-Burman et al. (1965) have shown that with DNA as primer E. coli polymerase may incorporate 5,6-dihydrouridine triphosphate even in the presence of the normal four ribonucleoside 5'-triphosphates. Analysis of the product shows the synthetic base to occur in a non-random fashion which would mean that the polymerase was capable of some auxilliary mechanism. A similar type of incorporation has been observed by Adman and Grossman (1967) using RNA-dependent RNA polymerase from M. lysodeikticus. With this system poly A may be prepared, the amount formed depending on the concentration of nucleotide present. The reiteration reaction seems to be directed by specific sites on the template in which sequences of uracil are terminated by other bases, with two uracil residues seeming to be the minimum required. The nature of the terminating base may be adenine, guanine or cytosine as well as 5,6-dihydrouracil or another uracil derivative. If the sequence of uracil residues is terminated by a natural base, the addition of the ribonucleoside

5'-triphosphate complementary to that of the terminating base, will depress reiterative poly A synthesis, and transcription to form a copolymer can then be observed. If however the uracil sequence is terminated by a modified uracil no depression of reiterative poly A synthesis by low concentrations of any of the three other normal ribonucleotides occurs. This RNA-dependent RNA polymerase purified from M.lysodeikticus is thought to be the same enzyme as the DNA-dependent counterpart since competitive inhibition can occur on addition of the second polynucleotide (Fox, Robinson, Haselkorn and Weiss (1964)).

Purified preparations of E. coli RNA polymerase in the absence of any primer have been shown by Smith et al. (1966) to be able to produce, after a variable lag phase, either poly A or poly U. Clark and Jaouni (1965) again using a purified E. coli preparation were able to show primed incorporation of $\left[^{14}\text{C}\right]$ guanosine monophosphate by an oligodeoxycytidylate as small as seven units long. RNA polymerase from E. coli can also be shown to accept poly dAT as primer and using this to produce a regularly alternating sequence of AMP and UMP residues (Chamberlin et al. (1963)).

To summarise it might be said that in purification of RNA polymerase i.e. on removal of the bound nuclear DNA there would seem to be a marked drop in template specificity which is emphasised by the variety of synthetic primers which the purified enzyme will accept. A similar change in specificity was noted on the purifica-

tion of the ascites enzyme (Table 7).

c. Physical properties of RNA polymerase

As no preparation of mammalian RNA polymerase has been shown to consist solely of this enzyme, the papers considered in this section describe the properties of the bacterial RNA polymerase from E. coli.

Colvill, Bruggen and Fernandez-Moran (1966) describe a form of RNA polymerase, with a sedimentation coefficient of 25s and an extinction ratio A_{280}/A_{260} of 1.63. The enzyme was prepared by a modification of the method of Chamberlin and Berg (1962).

Fuchs, Zillig, Hofschneider and Preuss (1964) prepared an RNA polymerase fraction of sedimentation coefficient 20s from E. coli by their method which has already been described (Section II.a.). Electron microscopy of this preparation showed a structure of six subunits arranged in a hexagon with a cross-section of 120 to 130 Å.

The DNA-dependent RNA polymerase from E. coli is strongly influenced by changes in the ionic strength. At low ionic strength (between 0.02 and 0.12) it has been shown to exist in a form sedimenting at 24s (Zillig et al. (1966), Richardson (1966a) and Stevens et al. (1966)) while at high ionic strength (up to 0.4) the enzyme reversibly dissociates into subunits of 13s (Priess and Zillig (1967), Richardson (1966a); Stevens et al. (1966)). The 13s form of the polymerase is thus probably the activated enzyme which has been obtained under assay conditions and the 13s form has

indeed been shown to demonstrate twice the ribonucleotide incorporation of the 24s form (Richardson (1966)). These results have of course been obtained with a purified bacterial enzyme, and several other factors will contribute to the rise in incorporation obtained in solutions of high ionic strength with the mammalian polymerase.

III. A consideration of the binding between DNA and protein with respect to RNA polymerase.

Using a purified preparation of RNA polymerase from E. coli, Anthony, Zeszotek and Goldthwait (1966) present evidence from which they deduce that RNA synthesis occurs in three steps :-

- a) association of DNA + ENZYME \rightleftharpoons DNA-ENZYME
- b) initiation : DNA-ENZYME + purine ribonucleotide
 \longrightarrow DNA-ENZYME - purine ribonucleotide
- c) polymer formation : DNA-ENZYME-purine ribonucleotide
+ ribonucleoside 5'-triphosphates
 \longrightarrow DNA-ENZYME-oligoribonucleotide + PPi

Maitra, Nakata and Hurwitz (1967) and others have shown that there is a significantly high proportion of RNA chains initiated by AMP or GMP incorporation. This incorporation is not dependent on the concentration of the four ribonucleotides present, the enzyme concentration, or the concentration of the DNA template. This would suggest that the DNA initiator is a pyrimidine. Mammalian systems would however appear more complex.

The demonstration of both a soluble and an aggregate form of

the mammalian enzyme poses two interesting questions. Firstly is it possible that the aggregate enzyme consists of the soluble form plus some material which is removed by exposure to 'high' salt concentrations? Secondly which is the active form during RNA replication in vivo?

Inhibition of the soluble RNA polymerase prepared by Ramuz et al. was observed at an ionic strength of 0.8. A similar result was also obtained at 0.8 with the soluble testicular enzyme purified by Ballard and Williams-Ashman (1966). They show an optimal stimulation of the enzyme at 0.35, with rising concentrations of ammonium sulphate. This closely resembles the results obtained by Fuchs et al. (1967) with the purified enzyme from E. coli, where the optimal ionic strength for ribonucleoside incorporation was found to be 0.36.

Richardson (1966b) has demonstrated that at an ionic strength of 0.5 the purified RNA polymerase from E. coli will not associate with DNA and if the ionic strength is gradually raised to this level the bound DNA will become detached and the free 13s form of the enzyme will appear in solution. If however the enzyme has begun to synthesise RNA it will not detach but the rate of synthesis will decrease.

In the ascites system the RNA polymerase is found to have an optimal ionic strength of 1.2; Widnell and Tata (1966), with an aggregate enzyme from rat liver nuclei, choose 0.6. In both of

these systems the RNA polymerase is bound to a considerable amount of native DNA and there are also other proteins present.

The similarity in ionic strength requirements between the soluble mammalian polymerase and that from bacterial sources suggests they may contain similar low concentrations of native DNA. The purified E. coli polymerase has been shown by starch-gel electrophoresis (Zillig et al. (1966)) to contain no DNA or contaminating protein and so its characteristics may perhaps be taken as those of the true enzyme. The soluble mammalian enzyme, on the other hand has not been purified to this degree and still contains low amounts of DNA (Ballard and Williams-Ashman (1966)). Using the purified polymerase from rat testes, Ballard and Williams-Ashman (1966) were however able to demonstrate variations in the base composition of the RNA produced corresponding to that of the primer DNA employed. Thus it seems possible that the RNA polymerase is operating under similar conditions in the purified bacterial and soluble mammalian systems.

Soluble preparations of RNA polymerase which have been examined and compared with the corresponding aggregate form, appear to possess a lower specific activity (e.g. Burdon and Smellie (1962)). However no aggregate enzyme has been prepared from rat testes, which may mean it is of lower specific activity than the corresponding form. It is possible that the soluble form of the polymerase is a degradation product, either natural or artificial, of the aggregate

enzyme. The properties of the two forms of the enzyme are almost identical, both giving higher levels of ribonucleoside triphosphate incorporation in the presence of all four nucleotides, Mn^{2+} in place of Mg^{2+} (except for the two aggregate forms demonstrated by Widnell and Tata (1966)). In addition, both soluble and aggregate forms of the enzyme show a marked preference for native DNA (Ballard and Williams-Ashman (1965), Furth and Ho (1965) and Jones and Berg (1966)). If the soluble enzyme were entirely an artefact these similarities would be unlikely. However it is still possible, if the mammalian enzyme like the bacterial, is composed of several units, that the soluble form of the enzyme could consist of several units linked to a minimum of DNA.

Deoxyribonucleoprotein consists of DNA and proteins. These proteins consist of largely of histones, but beyond those there are a number of proteins named collectively 'non-histone protein' as their exact composition is unknown. As described in the introduction, Gilmour and Paul (1967) have carried out a series of experiments to determine the nature of the material regulating transcription. They concluded that the repressor was contained in the non-histone protein fraction. Georgiev et al. (1967b) have carried out a similar series of experiments. They prepared chromatin from the nuclei of Ehrlich ascites tumour cells and exposed this chromatin to increasing concentrations of KCl. Up to 0.4M-salt solution deoxyribonucleoprotein appeared to be aggregated; in 0.5M-NaCl

(or KCl) it formed a gel and in 0.6M it gave a very viscous solution. The proteins dissociated from the deoxyribonucleoprotein complex were removed by centrifugation at 14,000g (after 0.3-0.5M-salt extraction) or at 105,000g (after extraction by more concentrated salt solution). The pellets obtained were washed, suspended and used as primer in the RNA polymerase reaction. Using a purified polymerase from E. coli they were able to demonstrate that increasing salt concentrations removed larger amounts of protein from association with the complex. The initial deoxyribonucleoprotein preparation revealed a very low template activity and extraction with salt solutions up to 0.4M does not increase its template activity significantly although much protein is removed during this procedure including part of the lysine rich histones and non-histone proteins. If the salt concentration rises to 0.6M only a relatively small part of deoxyribonucleoprotein (~15%) is additionally dissociated but the template activity of the deoxyribonucleoprotein rises to a large extent. It is significant that this increase in template activity cannot be ascribed to the increase in solubility of deoxyribonucleoprotein because although it is soluble in the presence of 0.6M-KCl, it is still insoluble in the medium used in assaying RNA polymerase activity. Further removal of protein from deoxyribonucleoprotein results in a further rise of template activity but it correlates with the increasing solubility of the material. The RNA produced in these experiments was isolated

and allowed to form hybrids with homologous DNA. Using deoxyribonucleoprotein untreated or exposed only to 0.4M-salt, the RNA produced did not complex with the DNA to any great extent. Extraction of the deoxyribonucleoprotein with 0.5-0.6M-salt solutions results in a large increase in the proportion of RNA produced which will form hybrids with the homologous DNA. This new level is as high as would be obtained if free DNA were used a primer. No higher level is obtained with further extraction of the deoxyribonucleoprotein. Thus the repressor of mRNA would seem to be contained in the protein extracted with 0.4-0.6M-salt. Continuing this type of experiment they were able to show that in vivo RNA polymerase was probably bound only to unmasked cystrons of the DNA.

The presence of histone and associated proteins in mammalian nuclei is undoubtedly a large factor distinguishing the environment of mammalian RNA polymerases from their bacterial counterpart. Soluble preparations of the mammalian enzyme may be a means of studying this more complex system, but there will remain the overshadowing problem of its true configuration.

SUMMARY

1. Experiments were carried out in vitro to characterise the RNA polymerase found in the nuclei of Landschutz ascites tumour cells.
2. The enzyme was found to be dependent on DNA as a primer. The level of incorporation of ribonucleoside triphosphates was found to increase when all four ribonucleoside triphosphates were present. This incorporation was stimulated by the presence of up to 1 μ mole/assay of Mn^{2+} and to a lesser extent by Mg^{2+} . Ammonium sulphate was shown to raise the specific activity of the enzyme, the extent of the rise being peculiar to this salt. A requirement was also shown for 2-mercapto-ethanol.
3. Attempts were made to purify the fraction containing RNA polymerase. These were restricted owing to the aggregate form of the enzyme. The preparation was treated with DNase or micrococcal nuclease to destroy the native DNA present. The treated preparations were then fractionated on DEAE cellulose but subsequent recoveries of RNA polymerase were not reproducible.

Treated and untreated polymerase preparations were fractionated by means of sucrose or glycerol gradients but no satisfactory separation was obtained.

4. Solutions of the aggregate preparation were made in various organic solvents. Sodium deoxycholate was found to give increased incorporation of ribonucleoside triphosphates and was therefore used in subsequent experiments.

Several fractionation techniques were tested using the detergent treated preparation. Addition of streptomycin sulphate followed by removal of the resulting precipitate was found to reduce the concentration of DNA present while increasing the specific activity of the RNA polymerase.

5. The characteristics of the streptomycin sulphate supernatant were established and compared with those of the crude preparation. The purified enzyme maintains the same characteristics with respect to Mn^{2+} , Mg^{2+} and ammonium sulphate, but no longer requires the presence of 2-mercapto-ethanol.

RNA as well as DNA can be accepted by the streptomycin preparation as primer, although the addition of DNase to the reaction mixture, in the absence of added polynucleotide, still causes a lower rate of incorporation.

6. Incorporation of ribonucleoside triphosphates by the crude enzyme was found to take place in the presence and absence of ammonium sulphate. This incorporation as already noted, was stimulated, in the presence of ammonium sulphate, by the addition of the other three ribonucleotides. In the absence of ammonium sulphate no such stimulation was obtained,

suggesting the formation of homopolymers. There was no incorporation of ribonucleotides by the purified preparation in the absence of ammonium sulphate, but in its presence addition of all four ribonucleotides caused an increase in enzyme activity.

7. In the presence of only one ribonucleoside 5'-triphosphate, the highest rate of incorporation by the crude enzyme was obtained with $[^3\text{H}]\text{UTP}$, the lowest rate with $[^3\text{H}]\text{GTP}$. Several experiments were performed using first $[^3\text{H}]\text{UTP}$ and later $[^3\text{H}]\text{GTP}$, to determine the effect of the presence of the other three ribonucleotides on the nature of the polyribonucleotide formed under these conditions. There was however no evidence to suggest that in using $[^3\text{H}]\text{UTP}$ as the labelled ribonucleotide there was any alteration in the heteropolymer formed.
8. Analysis on a sucrose density gradient of the polyribonucleotide product formed by the two enzyme preparations was also studied. That formed by the purified polymerase resembled more closely the product of the crude enzyme formed in the presence of ammonium sulphate than in the absence of the salt.
9. Both enzyme preparations were shown to be inhibited by the drug MPB. The nature of this inhibition was investigated and thought to be due to the formation of an enzyme - MPB - DNA complex. Both polymerase preparations were also shown to be inhibited by the presence of actinomycin D. This

inhibition was much greater in the presence of ammonium sulphate.

10. The methods of preparation and the characteristics of these RNA polymerase fractions are compared with those from other sources.

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A P P E N D I X

A Comparison of methods of preparing samples for scintillation counting.

Tritium labelled samples may be counted in a number of ways. The method chosen will depend on the nature of the labelled material and the facilities available. In all the subsequent experiments the samples were counted in either a Nuclear Chicago No. 725, or a Packard Series 4000 liquid scintillation spectrometer.

The material which was used in testing these conditions was a cold acid precipitate of the assay mixture in which labelled ribonucleoside triphosphate had been incorporated into RNA. The precipitate could be gathered by centrifuging at 600g. for 10 min.

The recovery of radioactive material was found to vary depending on the method of preparation employed. When the samples were hydrolysed in 98% formic acid and counted in a toluene based scintillation fluid containing absolute ethanol the recovery of counts was taken as 100%. The results obtained from the other samples are expressed as percentages of this.

I. Precipitation of the radioactive material on a suitable filter

It was relatively quicker to process a small number of samples using the filter technique. This consisted of precipitating the radioactive material with 5% cold trichloroacetic acid, a protein carrier also being present. The resulting precipitate was then collected on a range of filters supported on a Millipore filter holder. The precipitate was then washed with 20-25 ml. of cold 5% trichloroacetic acid and in the case of the RNA polymerase assays

Table A

The efficiencies and recoveries of radioactivity in RNA

polymerase assays using different filter membranes

| <u>Filter Material</u> | <u>Solubilising Agent</u> | <u>Scintillation Solvent</u> | <u>Efficiency</u> | <u>Percentage Recovery</u> |
|---|---------------------------|------------------------------|-------------------|----------------------------|
| Whatman No. 1 Filter Paper Coated with 2 ml. of Filter Aid | Hyamine Hydroxide | Toluene | 24% | 60 - 70% |
| Teflon Coated Glass Fibre Paper | Hyamine Hydroxide | Toluene | 22% | 75 - 85% |
| Glass Fibre/A (GF/81) | None | Toluene | 19.5% | 50 - 55% |
| Glass Fibre/B (GF/82) | None | Toluene | 18.5% | 55% |
| Glass Fibre/C (GF/83) | None | Toluene | 20% | 55% |
| * Glass Fibre/C | None | Toluene | 20% | 75 - 80% |

* Rectangles in a vertical position.

sodium pyrophosphate. This washing was followed with alcohol and the precipitate allowed to dry. The filter and dry precipitate were then transferred to a scintillation vial. The precipitate was rendered soluble by treatment with 0.5 ml. of hyamine hydroxide at 57° for 20 min. The samples were then counted in 10 ml. of toluene based scintillation fluid. This consists of 4 g. of P.P.O. (2,5-diphenyloxazole) and 200 mg. of P.O.P.O.P. (1,4-di-[2-(5-phenyloxazolyl)]-benzene) dissolved in 950 ml. of AnalaR toluene. Table A shows a summary of the filters used and the results obtained.

a) Filter aid

Filter aid as used here with Whatman No. 1 filter paper consisted of Hyflo-Super-Cel (prepared by Koch Light) 20 g./l. in 5% trichloroacetic acid. Although a relatively high counting efficiency was achieved with this method, the total counts obtained were low, suggesting a loss of radioactive material through the filter.

b) Teflon coated glass fibre paper

Teflon coated glass fibre paper (Fiberfilm filter material) although expensive to use, gave a higher retention of radioactive material. There was still some loss however.

c) Whatman glass fibres

Whatman glass fibres GF/81, GF/82, and GF/83 were also tested. They give similar results, GF/82 which is thicker (0.64 mm. as against 0.25 mm.) being harder to dry completely. The geometry of these glass fibre discs is very important as can be seen when the paper is erected

by spearing it with a short piece of capillary tubing. Preparing a large number of samples in this way is time consuming.

II. Methods of hydrolysing washed precipitates prior to liquid scintillation counting

The radioactive material was precipitated as before with 5% cold trichloroacetic acid in the presence of carrier protein. The precipitate was allowed to settle for 15 min. and then collected by centrifuging at 600g for 10 min. The supernatant was discarded and the precipitate resuspended in 4 ml. of 5% cold trichloroacetic acid and 0.2 ml. of saturated sodium pyrophosphate. After 10 min. this was centrifuged at 600g and the procedure repeated twice. The washed precipitate was then hydrolysed under varying conditions as is shown in Table B. The hydrolysed samples were counted in 5 ml. of dioxane based scintillation fluid, 10 ml. toluene based scintillation fluid or in toluene based scintillation fluid containing 6.8 ml. of toluene scintillator and 2.9 ml. of absolute ethanol. The volume of sample used was 0.3 ml. and in the case of the scintillator containing ethanol the volume was critical.

Dioxane used in the dioxane based scintillation fluid was either 'Scintillation grade' from Nuclear Enterprises, Edinburgh or AnalaR grade which had been purified by passage through a column of activated alumina (Type 'A' 16/32 mesh). The column was 6 cm. in diam. and 80 cm. long. No attempt was made to reactivate the alumina after use as this would involve the liberation of peroxides.

Table B

Influence of various methods of hydrolysis on the efficiencies
and recoveries of radioactivity in RNA polymerase assays

| <u>Agent</u> | <u>Scintillation Solvent</u> | <u>Efficiency</u> | <u>Percentage Recovery</u> |
|---|----------------------------------|-------------------|--------------------------------|
| Formic Acid | Dioxane | 15% | 85 - 90% |
| Perchloric Acid | Dioxane | - | - |
| Perchloric Acid neutralised with KOH | Dioxane | 14% | 70% |
| Trichloroacetic Acid | Dioxane | 5% | 20% |
| Trichloroacetic Acid neutralised with KOH | Dioxane | 8% | 55% |
| Trichloroacetic Acid extracted with ether | Dioxane | 12.5% | 80% |
| NCS | Toluene | 25% | 100% |
| Formic Acid | Toluene/Alcohol | 23% | 100% |

The dioxane scintillation fluid contained 0.7% P.P.O., 0.03% P.O.P.O.P. and 10% naphthalene.

Hydrolysis for 30 min. in a boiling water bath with 0.2 ml. of 98% formic acid yielded a high percentage count with dioxane scintillation fluid. However the efficiencies can be very low and dioxane is more expensive than toluene. In addition, dioxane is subject to peroxide formation and so must be freshly purified when used.

Some samples were hydrolysed with 0.2 ml. of 12N-perchloric acid in a boiling water bath for 60 min. The perchlorate ions were however found to cause severe quenching during counting and had to be removed by precipitation as the potassium salt. This adds a further step to routine procedures and introduces an additional source of error.

Experiments were also performed using 20% trichloroacetic acid for hydrolysis, in place of formic acid. Here the samples were heated in a boiling water bath for 60 min. Trichloroacetic acid also causes quenching but it could be adequately extracted with an equal volume of ether. Once more however an additional step is required.

Nuclear Chicago solvent (NCS) was tested as a means of solubilising the labelled material. 0.25 ml. of NCS were added to each sample which was then either heated at 57° for 30 min. or allowed to sit in the dark overnight at room temperature. It was

observed that exposure of NCS treated samples to artificial light gave rise to very high count rates which decayed rapidly when the samples were kept in the dark. Incubation overnight is therefore the preferable method of hydrolysis. When the samples were treated thus high efficiencies and high rates of recovery were obtained. This method was not used because of the care required to avoid high background counts.

Hydrolysis in formic acid seemed the most rapid, reliable method. Toluene scintillation fluid is the more economic so the last method tabulated was therefore the one chosen as standard.