POLYRIBONUCLEOTIDE SYNTHESIS BY CYTOPLASMIC ENZYMES

by J. R. WYKES

The possibility that the cell cytoplasm contains enzymes catalysing RNA biosynthesis was investigated in Landschutz ascites tumour cells. Enzyme fractions were obtained by differential centrifugation of disrupted cell homogenates. The incorporation of (α^{32}P) UTP into polyribonucleotides catalysed by such fractions was investigated in reaction mixtures containing primer RNA, ATP, GTP and CTP to stimulate conditions for synthesis of RNA. The microsomal fraction was most active in incorporating (α^{32}P) UTP into polyribonucleoti Experiments in which the microsomal fraction was analysed by sucrose density gradient centrifugation confirmed the particulate nature of the enzyme.

An acetone dried powder of the microsomal fraction was prepared and its properties further investigated. The incorporation of (α^{32}P) UTP was stimulated by an ATP regenerating system but not by ATP, GTP and CTP in the presence of the latter. The incorporation of (α^{32}P UTP showed a requirement for RNA as a primer and an absolute requirement for Mg^{2+} ions while Mn^{2+} ions, spermine and putrescene inhibited the reaction. Alkaline hydrolysis of the reaction products after

(α^{32}P) UTP incorporation showed mainly UMP residues to be labelled irrespective of whether ATP, GTP and CTP were present in the reaction mixture indicating synthesis of poly U chains.

^{3}H ATP, GTP and CTP were found to be incorporated into polyribonucleotides under the same conditions as (α^{32}P) UTP, though individual nucleotides were incorporated to different extents. The incorporation of ^{3}H ATP at increasing substrate concentration was inhibited by equal concentrations of GTP, CTP and UTP added to the reaction mixtures over the range of concentrations tested. These experiments indicate homopolyribonucleotide synthesi rather than the synthesis of RNA.

Extraction of RNA from the reaction mixtures after ^{3}H ribonucleoside 5'-triphosphate incorporation and analysis by sucrose density gradient centrifugation showed no labelling of the ribosomal RNA. The incorporat radioactivity appeared between the 4S region and the meniscus of the sucrose gradient. Determination of the chain length of the homopolyribonucleotide product showed only short sequences to be synthesised which is in agreement with the position of radioactive RNA on sucrose density gradients. Considerable phosphodiesterase activity was found to be associated with the microsomal

fraction which could account for the lack of labelling of ribosomal RNA and the short chain lengths of homopolyribonucleotide synthesised.

An attempt made to demonstrate homopolyribonucleotide occurrence *in vivo* proved negative though the possibility could not be ruled out that short chain homopolyribonucleotides were present.

The lack of ability of cytoplasmic fractions to catalyse *in vitro* the net synthesis of RNA is in agreement with *in vivo* experiments on the kinetics of nucleotide uptake into cells in the presence or absence of actinomycin D which indicate that synthesis of RNA occurs primarily in the nucleus on a DNA template. However limited homopolyribonucleotide synthesis does appear to be catalysed by the microsomal fraction, the possible significance of which is discussed.

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by

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ABBREVIATIONS

A	Adenine.
C	Guanine.
G	Cytosine.
U	Uracil.
AMP	Adenosine 5'-monophosphate.
CMP	Cytidine 5'-monophosphate.
GMP	Guanosine 5'-monophosphate.
UMP	Uridine 5'-monophosphate.
ADP	Adenosine 5'-diphosphate.
UDP	Uridine 5'-diphosphate.
ATP	Adenosine 5'-triphosphate.
dATP	Deoxyadenosine 5'-triphosphate.
CTP	Cytidine 5'-triphosphate.
GTP	Guanosine 5'-triphosphate.
TTP	Thymidine 5'-triphosphate.
UTP	Uridine 5'-triphosphate.
(^{32}P) UMP	Uridine 5'-monophosphate labelled with a radioactive phosphorus atom.
(α ^{32}P) UTP	Uridine 5'-triphosphate labelled with a radioactive phosphorus atom in the α position.

(α-^{32}P) TTP	Thymidine 5'-triphosphate labelled with a radioactive phosphorus atom in the α position.
(^{32}P) CEP	2-cyanoethyl phosphate labelled with a radioactive phosphorus atom.
tris	2-amino-2-hydroxymethylpropane-1,3-diol.
tris-HCl buffer	tris buffer adjusted to the required pH with concentrated HCl.
DNA	Deoxyribonucleic acid.
RNA	Ribonucleic acid.
sRNA	Soluble (or transfer) RNA.
poly A, etc.	Polyribonucleotides containing only adenylate residues; similarly for the other polyribonucleotides mentioned in the text.
poly (dAT)	A polydeoxyribonucleotide containing alternating deoxyadenylate and thymidilate residues.
pCpCpA	The terminal trinucleotide sequence of sRNA.
MAK	Methylated serum albumin on kieselguhr.
NMN	Nicotinamide mononucleotide.

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INTRODUCTION

INTRODUCTION

A combination of biochemical, cytological and genetical studies have lead to the realisation that two nucleic acids, RNA and DNA as well as protein are essential components of all living cells besides existing singly in combination with protein in viruses. The biochemical relationships between these three macromolecules are of fundamental importance in controlling the metabolism, growth and reproduction of living cells. The classical demonstrations (Avery, McLeod and McCarty, 1944) that DNA is the hereditary material, Brachet's observations that RNA is associated with the synthesis of proteins, which in turn as enzymes initiate cellular metabolic processes lead to the one gene, one enzyme hypothesis of Beadle and Tatum. This hypothesis in expanded form is the central hypothesis of molecular biology. Within such a framework the mediation of RNA in the genetic expression of protein synthesis is discussed.

1. The Physical and Chemical Structure of the Nucleic Acids.

The two nucleic acids RNA and DNA as found in nature are high molecular weight polymers; although chemically similar they exhibit different physical properties which reflect their different modes of synthesis and their

different biological functions.

Hydrolysis of nucleic acids, chemically or enzymically has allowed identification of the nucleotides and bases which form the repeating units of the polymer. The nucleotides are N-glycosides of a nitrogenous base and a phosphorylated pentose sugar. The latter is D-ribofuranose in RNA and D-2-deoxyribofuranose in DNA. The nitrogenous bases present in RNA are the purines, adenine and guanine, the pyrimidines cytosine and uracil, the latter being replaced in DNA by thymine. Besides these commonly occurring bases trace amounts of methylated purines have been identified in both RNA and DNA. (Alder, Weissman and Gutman, 1958; Dunn, Smith and Spahr, 1960; Dunn, 1959; 1961). In addition 5-methyl cytosine or 5-hydroxy methyl cytosine replace some or all of the cytosine residues in DNA from a variety of sources. (Wyatt, 1950; Wyatt and Cohen, 1952). Whilst pseudouridine and a number of methylated purine derivatives occur in sRNA. (Cohn, 1960; Hall, 1963).

With the development of ion exchange and paper chromatography, as methods for the separation of nucleotide mixtures obtained on hydrolysis of nucleic acids, it became possible to identify the mode of linkage between the nucleotide monomers. Thus, RNA treated with snake venom

phosphodiesterase gave a mixture of ribonucleoside 5'-monophosphates which were separated and identified chromatographically. (Cohn and Volkin, 1953). The 2', 3'-nucleoside phosphate mixture obtained on alkaline hydrolysis of RNA was similarly separated. Such experiments indicated the possibility of a 5', 2' or 5', 3' phosphodiester link. The problem was resolved in favour of the latter possibility by hydrolysis of RNA with spleen phosphodiesterase which produces a quantitative yield of 3'-nucleotides. This latter fact eliminates the possibility of alternating 5', 2'; 5', 3' or 5', 5'; 3', 3' links. (Whitfeld, Heppel and Markham, 1955). In the case of DNA the internucleotide linkage problem was more readily solved, there being no 2'-hydroxyl group. Similar experiments with specific nucleases have lead to the identification of either 3' or 5'-deoxyribonucleotides in quantitative yield from which it is deduced that a 3'-5' phosphodiester link is again involved. (Carter, 1951; Koerner and Sinsheimer, 1957). Such a structure is supported by electrometric titration experiments which show the hydroxyl groups of uracil and guanine are unsubstituted, and deamination experiments with HNO_2 which remove the amino groups of cytosine, adenosine and guanine without depolymerising the nucleic acid.

Apart from chemical differences naturally occurring RNA and DNA differ in that the latter has a one : one ratio of purines to pyrimidines. This causes profound differences in the physical properties of the two naturally occurring nucleic acids. X-ray analysis shows that DNA is a double stranded helical polymer, the 2 strands being held together by hydrogen bonds between G.C. pairs and A.T. pairs on opposite chains. The planes of adjacent base pairs are parallel to one another and perpendicular to the long axis of the helix. The sugar and phosphate groups are outermost and form the backbone of the molecule. The 3', 5' internucleotide links are oppositely polarised in complementary chains. (Watson and Crick, 1953). The validity of this structure was confirmed by Wilkins and co-workers, who were also able to add some refinements in interpretation (see Wilkins 1956 for a review). X-ray analysis of RNA on the other hand shows limited double helical regions with a DNA like structure. (Rich and Watson, 1954 a,b; Zubay and Wilkins, 1960). This is due to hydrogen bonding between base pairs formed by the RNA chain folding back on itself. Yeast sRNA has recently been crystalised which then shows a clear double helical structure similar to DNA. (Spencer, 1962). The precise nature of the folding of the RNA chain or of the hydrogen bonding arrangements cannot be ascertained from existing X-ray data. Double stranded native RNA has recently

been obtained from wound tumour and Reo viruses. These show diffraction patterns similar to DNA and sRNA. (Langridge and Gomatos, 1963). The double stranded structure is confirmed by electron-micrographs of the fibres. (Kleinschmidt and Dunnebacke, 1964). Similarly the replicative form of the RNA viruses R_{17}, TMV and MS2 has been demonstrated to have a double stranded helical configuration. (Langridge, Billeter, Borst, Durdon and Weissmann, 1964). However, differences exist between the double helical structures of RNA and DNA. It is known that actinomycin D complexes with DNA and prevents the B → A conformational change on lowering the humidity of the fibres. (Hamilton, Fuller and Reich, 1963). Thus it appears that actinomycin D binds only with the B configuration of the DNA helix. The actinomycin D — DNA complex also shows increased Tm values compared with untreated DNA. In the case of RNA, actinomycin D treated sRNA, (which has been shown to exist in the A configuration), T7 complementary RNA, or a hybrid of phage λDNA and RNA show no difference in Tm values compared with untreated controls. Hence any helical regions in RNA are likely to be in the A configuration. (Haselkorn, 1963).

The existence of hydrogen bonds in maintaining the secondary structure in RNA and DNA is confirmed by the

decrease in optical rotation and increase in ultraviolet absorption with temperature. The transitions are gradual and reversible in the case of RNA whilst with DNA they are sharp and irreversible, though in both cases the Tm is proportional to the G.C. content. The latter case represents the transition of a double helical rod like molecule to a single stranded randomly coiled molecule. In the case of RNA the transition represents the change of a compact flexible molecule folded back on itself to form helical hydrogen bonded regions to a randomly coiled molecule. These conclusions are supported by sedimentation coefficient and intrinsic viscosity measurements which indicate that in contrast to the rigid structure of DNA present in solution RNA behaves as a flexible molecule that gradually and reversibly changes its shape and dimensions with the ionic strength of the solution. (See Spirin, 1963 for a review). The helical content of RNA forming hydrogen bonded regions has been estimated to involve 70 - 80 per cent of the chain length. (Doty, 1961).

2. Cell Structure and the Intracellular Distribution of Nucleic Acids.

Two broad divisions of cellular organisation are recognised. Procaryotic cells represented by bacteria and

blue green algae and eucaryotic cells represented by all other types of plant and animal cells. Both types contain a nucleus embedded in the cytoplasm but no nuclear membrane is present in the former group.

The nucleus of the eucaryotic cell contains distinct chromosomes readily seen at mitosis and occasionally visualised as coiled threads by certain fixing procedures. Usually, however, the densely staining regions of the nucleus forming the chromatin and containing the DNA as nucleoprotein can be divided into two types; heterochromatin, densely staining condensed material and euchromatin which is less densely staining and more diffuse. Nucleoli, small densely staining spherical bodies, are often associated with the chromatin. In contrast, nuclei of procaryotic cells appear to contain a solid mass of DNA fibres, show no differentiation into chromosomes and contain no nucleoli.

The cytoplasm of eucaryotic cells contains numerous inclusions, the type and number depending on the species of cell. Most common among these are mitochondria, small rods or filaments between 0.5 - 2.0 μ long staining with janus green, lysozymes and chloroplasts, the latter occurring only in plant cells. Such inclusions are embedded in the basophilic ground substance or exgastoplasm.

Extensive electron-microscopy of many types of cell show the cytoplasm to be traversed by a three dimensional network of cavities bounded by a system of continuous membranes. (Palade, 1956 a,b). Small electron dense granules are found associated with the outer surface of the membranes. These structures, which also occur free in the cytoplasm, especially in tumour cells are known as ribosomes and the system of membranes and ribosomes is known as the endoplasmic reticulum. Not all the cytoplasmic membranes carry granules. Rough or smooth surfaced membranes may be distinguished according to the presence or absence of ribosomes. No membranes appear to be present in the cytoplasm of procaryotic cells though ribosomes have been obtained from such cells. Particles larger than the ribosomes, the chromatophores analogous to chloroplasts have been identified. No mitochondria or vacuoles appear to be present in such cells.

The distribution of nucleic acids in intact cells has been studied by cytological techniques. Specific stains such as the Feuglen stain (Feuglen and Rosenbeck, 1924) show that DNA is entirely located within the nucleus. Basic dyes such as toluidine blue which stains DNA and RNA, the Unna-Pappenheim stain, a mixture of methyl green (which stains DNA green) and pyronin (which stains RNA red) have

been used in locating nucleic acids. Such stains show basophilic granules over the whole cell. The Unna-Pappenheim stain has been used in conjunction with RNase pretreatment of fixed cell preparations. This removes the pyronin staining material from the cytoplasm, thus locating DNA in the cytoplasm and DNA in the nucleus. (Brachet, 1940, 1941). Similarly, DNA may be demonstrated in the nucleolus. An analogous experiment using DNase pre-treatment has shown DNA to be located in the chromosomes. (Kaufmann, McDonald and Kay, 1951).

Besides staining, ultraviolet micro-spectrophotometry before and after RNase or DNase treatment gives a similar distribution of nucleic acids (Davidson and Waymouth, 1946). Both methods have been used as the basis of a quantitative estimation of the nucleic acids. Ultraviolet micro-spectrophotometry measures the total amount of RNA, DNA and free nucleotides and may be used in conjunction with the Feulgen reaction, RNase or DNase pretreatment. (Caspersson, 1940, 1947). Micro-spectrophotometry of Feulgen stained nuclei has allowed the estimation of DNA in a single nucleus. (Mirsky and Ris, 1951; Pollister, 1955). Such methods show that the amount of DNA per cell is a function of its chromosome complement. Thus diploid cells contain double the amount of DNA in haploid cells, tetraploid cells

double the amount in diploid cells and so on. The amount of DNA in diploid cells of any given species is characteristic of that species. (Leuchtenberger, Leuchtenberger, Vendrey and Vendrey, 1952). The cellular RNA content, however, tends to vary from tissue to tissue and depends on the nutritional state of the organism. (Caldwell and Hinshelwood, 1950).

The distribution and location of nucleic acids has also been aided by the development of techniques allowing the separation of cell components. Claude, (1943; 1946) first used differential centrifugation of liver homogenised in 0.88 M sucrose. Centrifugation at 600 x g yielded a nuclear fraction, at 5,000 x g a mitochondrial fraction and at 25,000 x g a microsomal fraction. The technique has since been refined using higher gravitational fields. (Hogeboom, Schneider and Palade 1947, 1948). Subfractionation of the mitochondrial and microsomal fractions has also been achieved. Lysosomes have been separated from the mitochondria (de Duve, 1959), and ribosomes have been purified from the microsomal fraction after solubilising the membrane component with deoxycholate. (Petermann and Hamilton, 1961). Similarly, ribosomes have been isolated from the nucleus (Fogo, et al., 1962), from the nucleolus (Birnstiel, Chipchase and Hyde, 1963)

and from chloroplasts. (Clark, Mathews and Ralph, 1964).

Electron microscopic studies have been successful in correlating the cellular features observed by cytological staining procedures with the structures observed in electron-micrographs and with the subcellular particles obtained on disruption and differential centrifugation of cell homogenates. Thus the endoplasmic reticulum seen in electron-micrographs corresponds to the basophilic regions of the cytoplasm. Similarly, electron-micrographs of the microsomal fraction show this to consist of fragments of the endoplasmic reticulum, free and attached ribosomes. (Palade and Siekevitz, 1956 a,b). Analysis of the subcellular fractions has revealed the pattern of distribution of the nucleic acid in relation to cell structures. Cell fractionation experiments have confirmed that DNA is almost entirely confined to the nucleus and that the average amount per cell is constant for the somatic cells of any given species. They have also shown that RNA occurs mainly in the cytoplasmic ribosomes and soluble fraction of the cytoplasm, though small amounts occur in the nucleus, especially in the nucleolus and nuclear ribosomes.

It is now possible to obtain native RNA in an undegrade state largely as a result of the introduction of phenol as a deproteinising agent (Kirby, 1956) and the use of nuclease

inhibitors during the extraction process. Analysis of such material by ultracentrifugation has lead to the identification of three components having sedimentation coefficients of 23S, 17S and 4S from bacterial sources and 30 - 28S, 18S and 4S from plant and animal sources. These species of RNA have molecular weights 1.1 - 1.4 x 10^6; 0.5 - 0.7 x 10^6 and 2.5 - 3.0 x 10^4 respectively and correspond to the RNA in 50S, 30S ribosomes and the soluble or unsedimentable fraction of the cell. Spirin, (1963) has determined the S values of a variety of ribosomal RNAs under identical conditions and calculated their molecular weights. It appears that the total RNA from a variety of bacterial 70S ribosomes has a molecular weight of 1.7 x 10^6 though sub-units show considerable variation in molecular weight. This suggests that the ribosomal RNA may be formed as one unit and then divided into smaller units.

The species of RNA described above show no dissociation on heating or dialysis to remove metal ions though earlier preparations did, probably due to contamination with nucleases. (Spirin, 1961; Bogdanova, Gavrilova, Dvorkin, Kisselev and Spirin, 1962). Electron micrographs of the high molecular weight preparations of RNA show long continuous threads, in some cases up to 18,000 Å long corresponding to the continuous extended chain of the 23S

RNA. (Kisselev, Gavrilova and Spirin, 1961; Moller and Boedtker, 1962). Hence the cellular RNA appears to be a continuous unbranched polynucleotide chain. The chain length being 4000 - 4500, 1500 - 2000 and 80 - 100 nucleotides for the three species of RNA.

Ribosomal RNA forms 75 - 85 per cent of the total cellular RNA; sRNA 10 - 20 per cent and a rapidly labelled fraction known as messenger RNA having a DNA like base ratio, 1 - 2 per cent of the total. The latter fraction is heterogeneous in molecular weight. S values of 10 - 30S have been recorded in Escherichia coli and up to 45S in mammalian cells. In general the base composition of RNA is different from DNA except for the messenger RNA fraction. The base ratios of total cellular RNA are usually similar for RNA from different tissues of the same species. (Kit, 1960). sRNA shows a strict one : one ratio of purines to pyrimidines and this is often, though not necessarily the case with ribosomal RNA (see Magasanik, 1955, and Brown, 1963, for reviews). Analysis of 23S and 16S ribosomal RNA from a variety of bacteria showed little difference in base composition. (Midgley, 1962). However, heterogeneity of the base sequence in ribosomal RNA is indicated by fractionation of partially degraded preparations having sedimentation coefficients of 2.5 - 4S by countercurrent

distribution into fractions of different base composition. (Kirby, 1962). Even when less degraded DNA from rat liver and Drosophila has been studied there is evidence of both inter and intramolecular heterogeneity of the major species of the total RNA. (Kirby, Hastings and O'Sullivan, 1962). sRNA has been extensively fractionated by countercurrent distribution, (Apgar, Holley and Merrill, 1962), and specific amino acid sRNAs have been obtained which show different base compositions. (Tada and Tada, 1962).

The molecular weight of DNA *in vivo* remains uncertain because of the ease with which shear degradation occurs and the difficulty in accessing its size by the methods currently available. Chromatographic, autoradiographic and electronmicrographic techniques indicate molecular weights of more than 10^7 for the best preparations. It appears that the DNA isolated from T_2 bacteriophage of molecular weight 12×10^7 is sufficient to account for the total DNA of the bacteriophage particle which is almost certainly a single molecule. (Davison, Freifelder, Hede and Levinthal, 1961; Beams and Thomas, 1961). The DNA may also exist as a single molecule in bacteria. This is suggested by the single circular chromosome observed in electronmicrographs of DNA extracted from *E. coli* (Cairns, 1963). Similar high molecular weight preparations of DNA

have been reported from vertebrate sources. (Davison, 1960; Kuehl, 1962).

Heterogeneity of the base composition of DNA has been shown by fractionation on MAK columns (Cheng and Sueoka, 1962; Sueoka and Cheng, 1963), and by counter-current distribution (Kidson and Kirby, 1963). Since it is not known whether undegraded DNA is present differences in base composition can reflect both differences between individual molecules and differences in nucleotide sequences. CsCl density gradient centrifugation of DNA, however, from most vertebrate species gives a single band, though a minor or satellite band has been detected in several instances, e.g. in mouse and guinea pig DNA (Kit, 1962). These may be due to the mitochondrial DNA recently identified in chick embryos and algae (Nass and Nass, 1963 a,b). The satellite band appearing on centrifugation of crab DNA on CsCl density gradients has been identified as the naturally occurring poly dAT copolymer (Sueoka and Cheng, 1962, a).

3. In vivo Studies on RNA Metabolism.

RNA can be labelled both in mammalian systems and micro-organisms by small molecular precursors such as ^{32}P orthophosphate, ^{15}N ammonium citrate, ^{15}N glycine,

^{14}C formate, ^{14}C purines or ^{14}C pyrimidines (see Smellie, 1955, for a review). The pathways of purine riboside and pyrimidine riboside biosynthesis are now established from work on both micro-organisms and animal systems. Enzyme systems are also known for interconversions among each of the naturally occurring purine and pyrimidine nucleotides, their phosphorylation to nucleoside di and triphosphates and their reduction to deoxyribonucleotides. The details of such pathways are without the scope of this thesis (see Buchanan, 1960 and Crosbie, 1960 for reviews).

A. Metabolic relationships between species of RNA.

Numerous attempts have been made to demonstrate turnover of total RNA in mammalian cells and precursor product relationships between individual species of RNA. Care must be taken in the interpretation of *in vivo* experiments and several different experimental approaches are required to give a satisfactory indication of the intracellular events.

To show the metabolic stability of RNA L cells have been totally labelled with ^{32}P orthophosphate and then transferred to an unlabelled medium. In the following two generations 20 - 30 per cent of the labelling was lost from the RNA and none from DNA. No further loss of label from RNA occurred in the next five generations (Siminovitch

and Graham, 1956; Graham and Siminovitch, 1957). In a similar experiment ascites tumour cells grown *in vivo* and totally labelled with ^{14}C glycine or adenine were transferred to unlabelled mice. During the subsequent two generations 30 per cent of the label was lost from RNA and none in the succeeding generations. (Révész, Forssberg and Klein, 1956). Besides indicating metabolic stability of a fraction of the RNA retention of label could also be due to efficient reincorporation of mononucleotide breakdown products. However, a more detailed re-examination of the above experiments showed that radioactivity was conserved in the purines of RNA, that there was no change in the ratio of ^{14}C adenine/^{14}C guanine and that no transfer of radioactivity to DNA occurred; whereas during the initial two generations after transfer when 30 per cent of the RNA was degraded this ratio showed a considerable decrease. (Scott and Taft, 1958; Scott, Taft and Letourneau, 1962). In experiments of a different type the relative rates of incorporation of precursors into RNA and DNA have been determined in rapidly growing Hela cells. There is consistently more rapid incorporation into RNA bases than DNA bases suggesting a significant turnover of RNA under conditions of cellular multiplication. (Salzman, Eagle and Sebring, 1958; Salzman and Sebring, 1959)

Similar RNA turnover has been shown to occur in non growing cells. Rabbit macrophages which show no incorporation of ^{14}C thymidine into DNA and no net synthesis of RNA did however show incorporation of ^{14}C adenine into RNA which was linear with time. Transfer to a medium containing unlabelled adenine resulted in a decrease in the labelling of the RNA indicating that about 5 per cent was continuously turning over. (Watts and Harris, 1959).

The kinetics of RNA labelling have been studied in detail with cultures of mammalian and plant cells using autoradiographic procedures. In general such studies show incorporation of radioactive precursors initially in the nucleus and after a time lag in the cytoplasm. The time lag varies considerably from cell to cell but is roughly proportional to the length of the cell cycle. After initial labelling and transfer to a non radioactive medium the amount of label in the nucleus often continues to increase especially in growing cells where it is usually not possible to wash out or dilute labelled nucleotides with unlabelled nucleosides sufficiently rapidly. (Perry, Hell and Errera, 1961; Perry, Errera, Hell and Durwald, 1961; Prescott, 1962). After further incubation in an unlabelled medium nuclear labelling decreases and is often paralleled by an increase in labelling in the cytoplasm.

Harris, (1959) however, found that the nuclear loss was not great enough to account for the cytoplasmic gain, and that the nuclear fraction was turning over independently. Further experiments with Hela cells suggested that at least 3 different fractions turn over at different rates. (Watts, 1964 a,b).

More detailed autoradiographic studies involving short periods of incubation with a labelled precursor, removal of the labelled precursor and its replacement by unlabelled precursor, (i.e. pulse and chase experiments) have provided evidence of the sequence of labelling of nuclear RNAs, and of the relationships of these to cytoplasmic RNAs. Autoradiographic studies of the incorporation of ^{3}H cytidine and ^{3}H adenosine into the RNA of Hela cells show that the chromatin and nucleolar regions in the nucleus are both rapidly labelled, while the cytoplasm is more slowly labelled. Irradiation of the nucleolus by a microbeam of ultraviolet light prevents incorporation into the nucleolus and reduces cytoplasmic incorporation by 70 per cent. Nuclear incorporation outside the nucleolus is reduced 30 per cent after 1 hour but is not affected initially. Similar ultraviolet irradiation of the chromatin does not affect the nucleolar or cytoplasmic labelling. (Perry et al, 1961, a). Using the same system, the incorporation of nucleosides was shown to occur

independently into the chromatin and nucleolus. During a chase 50 per cent of this label migrated into the cytoplasm but the cytoplasmic gain appeared greater than the nuclear loss indicating some independent RNA synthesis in the cytoplasm and contrary to the results with ultraviolet irradiation. (Seinsvaara, Hiller-Bruner, Brunfaut and Errera, 1958). However, there is general agreement that the nucleolus and chromatin are rapidly labelled independently of one another and in constant ratio. (Amano and Leblond, 1960; Bather and Pirillo-Popper, 1961; Muramatsu, Hodnett and Busch, 1964).

It is now generally realised that further detailed kinetic analysis of the relationships between the various fractions of RNA turning over at different rates is not possible. Most autoradiographic experiments mentioned above tend to indicate turnover of nuclear RNA and transfer of a fraction to the cytoplasm and a low turn over of the latter fraction. Alternative explanations including the possibility of independent cytoplasmic synthesis are possible owing to lack of the necessary control experiments. Ambiguity is likely to arise because of interconversions between adenine and guanine, cytidine and uridine, difficulty in achieving rapid dilution of the nucleotide pools after pulse labelling experiments, feed back

inhibition, differences in the nuclear and cytoplasmic pool sizes and terminal labelling of sRNA.

Further evidence for metabolic differences between different RNA fractions has come from experiments on the fractionation of pulse labelled cells. Initial experiments with pulse labelled nuclei indicated the presence of several types of RNA that differ in extractability and metabolic activity. Thus fractionation of rat liver nuclei labelled in vivo showed the RNA of the nucleolar chromosomal complex to be most heavily labelled followed in order by low molecular weight RNA from the nuclear sap, the nuclear microsomes (56,000 x g per 1 hour) and the nuclear ultramicrosomes (105,000 x g for 2 hours). All these fractions were much more heavily labelled than the cytoplasmic RNA. (Georgiev and Samarina, 1961). Further analysis of the nucleolar chromosomal complex showed it to contain three distinct species of RNA. Treatment with 1.0 M NaCl removed nearly all the DNA and some associated RNA. Treatment of the residue in dilute salt solution with phenol released about 75 per cent of its RNA in the aqueous phase. This was termed nucleolar I RNA and had a high GC content showing similarities to ribosomal RNA. The residual RNA appearing at the phenol water interphase was also isolated and termed nucleolar II RNA. This had

a base ratio resembling DNA and ten times the specific activity of Nucleolar I RNA. With short periods of labelling RNA associated with DNA in the 1M NaCl extracts had still higher specific activity. (Sibitani, de Kloet, Allfrey and Mirsky, 1962). Experiments in which the total RNA was extracted using detergents from nuclei of pulse labelled rat liver and separated by sucrose density gradient centrifugation showed a polydisperse rapidly labelled RNA with sedimentation coefficients from 6 - 30S. The 23 - 30S, 16S ribosomal RNA and 4S transfer RNA were unlabelled. (Hiatt, 1962).

Many experiments have shown that when cytoplasmic fractions or whole cells are extracted with phenol in the absence of detergents RNA is obtained that can be shown by sucrose density gradient centrifugation to consist of 30S, 16S and 4S components giving virtually complete separation from any rapidly labelled RNA. (Sibitani, Yamana, Kimura and Okageki, 1959; Sibitani, Yamana, Kimura and Takahashi, 1960; Yamana and Sibitani, 1960; Harris and Watts, 1962). When the extraction is performed with phenol containing detergents or with hot phenol all species of nuclear RNA are extracted. Using this technique followed by centrifugation in sucrose density gradients labelled RNA from Hela cells incubated with

^{14}C uridine for 1 hour shows polydisperse labelling in the 10 - 30S region and additional labelled peaks of 35S and 45S. There is no definite correspondence in distribution of radioactivity and material absorbing in the ultra-violet at 260 mμ at such short time intervals. (Scherrer and Darnell, 1962).

Thus 4 fractions of RNA have been clearly recognised, though other fractions are not excluded; (a) RNA in the nuclear and cytoplasmic ribosomes with sedimentation coefficients of 30S and 18S (23 and 16S in bacteria), (b) a 4S component, both in the nucleus and cytoplasm, (c) RNA associated with the nucleolar chromosomal complex that has a high G.C. content and is similar to ribosomal RNA, (d) a second RNA associated with the nucleolar chromosomal complex that has a base ratio approximating to that of DNA. The species referred to in (c) and (d) are rapidly labelled species and correspond to the rapidly sedimenting peaks and the polydisperse labelling seen in sucrose gradients. Possibly a fifth type of RNA exists which is closely associated with the chromatin. The inter-relationships between these species of RNA have been indicated by pulse labelling experiments followed by transfer to an unlabelled medium in the presence and absence of actinomycin D.

Pulse labelled RNA that has been extracted from HeLa cells and analysed on sucrose density gradients shows 35S and 45S components of rapidly labelled RNA. If the cells were preincubated in 10^{-7}M actinomycin D the labelling in these peaks was considerably reduced though labelling in the 10 - 30S region was unaffected. In similar experiments in which incorporation of ^{3}H ribonucleosides was observed autoradiographically, the same actinomycin D treatment shows that nucleolar labelling is reduced without affecting the extent of labelling in the chromatin. Furthermore, phenol extraction of the pulse labelled RNA after a 4 hour "chase" showed the 30S and 18S components had become labelled. This labelling of ribosomal RNA was not observed if actinomycin D was present during the initial pulse though it did occur if actinomycin D was added during the "chase". Thus it appears that the rapidly labelled 35S and 45S RNAs are synthesised in the nucleolus and converted to 30S and 18S ribosomal RNAs. (Scherrer, Latham and Darnell, 1963). These experiments confirm the previous results of Perry (1962), with L strain fibroblasts.

Additional evidence confirms the nucleolar origin of ribosomal RNA. RNA from the two sources has been demon-

strated to have a similar base composition. (Georgiev and Mantieva, 1963; Edström, 1963) and a ribonucleoprotein has been extracted from a nucleolar preparation from pea seedlings with a sedimentation coefficient of 80S. (Bimstiel, Chipchase and Hyde, 1963). Whether such ribosomes are transferred into the nucleus and then into the cytoplasm has not been conclusively demonstrated. Evidence has been obtained that the rapidly labelled RNA in the 35S and 45S peaks can be converted into 30S and 20S ribosomal RNA. Some of the 20S RNA but not the 30S RNA appears in the cytoplasm. This may, of course, be a messenger RNA fraction. (Penwick, 1964).

Little information is available concerning the synthesis of sRNA or its stability once synthesised. Hela cells after 30 minutes incubation with ^{3}H cytidine show only nuclear labelling. Extraction and sucrose density gradient centrifugation of the RNA show that the 4S peak is labelled. Relatively high concentrations of actinomycin D inhibit this labelling, thus suggesting the nuclear origin of sRNA. (Perry, 1962).

B. <u>The nuclear origin and relation to DNA of RNA synthesis.</u>

The majority of the autoradiographic experiments described above indicate the nuclear origin of RNA synthesis.

Though they fail to show conclusive transfer of RNA to the cytoplasm they also provide little evidence of the independent cytoplasmic synthesis of RNA. Further evidence for the nuclear origin of RNA comes from experiments on nuclear transplantation and for DNA dependent RNA synthesis from experiments with actinomycin D.

Nuclei from ^{32}P labelled Amoeba proteus were transferred to non-labelled recipient cytoplasm and vice versa. Labelled products were detected in the non-labelled cytoplasm but not in non-labelled nuclei in the reverse transplantation, indicating transfer of RNA from the nucleus to the cytoplasm. However, the results could also be interpreted as transfer of degradation products of nuclear RNA to the cytoplasm and not vice versa. (Goldstein and Plaut, 1955). Incorporation of nucleosides into RNA has been investigated in enucleate cells. Of six types of cell tested, two species A. proteus and Acetabularia mediterranea showed RNA synthesis on enucleation. However, both of these contain extranuclear DNA, in rickettsial endosybionts in the former and in chloroplasts in the latter. No RNA synthesis could be detected in enucleate Hela cells, amphibian fibroblasts, Tetrahymena pyriformis or Acanthamoeba sp. (See Prescott, 1964, for a review). In Paramecium aurelia where cell division occurs to give one cell containing

a micronucleus and one a macronucleus RNA synthesis in the former is reduced by 97 per cent. Similarly in order to overcome the surgical objections to enucleation experiments RNA synthesis has been studied in mammalian reticulocyte formation where natural enucleation occurs on maturation of the normoblast. At the time of enucleation RNA synthesis stops though protein-synthesis continues for 1 - 2 days.(Pinheiro, Leblond and Droz, 1963).

The effect of actinomycin D on cellular RNA synthesis can be taken as a pointer to its DNA dependence. Actinomycin D has been shown to inhibit DNA dependent RNA polymerases in vitro by specifically binding with GMP residues in the primer DNA. (Goldberg and Rabinowitz, 1962; Hurwitz, Furth, Malamy and Alexander, 1962). In vivo actinomycin D has been shown to inhibit 99 per cent of the RNA synthesis in L strain fibroblasts though some protein and DNA synthesis was still able to occur. (Reich, Franklin, Shatkin and Tatum, 1962). Similarly actinomycin D completely inhibited the incorporation of ^{14}C uracil and guanine into RNA of ascites tumour cells (Haxbers and Muller, 1962) and into all species of RNA synthesised in Bacillus subtilis, including that produced in the presence of chloramphenicol. (Aes, Reich and Valanja, 1963).

Increasing concentrations of actinomycin D have been shown to inhibit progressively 28S and 18S ribosomal RNA then sRNA synthesis in L cells. Residual incorporation was due to turnover of the terminal pOpOpA sequences in sRNA. (Franklin, 1963).

Further DNA dependence of RNA synthesis is illustrated by specific hybridisation of all the major cellular RNA components with homologous DNA. T2 DNA was first shown to form specific RNase resistant hybrids with T2 specific RNA, indicating sequence homology between the base sequences in each. (Hall and Spiegelman, 1961). This observation has been used to show hybridisation between heated DNA and a rapidly labelled messenger RNA obtained from stepdown cultures of E. coli and Pseudomonas aeruginosa. RNase resistant hybrids are only formed with RNA and DNA from the same species. (Hayashi and Spiegelman, 1961). Similar experiments have shown that 23S and 16S ribosomal RNA of Bacillus megaterium forms hybrids specifically at different sites with its DNA suggesting two distinct cistrons for its synthesis. (Yanofsky and Spiegelman, 1962; 1963). sRNA has also been shown to form hybrids with its homologous DNA. (Goodman and Rich, 1962). Hybridisation experiments have been used to show homology between ribosomal and nucleolar RNA which is further evidence for the nucleolar

origin of the former. Thus ^{32}P labelled ribosomal RNA from pea seedlings forms RNase resistant hybrids with its homologous DNA, and the formation of hybrids is competitively inhibited by nucleolar RNA. (Chipchase and Birnstiel, 1963). A concentration of the DNA complementary to 28S ribosomal RNA has been demonstrated in the nucleolar associated chromatin of pea seedlings. (McConkey and Hopkins, 1964).

C. The transcription of the DNA helix by RNA.

Having established the nuclear origin and DNA dependence of RNA synthesis, an important question in the functioning of DNA in directing protein synthesis is whether messenger RNA is a copy of one or both strands of the double helix. It is known from *in vitro* experiments with purified RNA polymerases that both strands of DNA are copied. (Geiduschek, Nakamoto and Weiss, 1961, see also section 4B). Investigation of the transcription *in vivo* provides evidence that in some cases only one strand of DNA is replicated for the production of messenger RNA.

Examination of the effect of fluorouracil after T4 infection of *E. coli* on the phenotypic reversion of rII mutants showed about half of the T-A pairs estimated to be present in the mutant phage DNA were found to revert

with fluorouracil. This would be expected if about half the A, i.e. one strand could pair with fluorouracil. (Champe and Benzer, 1962). In the same system differences have been found between the base composition of messenger RNA and the T4 phage DNA, suggesting the former is not a copy of both strands of the DNA. (Bautz and Hall, 1962). A similar conclusion was reached from studies on the transforming ability of separated strands of pneumococcal DNA. One strand expressed its genetic information more rapidly than the other. (Guild and Robison, 1963).

More concrete evidence has been obtained by hybridisation of messenger RNA from phage ∝infected B. megaterium and SP.8 infected B. subtilis with the phage DNA. The DNA from these phages has been separated into heavy and light strands, the latter enriched in purines and the former in pyrimidines. (Cordes, Epstein and Marmur, 1961). Messenger RNA has been tested for its ability to hybridise with the heavy and light strands. Specific hybrids are only formed with the heavy strands of the homologous DNA in each case. (Tocchini-Valentini et al, 1963; Marmur et al, 1963). Similarly messenger RNA from ϕ x 174 infected E. coli does not hybridise with single stranded DNA of the mature phage but only with the heated double stranded DNA of the

replicative form and hence has an identical sequence to the mature phage DNA. (Hayashi, Hayashi and Spiegelman, 1963). In contrast to this, an experiment in which B. subtilis auxotrophic for histidine and tryptophan was transformed by a hybrid DNA made from strains singly auxotrophic for histidine and tryptophan, showed before DNA replication occurred enzymes synthesising both histidine and tryptophan appeared. This indicates both strands of the transforming DNA are producing messenger RNA. (Bresslor, Kreneva, Kushev and Mosevitskii, 1964).

Asymmetric synthesis has also been obtained in vitro using a crude supernatant fraction of B. megaterium and phage$\propto$ DNA as template. (Geiduschek, Tocchini-Valentini and Saxnat, 1964). The ability of DNA to serve as a template for asymmetric synthesis is dependent on the native configuration of DNA but not on the continuity of the phage chromosome. In contrast to this the asymmetric RNA synthesis occurring with nuclease free E. coli polymerase and double stranded ϕ x 174 DNA is dependent on the circular nature of the latter DNA. Breakage of the circular DNA template allows replication of both strands of DNA, indicating strand selection is a feature of the circular chromosome. (Hayashi, Hayashi and Spiegelman, 1964). The details of what causes strand selection for in vivo

transcription remain unknown. The anomalous results obtained *in vitro* may be due to the differences between the unpurified and highly purified RNA polymerases used, the latter being partially separated into subunits. (Fuchs, Zillig, Hofschneider and Preiss, 1964).

Besides asymmetric transcription of DNA the hybridisation technique has been used to show that non random reading of the genome occurs. On T2 infection of *E. coli* different messenger peaks could be identified at different time intervals indicating sequential non random reading of the genome. (Sueoka and Spiegelman, 1962). Similarly, in *B. subtilis* undergoing sporulation germination and stepdown transitions characteristic RNAs are formed which competitive hybridisation experiments show are derived from distinct genetic loci, indicating sequential transcription of the genome during morphogenesis. (Doi and Igarashi, 1964).

4. *The in vitro Synthesis of Nucleic Acids.*

The first enzyme isolated catalysing a net synthesis of nucleic acid was polynucleotide phosphorylase (EC 2.7.7.8) from *Azotobacter vinelandii*. (Grunberg-Manago, Ortiz and Ochoa, 1956). Ribonucleoside diphosphates are required as substrates and are incorporated into polyribo-

nucleotides from reaction mixtures containing single ribonucleoside diphosphates or mixtures of ribonucleoside diphosphates. In the latter case the base composition of the product reflects the base composition of the reaction mixture, with highly purified enzyme. The reaction occurs after a lag period in the absence of added polyribonucleotides as primer but this lag is abolished by the addition of primer polyribonucleotides. (See Grunberg-Manago, 1963 for a review). Although the enzyme occurs widely in bacteria, it is probably not involved in net RNA synthesis in vivo.

Since 1956 enzymes catalysing the net synthesis of DNA and RNA have been isolated from a variety of plant, animal and bacterialsources. These are DNA polymerase or DNA nucleotidyl transferase (EC 2.7.7.7) and RNA polymerase or RNA nucleotidyl transferase (EC 2.7.7.6). Both enzymes replicate the base sequence of a primer or template DNA in the formation of DNA or RNA products. Other enzymes are known which incorporate deoxyribonucleotides or ribonucleotides onto the 3'-hydroxyl end groups of existing polydeoxyribonucleotide or polyribonucleotide chains.

A. The biosynthesis of DNA.

DNA polymerase was first discovered in extracts of E. coli (Kornberg, Lehman and Simms, 1956) and subsequently

in animal tissues. (Bollum and Potter, 1957; Davidson, Smellie, Keir and McArdle, 1958). The *E. coli* polymerase was at first purified two thousand times and its properties investigated. (Lehman, Bessman, Simms and Kornberg, 1958). The enzyme catalyses the simultaneous incorporation of adenine, guanine, cytosine and uracil deoxyribonucleoside triphosphates into DNA in the presence of template DNA. Omission of any one of the triphosphates causes a hundred fold decrease in the reaction. The product DNA is double stranded, one strand being derived from the primer and the other being newly synthesised, has a high molecular weight and seemed to be indistinguishable from native DNA. (Bessman, Lehman, Simms and Kornberg, 1958). The molar proportions of the bases in the primer and product are always closely similar and more detailed analysis of the sixteen nearest neighbour frequencies shows the product DNA best fits a model in which the chains of the double helix have opposite polarity as predicted from X-ray data. (Josse, Kaiser and Kornberg, 1961).

Recently DNA polymerases have been further purified from *E. coli* and *B. subtilis*. (Richardson, Schildkraut, Aposhian and Kornberg, 1964; Okazaki and Kornberg, 1964). Experiments with these highly purified enzymes have thrown further light on the mode of DNA replication. Using

native DNA as a template it was found that the product DNA, unlike native DNA readily renatures after heating and shows a branched structure in electronmicrographs. No evidence could be found for covalent linkage of product to primer and it is suggested that replication of the product DNA occurs before the primer DNA is completely replicated. (Schildkraut, Richardson and Kornberg, 1964). If native DNA converted to a partially single stranded form is used as template, DNA polymerase activity first repairs the single stranded regions reconverting the template to a double strand with the same properties as native DNA. The product strand is covalently bound to primer and any further synthesis results in the production of DNA showing non-denaturability and a branched structure in electron-micrographs. (Richardson, Inman and Kornberg, 1964). Such experiments indicate the lack of control mechanisms for DNA synthesis in vitro and are further evidence for a semiconservative mode of replication.

DNA polymerases from mammalian systems have been less extensively purified, though in general show the same characteristics as the bacterial polymerases. A partially purified DNA polymerase has been obtained from calf thymus which shows an absolute requirement for heat denatured DNA. (Bollum, 1959). This enzyme has been obtained nuclease free on further purification. (Bollum, 1963). DNA poly-

merases have also been partially purified from Ehrlich ascites tumour cells and bone marrow (Smellie, Keir and Davidson, 1959), from regenerating rat liver (Mantsavinos, 1964) and mouse fibroblasts (Gold and Holleiner, 1964).

Besides replicative synthesis of template DNA enzymes have been identified which incorporate single deoxynucleotides onto the 3'-hydroxyl ends of DNA. Such a system was first purified from calf thymus nuclei. (Krakow, Couregeorgopoulos and Canellakis, 1961). These enzymes have been further investigated and shown to be distinct from the DNA polymerase. (Keir and Smith, 1963; Bollum, Groeniger and Yoneda, 1964).

B. The biosynthesis of RNA.

Considerable evidence from experiments in vivo shows that RNA synthesis is DNA dependent. It is therefore not surprising to find that most enzymes from cellular sources catalysing the net synthesis of RNA are DNA dependent.

DNA dependent RNA polymerases recognised by sensitivity of incorporation to RNase and DNase have been found widely distributed in nature and have been partially purified from several sources. Huxwitz, Bresler and Diringer (1960); Ochoa, Burma, Kroger and Weill (1961); Burma, Kroger, Ochoa, Warner and Weill (1961); Furth, Huxwitz and Goldman (1961a, b); have described bacterial systems simultaneously incorporating the four ribonucleoside

triphosphates. Similar systems have been described from plant sources by Rho and Bonner (1961); Huang, Maheshwari and Bonner (1960); Mans and Novelli (1964); Semal, Spencer, Kim and Wildman (1964), and from animal sources by Weiss and Gladstone (1959); Weiss (1960); Burdon and Smellie (1961); Straus and Goldwasser (1961); and Goldberg (1961). In many of these preparations the enzyme is particulate and appears to be associated with nucleic acid. In the cell free preparation the enzyme is coprecipitated with nucleic acid by streptomycin or protamine sulphate (Furth et al, 1961, b) and on gradient centrifugation the enzyme sediments with DNA. In rat liver preparations the enzyme appears to be specifically located in the nucleus (Weiss, 1960), in plant preparations it is associated with the chromatin (Huang and Bonner, 1962) and in mammalian cells a nucleolar origin is suggested (Ro and Busch, 1964). Recently, a DNA primed RNA polymerase has been found associated with the mitochondrial DNA of Neurospora crassa. (Luck and Reich, 1964).

The RNA polymerase from E. coli was originally purified 300 times and its properties extensively investigated. (Furth, Hurwitz and Anders, 1962). Similar enzymes have been purified from Micrococcus lysodeikticus (Nakamoto and Weiss, 1962) and A. vinelandii (Krakow and

Ochoa, 1963). Such enzymes show a requirement for a DNA primer which acts catalytically. Up to a sixty fold excess of product over primer has been obtained. (Chamberlin and Berg, 1962; Hurwitz, Furth, Anders and Evans, 1962). No degradation or alteration in the structure of DNA occurs during the reaction as judged by the identity of buoyant density of ^{32}P DNA and retention of transforming activity before and after use for priming the RNA polymerase reaction. (Goldthwait et al, 1961; Hurwitz et al, 1962,a). Unlike the DNA polymerase reaction native DNA is a better primer than heated DNA. The base composition of the product RNA is dictated by the DNA used to prime the reaction over a thousand fold range of RNA synthesis. Base analogues of the normal ribonucleoside triphosphates substitute for the normal substrates in a manner identical to that found in DNA synthesis. Furthermore, the nearest neighbour frequencies of RNA product are in close agreement with those of the template DNA. (Hurwitz et al, 1962,a). This, in conjunction with the formation of RNA-DNA hybrids between product RNA and heat denatured primer (Goldthwait et al., 1961), provides good evidence that the base sequence of DNA is rigorously copied in the synthesis of RNA. The RNA polymerase is inhibited by actinomycin D which specifically binds with GMP residues in DNA. In this

respect RNA synthesis is about ten times more sensitive to the antibiotic than DNA synthesis. (Hurwitz et al, 1962, b; Kahan, Kahan and Hurwitz, 1963).

In addition to DNA, synthetic polydeoxynucleotides and polyribonucleotides can act as primers. Poly A, U and C have been shown to separately prime the incorporation of the complementary nucleotides. Competitive inhibition occurs between a mixture of polynucleotides and their substrates, suggesting that a single enzyme is involved. (Krakow and Ochoa, 1963). Similarly, poly dAT acts as a primer for the incorporation of ATP and UTP as the only substrates. No reaction occurs with one substrate and the product as shown by alkaline hydrolysis after α^{32}P nucleotide incorporation consists of alternating A and U residues in a double helical configuration. (Chamberlin and Berg, 1963).

Further purification of a DNA primed RNA polymerase from M. lysodeikticus has resulted in a preparation free of pyrophosphatases, phosphatases, DNase, polynucleotide phosphorylase and DNA polymerase. (Nakamoto, Fox and Weiss, 1964). With this purified enzyme two reactions are detected, one involving the complementary synthesis of RNA as described above, the other involving homopolymer formation at a single higher ribonucleoside triphosphate

concentration. The latter reaction is inhibited by the presence of the other ribonucleoside triphosphates and by polyamines. Both reactions can be primed by natural and synthetic polyribonucleotides as well as by DNA. The complementary synthesis requires Mn^{2+} and the ribonucleoside triphosphate complementary to the primer. Polyamines are inhibitory though stimulation occurs with DNA primers. With polyribonucleotides as primer heating of the primer, or actinomycin D have no effect. DNA competitively inhibits the reaction and it appears that only one enzyme is involved in both RNA and DNA primed reactions. (Fox and Weiss, 1964; Fox, Robinson, Haselkorn and Weiss, 1964). A further purification of the E. coli RNA polymerase has been obtained by Stevens and Henry (1964). Similar complementary synthesis with natural or synthetic polydeoxyribonucleotides or polyribonucleotides and homopolymer synthesis has been demonstrated. (Stevens, 1964). The size of the product RNA appears to depend on the size of the primer, the enzyme preparation and the method of isolation of product. Sedimentation coefficients of 4 - 7 S have been reported. (Geiduschek et al, 1961; Chamberlin and Berg, 1962). End group analysis indicates chains of 300 residues. (Furth et al, 1962). These chain lengths and sedimentation coefficients are much lower than

for naturally occurring RNAs and are probably due to nuclease contamination of the enzyme preparations. However, using the purified M. lysodeikticus polymerase and an E. coli DNA primer, product RNA is obtained which forms hybrids specifically with E. coli ^{32}P labelled 23S, 16S and 4S RNA indicating specific synthesis of high molecular weight species of RNA. (Robinson, Hsu, Fox and Weiss, 1964).

Though replication of DNA appears to occur semi-conservatively *in vivo* and *in vitro* (Meselson and Stahl, 1958; Wake and Baldwin, 1962), this is not so with the RNA polymerase reaction. *In vitro* both strands of a native DNA primer appear to be replicated. (Hurwitz *et al*, 1962, a). Identical RNA product DNA primer nearest neighbour frequencies are obtained whether single stranded or double stranded DNA acts as primer. With the latter primer the frequency of dinucleotide pairs indicates synthesis of each strand proceeds in the opposite direction to that of the complementary DNA strand. The RNA product is thus a replica of the DNA template, the two strands being self complementary and of opposite polarity. Using poly dAT as a primer the product poly AU has similar physical properties to the primer indicating a rigid rod-like double helical structure. No evidence could be obtained for the formation of a hybrid dAT-AU copolymer during the reaction though this

may be due to the instability of such a hybrid, or its destruction during isolation or centrifugation in CsCl. (Chamberlin and Berg, 1963). Hence in the absence of detectable hybrid formation a conservative mode of replication was proposed. However, using single stranded ϕ x 174 DNA as template a DNA-RNA hybrid is first formed and any further replication is semi-conservative, the growing strand replacing the existing strand before any free RNA appears. (Chamberlin and Berg, 1964).

Recently, a hybrid DNA-RNA polymerase has been isolated from E. coli which incorporates ribonucleotides and deoxyribonucleotides into the same polymer chains. The reaction is primed by double stranded polyribonucleotides, polydeoxyribonucleotides and natural DNA. Both chains of the product must be synthesised simultaneously suggesting the hybrid polymerase is bifunctional or dimeric. (Huang and Cavalieri, 1964). The enzyme may be an artifact of the isolation procedure due to combination of subunits of both RNA and DNA polymerases and hence is evidence for the subunit nature of these polymerases. That subunits make up the RNA polymerase is supported by the appearance of a hexagonal structure in electron micrographs of the E. coli polymerase when prepared by methods avoiding protamine or streptomycin sulphate precipitation. (Fuchs,

et al, 1964).

Besides DNA primed RNA polymerases another important class of enzymes catalysing the net synthesis of RNA occur during intracellular virus growth. Possibly DNA viruses induce a DNA primed RNA polymerase or utilise the host polymerase but RNA viruses processing either single or double stranded RNA induce a RNA primed RNA polymerase. Such an enzyme has been detected in mengo virus infected L cells. (Baltimore and Franklin, 1963). The system incorporates the four ribonucleoside triphosphates in the presence of Mg^{2+} or Mn^{2+}, is inhibited by RNase and not by DNase or actinomycin D. The activity is present in the microsomal fraction of virus infected cells but not in a similar preparation from uninfected cells. A similar enzyme has been detected in Hela cells infected with polio virus (Baltimore, Eggers, Franklin and Tamm, 1963), and in E. coli infected with MSØ2 bacteriophage (Haruna, Nozu, Ohtaka and Spiegelman, 1963). The latter enzyme has been partially purified and the preparation contains viral RNA in a double stranded replicative form. RNA synthesis occurs in the absence of exogenous primer by an asymmetric semi-conservative replication of the natural primer. (Weissmann, Borst, Burdon, Billeter and Ochoa, 1964). The same enzyme has been obtained free of the natural template which then

shows a specific requirement for the homologous RNA. (Haruna et al, 1963). Ochoa and coworkers, however, are unable to repeat these results and consider the viral RNA polymerase complex as a holoenzyme.

The above systems are examples of enzymes catalysing the net synthesis of RNA. Many enzyme fractions have also been obtained which incorporate single ribonucleoside triphosphates forming homopolymers. Such systems usually require RNA as a primer and are inhibited by more than one ribonucleoside triphosphate. A system incorporating AMP residues from ATP into RNA has been described in the soluble fraction of chick-embryos. (Chung, Mahler and Enriomo, 1960). The system has a pH optimum of 9, requires Mg^{2+} and RNA as primer. Terminal incorporation onto the primer chains occurs. A similar system has been partially purified from the chorioallantoic membranes of eleven day old chick-embryos. Chains of 8 - 11 residues are formed. (Venkataraman and Mahler, 1963). A system from the pH5 precipitate of the 105,000 x g supernatant fraction of rat liver has been partially purified. (Klemperer, 1963a). In the presence of UTP and Mg^{2+} UMP residues are terminally incorporated into the primer giving chains 3 - 5 residues long. Terminal CMP or UMP groups in the primer act as acceptors. If the metal ion used is Mn^{2+} then AMP

residues from ATP are terminally incorporated giving chains 20 residues long. (Klemperer, 1963b). The primer requirement is satisfied by ribosomal RNA, sRNA, poly C poly U and small oligonucleotides, especially tri and tetra oligonucleotides with free 3'-hydroxyl groups. Heated DNA and poly A inhibit the reaction. (Klemperer, 1964). Cytoplasmic fractions have been obtained from ascites tumour cells which incorporate UTP into poly U. (Burdon and Smellie, 1961; 1962). Nuclear ribosomes from such cells appear to incorporate ATP and UTP. (Burdon, 1963a). An enzyme synthesising poly A chains up to 100 residues long has been purified from calf thymus nuclei. CTP is incorporated to one tenth the extent of AMP whilst GMP and UMP are not incorporated at all. Initial observations showed apparently no primer requirement for this reaction. (Edmonds and Abrams, 1960). Further work has identified a naturally occurring poly A in calf thymus nuclei and in the purified enzyme preparation. (Edmonds and Abrams, 1962; 1963).

Besides the above RNA primed incorporation of single nucleotides and similar DNA primed incorporation by the highly purified bacterial polymerases, an enzyme has been found in the particulate fraction of hen liver which incorporates ATP into poly A in the presence of Mg^{2+} DNA

and NMN. RNA does not replace DNA and the enzyme is not inhibited by actinomycin D or the other ribonucleoside triphosphates. (Chambon, Weill and Mandel, 1963). A similar enzyme from the nuclei of ascites tumour cells requires DNA for the incorporation of UMP residues into poly U. (Burdon, 1963,b).

Also of note is an enzyme system that incorporates ribonucleoside triphosphates into polydeoxyribonucleotides. (Krakow, Kemmen and Cannellakis, 1961; Cannellakis and Krakow, 1962). Any one of the 4 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 ribotidyl-DNA will then act as a primer in the rat liver system which incorporates UMP residues into poly U.

Whilst the function of the above homopolymer forming systems are not known and may even represent artifacts the terminal formation of a pCpCpA sequence for sRNA molecules is of biological importance for the latter's function as an amino-acid acceptor in protein synthesis. Heidelberger, Harbers, Leibman, Takagi and Potter (1956), showed that a cytoplasmic fraction of rat liver homogenate which retained a capacity for oxidative phosphorylation could incorporate ^{32}P AMP into RNA. Digestion of the

product with 5' phosphodiesterase results in recovery of the radioactivity as 5'-AMP whereas hydrolysis with alkali results in recovery of labelled 2'3'-CMP. This suggests AMP is incorporated terminally adjacent to CMP residues. More detailed investigations using partially purified preparations from the pH5 precipitates of 105,000 x g supernatant fractions have shown that CMP residues are also incorporated. If the enzyme is first preincubated in the absence of substrate its capacity subsequently to incorporate AMP residues is reduced. This incorporation can be restored by the presence of CTP and it appears the two nucleotides are incorporated in a ratio of one to two respectively, in a terminal sequence. No other ribonucleoside triphosphates are incorporated and it appears that sRNA is a specific acceptor for these enzymes. (See Smellie 1963 for a review).

The above enzyme systems are sufficient to account for the net synthesis of the nucleic acid components of the cell. However, both RNA and DNA contain methylated bases and some bacteriophage DNAs contain O-glycosyl residues. Such modifications of the nucleic acids probably occur after synthesis of the polynucleotide chain even though methylated bases can be incorporated by the enzymes <u>in vitro</u>. This hypothesis gains support from the identification of enzymes methylating sRNA and DNA.

(Fleissner and Borek, 1962; Gold and Hurwitz, 1964, a). These enzymes have been partially purified and shown to be specific for a particular base of the homologous sRNA or DNA. S-adenosyl methionine acts as the methyl donor and the reaction is irreversible. (Hurwitz, Gold and Anders, 1964, a, b; Gold and Hurwitz, 1964, b).

5. The Aims of the Present Work.

In vivo experiments on the kinetics of incorporation of ribonucleotides or pulse labelling experiments followed by a "chase" in the presence or absence of actinomycin D indicate, while not definitely proving, that all RNA synthesis is directed by a DNA template in the nucleus. Harris (1959), Harris and Watts (1962), however, interpret their experiments as showing no transfer of nuclear RNA to the cytoplasm. Thus cytoplasmic RNA synthesis may occur which requires a RNA template or primer. Burdon and Smellie (1962) have described an enzyme system from the cytoplasm of Ehrlich ascites tumour cells which incorporates (α^{32}P) UMP residues into RNA utilising UTP as a substrate. The present work is concerned with the further investigation and characterisation of such a system to establish if the

necessary cytoplasmic enzymes are present for the non-nuclear synthesis of RNA.

METHODS

METHOD

1. Preparation of Ehrlich's Ascites Carcinoma.

Ehrlich's ascites carcinoma was maintained by serial transplantation in Swiss albino mice of the departmental colony. Tumour was withdrawn from mice after 7 days growth and 0.2 ml portions were injected into the peritoneal cavity of fresh mice under aseptic conditions.

2. Preparation of Extracts from Ehrlich's Carcinoma Cells.

Mice containing 7 - 8 day old tumour were anaesthetized with ether and killed. The tumour was removed from the peritoneal cavity with a pasteur pipette and collected in ice cooled pondered acetrifuge tubes. 4 - 5 ml of tumour was obtained from each mouse in this way and any tumour containing blood was discarded. The ascitic fluid was separated by centrifugation of the tumour suspension at 600 x g for 10 minutes at 0° in a PBS medium recentrifuged centrifuge. All further operations were performed at 1 - 3° in ice cooled apparatus. The cells were broken by a modification of the procedure of Fuller and Hamm (1962) in 5 volumes of a medium containing 0.01M tris-HCl buffer pH 8.0, 0.0005M $MgCl_2$ and 0.2 per cent (v/v) tween 80, by repeated passage through a Potter homogeniser until

microscopic examination of the smears stained with crystal violet showed that at least 95 per cent of the cells were broken and clumping of the nuclei was observed. When required, nuclei were collected by centrifugation at 600 x g for 10 minutes and washed free of cytoplasmic contamination by repeated resuspension and recentrifugation in 25 per cent sucrose (w/v), 0.01M with respect to tris-HCl buffer pH 8.0 until a clear supernatant was obtained (Usually 3 resuspensions were necessary). After removal of the nuclei, mitochondria were prepared by centrifuging the supernatant fluid at 10,000 x g for 20 minutes in a Sorvall refrigerated centrifuge. The nuclear and mitochondrial pellets were resuspended by gentle homogenisation in a small volume of 0.01M tris-HCl buffer pH 8.0 to give a protein concentration of 2 - 3 mgs/ml. Microsomes were then prepared by centrifugation of the 10,000 x g mitochondrial supernatant fraction at 105,000 x g for 2 hours in a Spinco model L or a MSE super speed centrifuge. Usually nuclei and mitochondria were separated from the disrupted cell suspension in one operation by centrifugation at 15,000 x g for 20 minutes. Microsomes were prepared from this supernatant fraction as described above and the microsomal pellet was resuspended by gentle homogenisation in 0.01M tris-HCl buffer pH 8.0 to give a

protein concentration of 2 - 3 mg/ml. In some experiments the 105,000 x g supernatant fraction was recentrifuged at 150,000 x g for 2 hours giving a pellet, resuspended as before and a 150,000 x g supernatant fraction.

In some experiments subfractionation of the microsomal fraction was performed using the method of Holdiman and Munro (1965). The packed cells tissue cells were initially broken by homogenization in 2.5 volumes of the tris-HCl, $MgCl_2$, Triton GO buffer used before, and nuclei and mitochondria were removed by centrifugation at 15,000 x g for 20 minutes. An equal volume of 60 per cent (w/v) sucrose in 0.05M tris-HCl buffer pH 8.0 was slowly added to the supernatant fluid. A rough microsomal fraction was then obtained as a sediment by centrifugation at 70,000 x g for 1 hour. The supernatant fluid was treated with 0.5 volumes of iso-octane and the tissue dispersed in a Potter homogenizer until viscous. The microsomal was centrifuged at 150,000 x g for 2 hours which caused sedimentation of the free subpernae and flotation of a membrane component. The latter component was floated out of the centrifuge tube with 20 per cent (w/v) sucrose and recentrifuged at 600 x g for 10 minutes to remove excess iso-octane. The membrane material was washed by homogenization with a small volume

of tris-HCl buffer followed by recentrifugation at 600 x g for 10 minutes. The membrane material and sediments of the other fractions were resuspended by gentle homogenisation in tris-HCl buffer as before.

3. Preparation of an Acetone Dried Powder of the Microsomes.

Microsomes prepared as described above were homogenised in a small volume of 0.01M tris-HCl pH 8.0 to give a thick suspension which just flowed on pouring. Usually the microsomal pellet from 200 ml of disrupted cell homogenate was homogenised in 3 ml of buffer. 1 ml of the microsomal suspension was poured into 30 ml of acetone cooled to -30^{0} in a solid CO_2, ethanol mixture. The suspension was rapidly dispersed with a Nelco blender at full speed for 1.5 minutes. The suspension so obtained was collected by centrifugation at 600 x g for 5 minutes at -15 to -20^{0} and washed 3 times by resuspension in 30 - 100 ml of acetone at the same temperature. Finally the sediment was dried under vacuum giving a white powder which was stored at -15^{0}. For assay purposes it was suspended by homogenisation in 0.01M tris-HCl buffer pH 8.0.

4. Assay Procedures.

All assays were performed in 5 x 0.5 in. or 5 x 0.25 in.

test tubes and incubated with shaking at 37° in a constant temperature water bath. Where possible a special mixture was made up to include all reagents common to a given assay. This was then pipetted into ice cooled tubes followed by any variable reagents and finally the enzyme fraction to give a total volume of 0.5 ml. After incubation, the samples were either processed immediately or the tubes were rapidly frozen in a mixture of solid CO_2 in ethanol and stored at -15° until required.

5. Washing Procedures.

In initial experiments RNA was extracted from the reaction mixtures with NaCl by the procedure of Weiss (1960). The reactions were either stopped by adding 4 ml of cold 5 per cent (w/v) trichloroacetic acid containing 10 per cent (v/v) saturated $Na_4P_2O_7$ or frozen tubes were thawed into the same mixture. 1 mg of carrier RNA was added and the precipitates were collected by centrifugation at 600 x g for 10 minutes. The precipitate was resuspended in 4 ml of the trichloroacetic acid $Na_4P_2O_7$ mixture and the procedure repeated 2 times after which it was dried by washing with 3 ml of ethanol followed by 3 ml of ether. RNA was extracted from the precipitate by adding 2 ml of 10 per cent (w/v) NaCl adjusted to pH 8.0, and heating in

a water bath at 90° for 20 minutes. After centrifugation the supernatant fluid was decanted and a further 1 mg of carrier RNA added before the extraction was repeated. The combined extracts were treated with 2 volumes of ethanol, cooled for 30 minutes at -15° to precipitate the RNA which was collected by centrifugation at 600 x g for 30 minutes. The precipitate was dissolved in 2 ml of water and reprecipitated with 2 volumes of ethanol, cooled and recentrifuged as before. This was repeated twice to ensure complete removal of unincorporated ribonucleotides.

In later experiments the following washing procedure based on the method of Baltimore and Franklin (1963) was used. All operations were performed at 1 - 3°. 1 mg of carrier protein (0.5 ml 0.2 per cent w/v) bovine serum albumin was added to the reaction mixture followed by 5 ml of 5 per cent (w/v) trichloroacetic acid containing 10 per cent (v/v) saturated $Na_4P_2O_7$ neutralised to pH5 - 6 with H_3PO_4 (Solution A) and the precipitate was collected by centrifugation at 600 x g for 10 minutes. The sediment was dissolved in 0.5 ml, 0.5N NaOH and immediately reprecipitated with solution A. Subsequent washing was performed by suspending the sediment in 1 ml of solution A with a Vortex mixer (Scientific Instruments Ltd.) adding a further 4 ml of solution A and allowing to equilibrate in

on ice bath for 10 minutes before centrifugation. For experiments in which the incorporation of (α^{32}P) UTP was being measured the washing was repeated 4 times and the RNA present in the final pellicant was then hydrolysed in 1 ml 0.3 M KOH at 37° overnight. The excess KOH and protein were precipitated by neutralisation with 0.025 ml 72 per cent (v/v) $HClO_4$ and after removal of the precipitate by centrifugation a fraction of the supernatant fluid was washed and dried on steel planchettes for radioactivity measurements. If incorporation of ^{35}S radioactivity was being measured the washing procedure was performed 5 times and the RNA was hydrolysed with 0.6 ml of 5 per cent trichloroacetic acid by heating at 95 - 100° for 20 minutes in a water bath. The protein was centrifuged by centrifugation and aliquots of the clear supernatant fractions were transferred to vials for radioactivity measurements. In some experiments with (α^{32}P) UTP incorporation it was found convenient to wash the sediment 5 times and finally dissolve it in 1 ml of concentrated formic acid, before transferring to stainless steel planchettes for radioactivity measurements. These methods give low background counts in zero time controls and were found to be superior to washing procedures using $HClO_4$ with or without $Na_4P_2O_7$, precipitation of a fraction

of the reaction mixture on filter paper discs followed by washing with several changes of solution A, or washing on millipore filters which tended to clog with the large amount of protein present in the reaction mixtures.

6. Measurement of Radioactivity.

^{32}P was measured in a Nuclear Chicago gas flow counter usually fitted with a micromil window. This gave an efficiency of nearly 50 per cent with a background of 15 - 18 counts per minute. ^{32}P on chromatograms was detected using a Nuclear Chicago Actigraph fitted with a windowless gas flow detector. A chart recorder moved synchronously with the chromatogram being scanned.

^{3}H and ^{14}C were measured in a 3 channel Nuclear Chicago model 725 liquid scintillation counter with efficiencies of 10 - 15 per cent and 75 - 80 per cent respectively as calculated from the channels ratios. Samples for counting were prepared from 1 volume of test solution, usually 0.5 ml, and 10 volumes of scintillation fluid. The latter consisted of scinstant NE572 (Nuclear Enterprises, Edinburgh) dissolved in scintillation grade dioxane. Double counting of ^{3}H and ^{14}C was also performed in the scintillation counter, the amounts of each isotope being calculated from the channels ratios.

7. Analysis of Ribonucleotides.

A. Electrophoresis.

The acid washed insoluble material described in section 5 was hydrolysed in KOH, neutralised as before and applied as a narrow band to strips of Whatman No. 3 chromatography paper 70 cms by 2 cms. Usually 0.15 - 0.2 ml was dried on the paper in a stream of warm air from a hair drier. The paper was moistened in 0.05M citrate, citric acid buffer pH3.4 (2 litres of 0.05M citric acid and 700 ml of 0.05M sodium citrate) and electrophoresis was carried out at 4Kv for 80 minutes. 3 strips drawing a current of approximately 20 mAs could be run simultaneously in a Shandon high voltage electrophoresis apparatus. The ultraviolet absorbing spots were located with the aid of a "Chrometolite" low pressure mercury vapour lamp, the radiation from which was filtered so that most of the emission was at 253.5 mμ. The ultraviolet absorbing spots were cut out and the radioactivity was measured directly in a Nuclear Chicago gas flow counter. Alternatively the strip was scanned in a Nuclear Chicago Actigraph (see section 6).

B. Chromatography.

Acid soluble nucleotides in the supernatant fraction after the initial precipitation of reaction mixtures were

separated by descending chromatography in an ammonium isobutyrate system consisting of 100 ml isobutyric acid, 55.8 ml water 4.2 ml 0.88 NH_4OH and 1.6 ml 0.1M versene. 0.02 ml of the acid soluble nucleotide fraction and 2 drops of a mixture of UTP, UDP and UMP as markers were dried on Whatman No. 3 chromatography paper in a stream of cold air from a hair drier. The chromatogram was developed for 20 hours, and ultraviolet absorbing spots were located as described in section 7A. The chromatogram was cut into strips and scanned in an Actigraph (see section 6). The total radioactivity corresponding to the various peaks was determined by weighing the chart paper under the peaks.

Separation of nucleosides from nucleotides obtained in neutralised KOH hydrolysates of the acid washed insoluble sediments was achieved by descending chromatography in an aqueous isopropanol ammonia system containing 15 ml water 85 ml isopropanol and 1 ml 0.88 NH_4OH. The neutralised hydrolysate was dried on Whatman No. 3 chromatography paper with the appropriate nucleoside markers and the chromatogram was developed for 16 hours. The ultraviolet absorbing spots were located as described in section 7A and the paper cut into strips to allow elution of the spots with 0.01M formic acid directly into scintillation vials. About 1 ml of eluate was collected and 10 ml of scintillator

fluid were added for radioactivity measurements.

0.5 mg samples of ^{14}C labelled RNA were hydrolysed with 0.5 ml 72 per cent (w/w) $HClO_4$ by heating in a boiling water bath for 1 hour followed by partial neutralisation with KOH and dilution to 3 ml. The purine and pyrimidine bases were separated by descending chromatography in a system containing 30 ml isopropanol 37 ml water 33 ml concentrated analar HCl. 0.05 ml of the hydrolysate was dried on Whatman No. 1 chromatography paper with the appropriate purine and pyrimidine markers and the chromatogram was developed for 18 hours. Ultraviolet absorbing spots were located and eluted into counting vials as described in section 7A.

8. Preparation of RNA.

A. Preparation of ascites tumour whole cell RNA.

The method of Eason, Cline and Smellie (1963) was followed. 10 ml of packed ascites tumour cells were suspended in 2 ml of 5 per cent (w/v) bentonite in 0.01M sodium acetate buffer pH 6.0, 6 ml 0.5 per cent sodium dodecylsulphate and 60 ml 0.01M sodium acetate buffer pH 5.2. The mixture was passed through a Potter homogeniser several times to ensure disruption of the cells. 60 ml of 90 per cent (w/v) aqueous phenol was

added and the mixture shaken mechanically for 10 minutes at room temperature. The resulting emulsion was centrifuged at 10,000 x g for 10 minutes and the aqueous phase was transferred with a pasteur pipette into a chilled flask containing 0.05 ml of 5 per cent (w/v) bentonite suspension. The phenol interphase residue was re-extracted with 30 ml of 0.01M sodium acetate pH 5.2. After shaking for 10 minutes, centrifugation and removal of the aqueous phase as before, the combined aqueous extracts were shaken for a further 10 minutes with an equal volume of 90 per cent (w/v) phenol and centrifuged again at 10,000 x g for 10 minutes. The aqueous phase was removed and 2 volumes of chilled ethanol containing 1 per cent potassium acetate were added. The precipitate was collected by centrifugation at 600 x g for 30 minutes at 0^{o} and dissolved in 50 ml of buffer A (0.01M tris-HCl 0.001M $MgCl_2$ pH 7.5), 50 µg of crystalline bovine pancreatic DNase were added and the mixture was incubated at 37^{o} for 15 minutes. The mixture was cooled on ice and RNA precipitated with 2 volumes of ethanol containing 1 per cent potassium acetate and collected by centrifugation as before. The RNA was then dissolved in 20 ml of buffer B (0.01M sodium acetate, 0.05M NaCl, 0.0005M $MgCl_2$ pH 5.2) and dialysed against 2 changes of 7 litres of the same buffer for 18 hours.

The dialysed solution was clarified by centrifugation at 10,000 x g to remove insoluble material and the RNA was precipitated as before. The final RNA precipitate was dissolved in a small quantity of buffer B, the solution was extracted twice with an equal volume of ether and the last traces of ether were removed with a stream of nitrogen. The RNA concentration was measured spectrophotometrically and adjusted to 10 mg/ml assuming an extinction of 1 to correspond to 50 µg of RNA at 256 mµ. Analysis by sucrose density gradient centrifugation showed the RNA to be of high molecular weight containing the characteristic 30S, 18S and 4S peaks.

B. Preparation of ^{14}C labelled RNA.

5 mgs of solid sodium formate containing 1 mcurie ^{14}C were dissolved in 2 ml of water and 0.4 ml aliquots were injected into 5 mice with 6 day old tumours. Cells were harvested 18 hours later, 10 ml of packed cells were obtained and the RNA was extracted as described above.

Hydrolysis with concentrated $HClO_4$ and separation of the purines and pyrimidines by chromatography showed the pyrimidine bases to be labelled to less than 1 per cent of the purine bases. Analysis by sucrose density gradient centrifugation showed the characteristic 30S, 18S and 4S peaks which showed a constant ratio of radioactivity

to extinction at 256 mμ.

O. Extraction of RNA from reaction mixtures.

In experiments in which RNA was to be extracted from reaction mixtures a sufficient amount of microsomes to give 1 mg RNA per assay tube was used. 0.5 ml of 0.1M sodium acetate buffer pH 5.2, 0.05 ml of 0.5 per cent (w/v) sodium dodecylsulphate and 0.05 ml of 0.1 per cent (w/v) bentonite suspension were added to the incubated reaction mixtures which were then mixed with a vortex mixer and shaken with 0.7 ml of 90 per cent (w/v) phenol for 10 minutes at room temperature. The resulting emulsion was then broken by centrifugation at 10,000 x g for 5 minutes and the aqueous layer transferred with a pasteur pipette into a chilled 15 ml centrifuge tube containing 50 μg bentonite. The phenol interphase residue was re-extracted twice more by adding 0.7 ml 0.01M sodium acetate buffer pH 5.2 shaking for 10 minutes and centrifuging at 10,000 x g for 5 minutes. The combined aqueous layers were extracted with 1 ml of ether and RNA was precipitated with 2 volumes of ethanol containing 1 per cent potassium acetate. After cooling at -15^{0} for 30 minutes the precipitate was collected by centrifugation at 600 x g for 30 minutes and washed twice by redissolving in buffer B and reprecipitating with ethanol-potassium

acetate. After the initial precipitation RNA from duplicate tubes was usually combined. The RNA was then dissolved in 2 ml buffer B and dialysed against 2 changes of 7 litres of the same buffer for 18 hours. The dialysed solution was clarified by centrifugation at 600 x g for 10 minutes and the RNA reprecipitated from the supernatant as before. The final precipitate was dissolved in 0.4 ml of buffer B and extracted twice with an equal volume of ether. The last traces of ether were removed with a stream of nitrogen. The solutions which contained 0.5 - 0.75 mg of RNA were analysed by sucrose density gradient centrifugation.

9. Sucrose Density Gradient Centrifugation.

A. Analysis of RNA.

Sedimentation analyses of RNA solutions were performed by centrifugation in linear sucrose density gradients. A mixing device was used to deliver 4.6 ml of sucrose in buffer B linearly graded from 20 per cent (w/v) to 5 per cent (w/v) in a 0.5 x 2 ins. cellulose acetate centrifuge tube (Bock and Ling, 1954). 0.4 ml of a solution containing 0.5 - 0.75 mg RNA (i.e. having an extinction of 10 - 15 at 256 mμ) was layered on the sucrose gradients and the tubes were centrifuged in a

swinging bucket rotor (SW 39) of a Spinco model L ultracentrifuge. Usually centrifugation was performed at 41,000 x g for 12 hours.

30 - 35 fractions were collected by puncturing the base of the tube with a hypodermic needle and allowing the contents to drip out into test tubes. 2 drops per tube were collected giving a volume of about 0.2 ml. 0.8 ml of water was then added and the ultraviolet absorption of each fraction was measured. 0.5 ml of each fraction was used for radioactivity measurements.

B. Analysis of microsomal preparations.

Microsomal preparations treated in various ways were fractionated by sedimentation in sucrose density gradients. A gradient was prepared in 1 x 3 ins. cellulose acetate centrifuge tubes by adding 5 ml of 30 per cent (w/v) sucrose in 0.01M tris-HCl buffer pH 8.0 and layering on successively 3 ml of 25, 20, 15, 10, 5 per cent (w/v) sucrose solutions in the same buffer. After equilibrating at 2 - 4° for 12 hours, 1.5 - 2 ml of a microsomal fraction containing 20 - 25 mg of protein was layered on the sucrose and the gradients were centrifuged in a swinging bucket rotor (SW 25) in a Spinco model L preparative ultracentrifuge for 6 hours at 27,000 x g.

The base of the tube was punctured with a hypodermic

needle and 1 ml fractions were collected in ice cooled test tubes. Usually 11 - 13 drops gave a fraction of approximately 1 ml. 0.25 ml of each fraction was diluted to 5 ml for ultraviolet absorption measurements at 260 mμ and 280 mμ and a further 0.38 ml was used to measure the incorporation of ^{3}H or ($\alpha^{32}P$) UTP.

10. Spectrophotometric Estimations.

The ultraviolet absorption of 1 ml RNA fractions were obtained at a wavelength of 256 mμ using silica microcells in a Unicam SP 500 spectrophotometer fitted with a microcell attachment.

The ultraviolet absorption of 5 ml microsomal fractions were obtained at wavelengths of 260 or 280 mμ in either a Unicam SP 500 or a Beckman DB spectrophotometer.

11. Protein Estimations.

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

Reagent A contained 2 per cent (w/v) Na_2CO_3 in 0.1 N NaOH. Reagent B contained 0.5 per cent (w/v) $CuSO_4$, $5H_2O$ in 1 per cent sodium or potassium tartrate. Reagent C was a mixture of 100 ml of reagent A and 2 ml of reagent B. 1 ml of protein solution was treated with 5 ml of reagent C and after 10 minutes, 0.5 ml of a dilute Folin

reagent was added. (Folin and Ciocalteu phenol reagent was titrated to a phenolphthalein end point and diluted to make it 1 N in acid). The mixture was shaken immediately and allowed to stand for at least 30 minutes before the colour developed was read against a reagent blank at a wavelength of 750 mμ in a Unicam SP 600 spectrophotometer. A calibration curve was prepared using a standard solution of bovine serum albumin.

12. Preparation of MAK columns.

Methylated albumin on kieselguhr was prepared by the method of Sueoka and Cheng (1962b). 5 grams of albumin were suspended in 500 ml of methanol and 4.2 ml of 12N HCl were added. The mixture was allowed to stand for 3 days and shaken occasionally. The precipitate was collected by centrifugation and washed twice with methyl alcohol and twice with anhydrous ether. After decanting the ether the precipitate was dried under a vacuum and stored as a powder. Protein coated kieselguhr was prepared as follows. 20 grams of kieselguhr were boiled to expel air in 100 ml of 0.1M NaCl in 0.05M phosphate buffer pH 6.7 and after cooling 5 ml of a 1 per cent solution of esterified albumin was added followed by a further 20 ml of the salt phosphate buffer. The methylated albumin on kieselguhr (MAK)

suspension so obtained was stored at 1 - 2°.

A column was prepared from 10 ml of the MAK suspension in a 1 cm glass tube. The column was packed and excess buffer was driven down to the top of the packed material under air pressure (2 lbs./sq. in.). The top of the column was covered with a 2 - 3 mm layer of kieselguhr and then with a piece of filter paper. After washing with 0.1M NaCl in 0.05M phosphate buffer pH 6.7, 0.5 mg of RNA were applied to the column at a concentration of 20 µg/ml in the same buffer which was again driven through under air pressure. Gradient elution was then performed using a mixing vessel containing 150 ml of 0.1M NaCl in 0.05M phosphate buffer and a reservoir containing 2.0M NaCl in 0.05M phosphate buffer pH 6.7. This was pumped through the column giving a gradient from 0.1 - 1.2M NaCl and 50, 5 ml fractions were collected using an Ahmer fraction collector. The extinction of each fraction was recorded and, after adding 0.5 mg of carrier RNA to each, RNA was precipitated with 2 volumes of cold ethanol. The precipitate was dissolved in 1 ml of water and transferred to scintillator vials for radioactivity measurements.

13. Nuclease Assay.

Nuclease activity was determined using the same

reaction conditions as for nucleotide incorporation except that ribonucleoside triphosphates were omitted. Endogenous RNA in the microsomes, added primer RNA, p-nitrophenyl thymidine 5'-phosphate or p-nitrophenyl thymidine 3'-phosphate were used as substrates. (Raszel and Khorana, 1959, 1961). When RNA was used as the substrate, the reaction was stopped by the addition of 3.5 ml 1.0N $HClO_4$, the precipitate was centrifuged down at 600 x g for 10 minutes and the extinction of the supernatant determined at 260 mμ. The amount of nucleotide hydrolysed was calculated assuming ϵ_{max} 10,600. When p-nitrophenyl thymidine 3'-phosphate or p-nitrophenyl thymidine 5'-phosphate were used as substrates the reaction was stopped with 3.5 ml 0.1N NaOH, any insoluble material was centrifuged down at 600 x g for 10 minutes and the extinction of the supernatant was read at 400 mμ. The amount of nucleotide hydrolysed was calculated assuming ϵ_{max} 12,000.

MATERIALS

1. Chemicals and Enzymes.

ATP, GTP, CTP and UTP were purchased from Pabst Laboratories, the Sigma Chemical Company or British Drug Houses Limited. Phosphocreatine was obtained from Calbiochem, streptomycin sulphate from Glaxo Laboratories Limited; highly polymerised yeast RNA used for routine assays from British Drug Houses Limited; putrescine and spermine hydrochlorides, phosphocreatine kinase, DNase and RNase from the Sigma Chemical Company.

Actinomycin D was a gift from Merck, Sharp and Dohme, Inc. Purified yeast sRNA was a gift from Dr. D. Bell.

^{3}H ribonucleoside triphosphates and ^{3}H dATP were obtained from Schwarz Bioresearch Inc. ^{14}C formate, carrier free ^{32}P orthophosphate and ^{32}P cyanoethylphosphate were obtained from the Radiochemical Centre, Amersham, England. (αP^{32}) TTP was a gift from Mr. J.B. Shepherd.

Iso-octane or 2,2,4,-trimethylpentane was obtained from British Drug Houses Limited and tween 80 or polyoxyethylene sorbitan mono-oleate was obtained from Light and Company Limited.

MATERIALS

p-Nitrophenyl thymidine 5'-phosphate was obtained from Calbiochem and p-nitrophenyl thymidine 3'-phosphate was a gift from Mr. P.J. Curtis.

2. The preparation of Bentonite.

The bentonite suspensions were prepared from commercial bentonite obtained from British Drug Houses Limited. 2 grams of crude bentonite were suspended in 40 ml of water and centrifuged at 750 x g for 15 minutes. The sediment was discarded and the supernatant fraction was centrifuged at 8,700 x g for 20 minutes. This sediment was resuspended in 0.1M versene pH 7.0 and stored at room temperature for 48 hours. The suspension was recentrifuged at 750 x g, the sediment discarded and the supernatant again centrifuged at 8,700 x g for 20 minutes. The sediment was finally resuspended in 0.01M sodium acetate buffer pH 6.0 at a concentration of 2 - 6 per cent (w/v).

3. The Preparation of (α^{32}P) UTP.

(^{32}P) UMP was prepared from (^{32}P) orthophosphate by the method of Tener (1961) and was phosphorylated by the method of Smith and Khorana (1958) to yield (α^{32}P) UTP.

A. The preparation of (^{32}P) CEP.

A solution of ^{32}P-labelled phosphoric acid (100 mcuries carrier free ^{32}P phosphate and 1mmole of cold phosphoric acid in aqueous solution) was concentrated to dryness *in vacuo* at 40° to remove traces of HCl. 10 ml of anhydrous pyridine (prepared by storing over calcium hydride) and 1 ml cyanoethanol were added and the solution was concentrated *in vacuo* to an oil at 40°. A second portion of anhydrous pyridine was added and the solution was again concentrated to an oil. 5 ml of anhydrous pyridine and 2.1 g (2.3 ml) of dicyclohexylcarbodiimide (DCC) were added and the reaction was left overnight at room temperature in a well-stoppered flask.

5 ml water were added to stop the reaction and the resulting solution was heated in a boiling water bath for 30 minutes. The solution was concentrated to dryness *in vacuo* and 10 ml water and 10 ml of saturated $Ba(OH)_2$ were added to the residue. After 5 minutes at room temperature the solution was adjusted to pH 7.5 with glacial acetic acid and filtered to remove dicyclohexylurea (DCU) and barium phosphate. Two volumes of ethanol were added to precipitate the barium 2-cyanoethylphosphate which was collected after 1 hour at 0° by centrifugation. The The crystals were redissolved in 5 ml water by adding a

minimum volume of glacial acetic acid, neutralised with $Ba(OH)_2$, centrifuged to remove a trace of insoluble material and recrystallised by adding 10 ml ethanol. The product was collected by centrifugation in a preweighed tube, washed with ethanol, acetone and finally ether. The yield at this stage was usually about 60 per cent of the starting material. The product was dissolved in 20 ml 20 per cent (v/v) acetic acid and passed through a Dowex-50-H$^+$ column (8 x 2 cm). The effluent was taken to dryness and dissolved in 10 ml anhydrous pyridine to which isopropylidene uridine had been added in the ratio of 1 mmole isopropylidene uridine to 0.5 mmoles cyanoethyl-phosphate.

B. The preparation of ^{32}P UMP

The above solution was concentrated to an oil *in vacuo* at 40°. 10 ml anhydrous pyridine was added and the solution again concentrated to dryness. The process was repeated once more and the residue was dissolved in 5 ml anhydrous pyridine and DCC added in the proportion 2.0 mmole DCC to 1 mmole isopropylidene uridine.

After 20 hours at room temperature, the well-stoppered flask was opened and 10 ml of water were added. After 1 hour, the mixture was concentrated to dryness *in vacuo*. The residue was hydrolysed for 90 minutes in 10 per cent

(v/v) acetic acid (40 ml) at 100° to remove the isopropylidene group and cleave phosphoamide bonds. The acetic acid was removed by evaporating the solution to dryness, the last traces being removed by a second evaporation after adding 10 ml of water. The residue was heated with 40 ml of 9N ammonium hydroxide (to remove cyanoethyl groups) at 60° for 90 minutes and the ammonia removed by concentrating the mixture to dryness. 10 ml of water was added to the residue and the insoluble DCU was removed by filtration under reduced pressure.

The precipitate was washed with a small volume of water and a sample of the filtrate taken for paper chromatography of the reaction products. The precipitate was then washed very thoroughly with 100 - 200 ml of water. Descending chromatography was performed on Whatman No. 1 chromatography paper using UMP, uridine and isopropylidene uridine as markers. The chromatogram was developed in the isobutyric acid-ammonia water system for 18 hours (see Methods, section 7B). Ultraviolet absorbing spots were scanned for radioactivity.

The combined filtrates were diluted to about 250 ml then applied to a Dowex-1-Cl⁻ column and washed with water till the extinction at 262 mμ was less than 0.05. The ^{32}P UMP was eluted with 0.05N HCl (500 ml).

The total extinction of the UMP fraction was determined at this stage and the yield of UMP calculated. The eluate was concentrated to an oil in vacuo at 40°.

C. The preparation of (α^{32}P) UTP.

The reaction mixture for UTP preparation contained the following components for each 100 μmoles of ^{32}P UMP:- 1.2 ml tri-n-butylamine, 6 ml pyridine, 0.2 ml 85 per cent (v/v) phosphoric acid and 5 g DCC. The mixture was allowed to stand at room temperature for 48 hours. At the end of the reaction a thick precipitate of DCU had formed. About 2 volumes of water were added to precipitate DCU from unreacted DCC. The flask was shaken and left for 1 hour at 0°. The DCU was filtered off under reduced pressure and washed with water. The eluate was extracted with four 50 ml portions of ether to remove pyridine and the ether washes were extracted with two 10 ml portions of water and these water washes were added to the main aqueous phase.

The combined aqueous phase was concentrated at 40° in vacuo. The material was applied to a Dowex-50-Na$^+$ column (4 x 10 cms.) to remove tri-n-butylamine. The uridine derivatives were eluted by washing with water until the extinction of the eluate at 262 mμ was less than 0.05. The total extinction of the eluate at 262 mμ was

determined and the amount of uridine derivatives calculated. The effluent was diluted to 1 litre and absorbed on Dowex-1-Cl⁻ column (2 x 20 cm.). The column was washed with water until the extinction at 262 mμ was less than 0.05. Gradient elution from the Dowex-1-Cl⁻ column was then carried out with 1.5 litres of 0.01N HCl in the mixing vessel and 2 litres of 0.3M LiCl in 0.01N HCl in the reservoir. The eluate was collected in 20 ml fractions which were scanned automatically for radioactivity. The fractions containing (α^{32}P) UTP were pooled and the total yield determined. LiCl, HCl and inorganic phosphate were removed on an active charcoal column which was prepared in the following manner. A charcoal column (1 x 10 cms.) was washed with ethanol-ammonia (70 per cent v/v aqueous ethanol containing 10 ml of concentrated ammonia per litre) until the extinction at 262 mμ fell to less than 0.1. The column was then washed with water, 0.01M $NaHCO_3$ and finally with 5 column volumes of 1N HCl. The column was then resuspended in water, fine particles were decanted off and washing was continued until neutral. The column was then repacked.

The (α^{32}P) UTP fraction from the Dowex-1-Cl⁻ column was absorbed onto the charcoal column which was washed with water until no chloride ions could be detected in

the effluent. Washing was continued with small volumes of 0.01M $NaHCO_3$ to remove inorganic phosphate until ultraviolet absorbing material began to be eluted. The column was allowed to drain and then washed with 2 column volumes of water. ($\alpha^{32}P$) UTP was eluted with about 1 litre of ethanol-ammonia and was concentrated *in vacuo* at 25°. The material was redissolved in water, passed through a small Dowex-50-Na^+ column and checked for purity by chromatography in the isobutyric acid ammonia water system (see Methods, section 7B). The amount of ($\alpha^{32}P$) UTP in the final eluate was estimated spectrophotometrically, assuming a molecular extinction coefficient at 262 mμ of 10^4, the concentration of the solution was adjusted to 10 μmoles per ml and stored at -15° in 2 ml. aliquots.

R E S U L T S

RESULTS

1. Preliminary Investigations.

Polyribonucleotide synthesis was measured by following the incorporation of radioactive ribonucleotides into acid precipitable material. In initial experiments to detect net synthesis of RNA, the reaction mixture contained equal amounts of (α^{32}P) UTP and unlabelled ATP, GTP and CTP which should stimulate (α^{32}P) UTP incorporation if synthesis of a hetero-polyribonucleotide occurs. Highly polymerised RNA was included as a primer, Mg^{2+} to fulfil any metal ion requirement and bentonite as an RNase inhibitor. The reaction mixture was buffered with tris-HCl and studies on the effect of buffer concentration showed this to be without effect on the reaction.

The incorporation of (α^{32}P) UTP by subcellular fractions of Lendschutz ascites tumour cells was first determined. The time course of incorporation of ^{32}P UMP residues by broken nuclei, a mitochondrial fraction, a microsomal fraction and a 105,000 x g supernatant fraction is shown in Figure 1. Only the microsomal fraction shows appreciable activity and the reaction appears to be complete after 15 minutes. This fraction was characterised in further experiments.

Figure 2 shows the pH dependence of the system. Further assays were performed at the optimum pH of 8.5. Table 1 shows the effects of omission of ribonucleoside 5'-triphosphates from the reaction mixture. A decrease in the incorporation of (α^{32}P) UTP was observed on removing ATP and GTP from the system. The removal of CTP, however, caused some stimulation, possibly by allowing some conversion of UTP to CTP and simultaneous incorporation of both. A further decrease in the incorporation of (α^{32}P) UTP was observed on removing ATP, GTP and CTP. The system was also shown to be stimulated by added RNA and inhibited by RNase. It was not inhibited by actinomycin D which suggests that the observed incorporation cannot be accounted for by utilisation of an endogenous DNA primer, and this, together with the stimulation by RNA, suggests a RNA template or primer may be used. Bentonite appears to have no effect over the incubation time employed. Table 2 shows the stimulating effect of adding an ATP regenerating system to the reaction mixtures. Phosphocreatine and phosphocreatine kinase (EC 2.7.3.2) were used in the approximate ratios given by Krakow et al (1961). Phosphocreatine kinase catalyses the transfer of the phosphate group of phosphocreatine specifically to ADP as an acceptor. (Kuby, Noda and Lardy, 1954; Morrison, O'Sullivan and Ogston,

FIGURE 1

The time course of the incorporation of (α^{32}P) UTP by subcellular fractions of Landschutz ascites tumour.

The reaction mixture contained 50 µmoles tris-HCl pH 8.5, 2 µmoles $MgCl_2$, 0.1 µmoles each of (α^{32}P) UTP (5×10^6 counts/min/µmole) CTP, ATP, GTP, 20 µg RNA, 20 µg bentonite, and 0.25 - 1.0 mg of protein in a total volume of 0.5 ml.

Incubations were performed at 37° for 15 minutes.

RNA was extracted from the reaction mixture by the Weiss procedure.

—▲— Microsomal fraction.

—●— Nuclei.

—■— Mitochondria.

—○— 105,000 x g supernatant fraction.

FIGURE 2

The effect of pH on the incorporation of (α^{32}P) UTP by a microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles tris-HCl at the pH indicated. 2 µmoles $MgCl_2$, 0.1 µmoles each of (α^{32}P) UTP (5×10^6 counts/min/µmole) CTP, ATP, GTP, 20 µg RNA; 20 µg bentonite and 1.3 mg microsomal protein in a total volume of 0.5 ml.

Incubations were performed at 37° for 15 minutes.

RNA was extracted from the reaction mixture by the Weiss procedure.

Figure 1

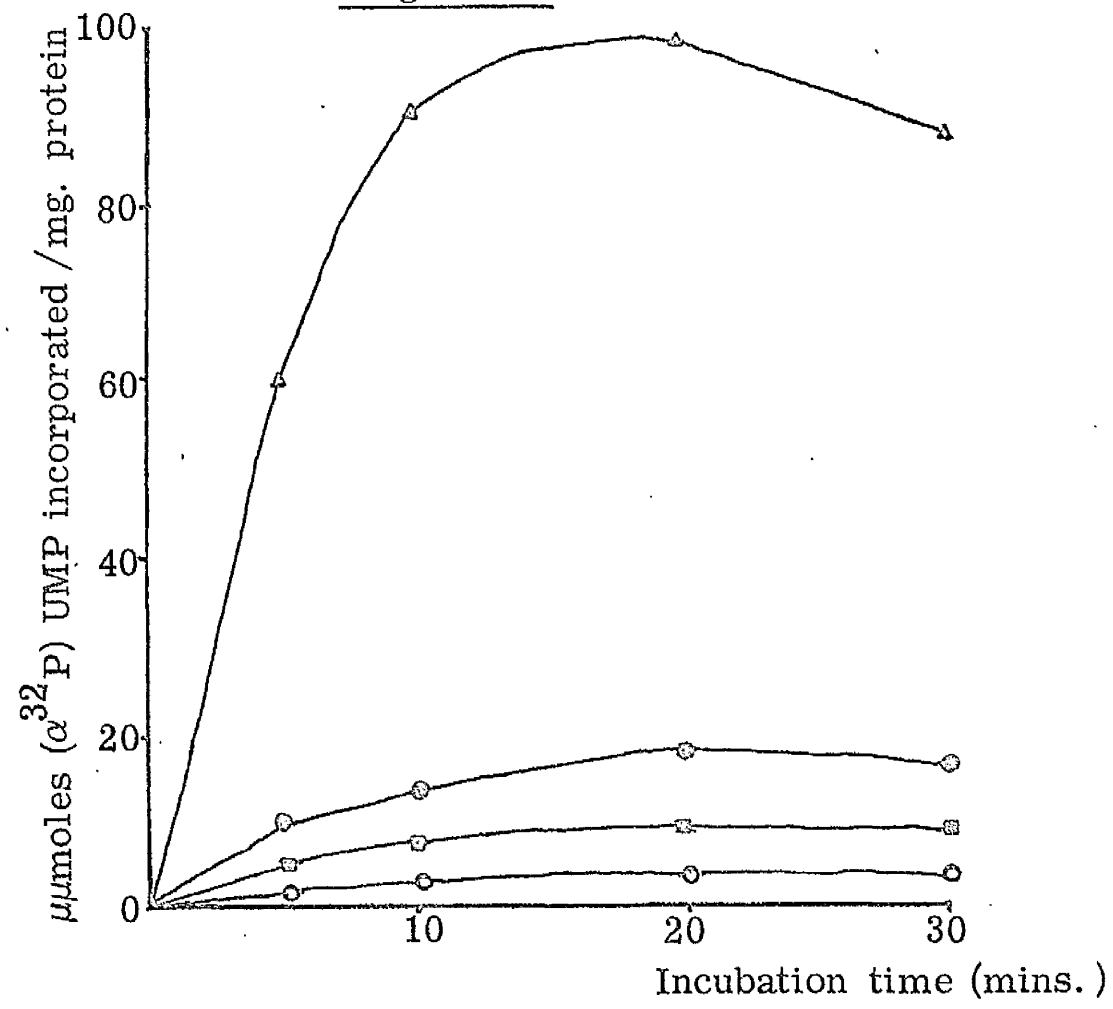


Figure 2

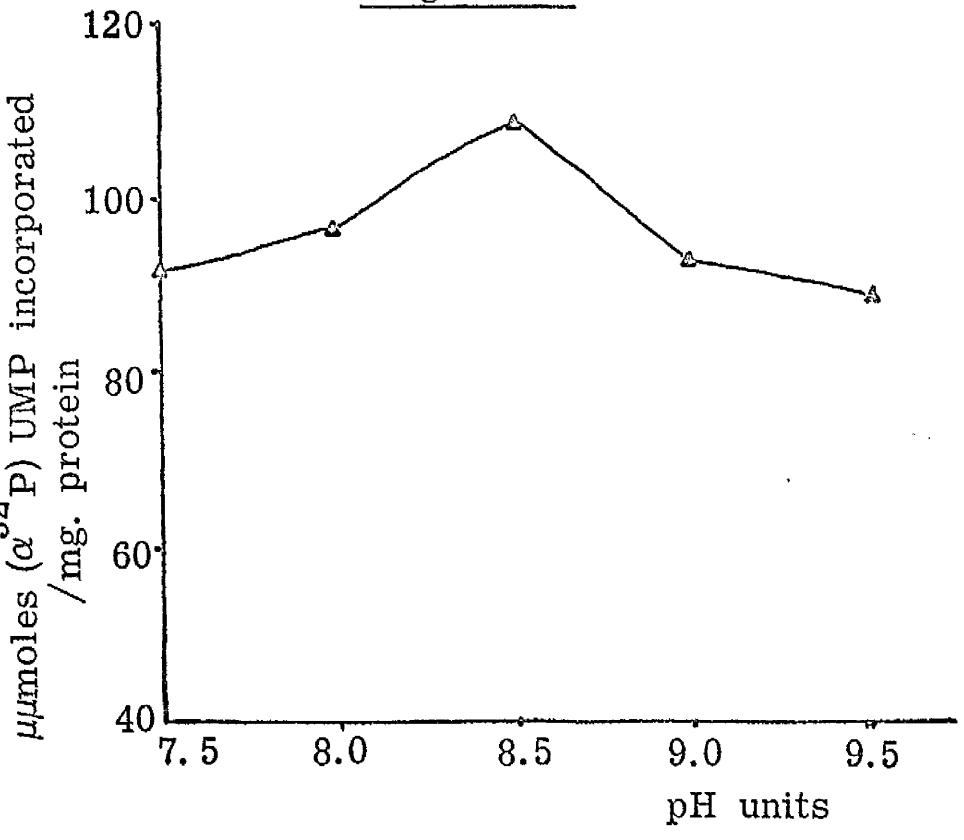


TABLE 1

Factors affecting the incorporation of (α^{32}P) UTP into a microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles tris-HCl buffer pH 8.5, 2 µmoles $MgCl_2$, 0.1 µmoles each of (α^{32}P) UTP (5×10^6 counts/min/µmole) ATP, GTP, CTP, 20 µg RNA, 20 µg bentonite and 1.65 mg microsomal protein in a total volume of 0.5 ml.

Where indicated 5 µg actinomycin D and 0.5 µg RNase were included.

The incubations were performed at 37^0 for 15 minutes.

RNA was extracted from the reaction mixture by the Weiss procedure.

Table 1

Incubation mixture	$\mu\mu$moles (α^{32}P)UMP incorporated /mg. protein
Complete system	95
-RNA	65
- ATP	74
-GTP	87
- CTP	117
- ATP, GTP, CTP	55
+RNase	7
+Actinomycin D	122
-Bentonite	95

TABLE 2

The effect of phosphocreatine and phosphocreatine kinase on the incorporation of (α^{32}P) UTP by a microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles tris-HCl buffer pH 8.5, 2 µmoles $MgCl_2$, 0.1 µmoles (α^{32}P) UTP (5×10^6 counts/min/µmole) 20 µg RNA, 20 µg bentonite and 1.3 mg of microsomal protein.

Where indicated 0.1 µmoles of each of ATP, GTP and CTP, 2.0 µmoles of phosphocreatine (PC)and 50 µg of phosphocreatine kinase (PCK) were included in a total volume of 0.5 ml.

Incubations were performed at 37^0 for 15 minutes.

RNA was extracted from the reaction mixture by the Weiss procedure.

Table 2

Incubation mixture	$\mu\mu$moles (α^{32}P)UMP incorporated /mg. protein
(α^{32}P)UTP	49
(α^{32}P)UTP+ATP, GTP, CTP	114
(α^{32}P) UTP+PC/PCK	262
(α^{32}P)UTP+ATP, GTP, CTP, PC/PCK	213

1961). The results show that incorporation of (α^{32}P) UTP was stimulated by phosphocreatine and phosphocreatine kinase. Thus, either UDP can act as a phosphate acceptor at the much higher enzyme concentrations used in the assay compared with the amounts used by Kuby et al (1954) or trace amounts of ADP in the presence of ribonucleoside diphosphate kinases (EC 2.7.4.6) present in the microsomal fraction act catalytically in rephosphorylating UDP. The phosphate regenerating system causes a marked increase in the incorporation of (α^{32}P) UTP alone, and while it also stimulates the incorporation of (α^{32}P) UTP in the presence of ATP, GTP and CTP, the overall incorporation of UTP in the latter case is lower than in the former. Hence it is possible that stimulation of incorporation of (α^{32}P) UTP by ATP, GTP and CTP, previously observed in the absence of a phosphate regenerating system, is due to a higher concentration of (α^{32}P) UTP in the reaction mixture when ATP, GTP and CTP are present than when (α^{32}P) UTP alone is present.

2. Further Characterisation of the Incorporation of (α^{32}P) UTP.

Further characterisation of the incorporation of (α^{32}P) UTP was performed using an acetone dried powder of

the microsomal fraction which could be stored for up to 2 weeks at -15° without loss of activity. The acetone dried powder had about 80 - 90 per cent of the activity of the original microsomal fraction depending on the preparation, though the overall activity varied from preparation to preparation. In the following experiments the second washing procedure was used for removing unincorporated ribonucleoside 5'-triphosphate (see Methods, section 5).

Table 3 shows the substrate requirements in the presence and absence of phosphocreatine and phosphocreatine kinase for the acetone dried powder. The incorporation of (α^{32}P) UTP is not stimulated by CTP or ATP, GTP and CTP, but is stimulated by phosphocreatine and phosphocreatine kinase. In the latter case, addition of CTP or ATP, GTP and CTP inhibit the incorporation. Figure 3 shows the pH requirement for the incorporation of (α^{32}P) UTP by the acetone dried powder in the presence of ATP, GTP, CTP, phosphocreatine and phosphocreatine kinase. The pH curve is sharper than for the untreated microsomes and the maximum appears to have shifted slightly to pH 8.3, possibly because of removal of interfering enzymes or the presence of phosphocreatine and phosphocreatine kinase. However, both the similarity of the substrate requirements and the pH optimum indicate essentially no change in the

pattern of incorporation of (α^{32}P) on preparing an acetone dried powder of the microsomes. ~~Though~~ In the absence of an ATP regenerating system ATP, GTP and CTP stimulate the incorporation of (α^{32}P) UTP catalysed by the microsomal fraction but not when catalysed by the acetone dried powder (see Tables 2 and 3). The time course of the reaction is shown in Figure 4, incorporation is still continuing after 40 minutes, though at about 25 per cent of the rate at 10 minutes.

Experiments were also performed using a reaction mixture containing ATP, GTP and CTP and bentonite as a nuclease inhibitor to study the effects of varying concentrations of RNA, DNA, RNase, DNase, actinomycin D and Mg^{2+} ions on the incorporation of (α^{32}P) UTP. Figure 5 shows the effect of increasing concentrations of RNA and DNA. Stimulation is observed with the former up to about 60 µg per assay tube and thereafter the incorporation remains constant. The maximum activity in the presence of added RNA is only 35 - 40 per cent above that obtained in its absence. DNA neither stimulates nor inhibits the reaction. The requirement for an RNA primer is also indicated by the effect of adding increasing concentrations of RNase to the reaction mixture. Figure 6 shows as little as 0.25 µg RNase causes a 70 per cent inhibition of the incorporation.

TABLE 3

The effect of ribonucleoside 5'-triphosphates and phosphocreatine kinase on the incorporation of (α^{32}P) UTP by an acetone dried powder of a microsomal fraction from Landschutz ascites tumour cells.

The reaction conditions were as for Table 2 except 1.7 mg of acetone powder, containing 0.86 mg protein and 0.1 μmoles (α^{32}P) UTP (2.5×10^6 counts/min/μmole) were added.

The incubations were carried out at 37° for 15 minutes.

RNA was extracted from the reaction mixture by the Weiss procedure.

Table 3

Incubation mixture	$\mu\mu$moles (α^{32}P)UMP incorporated /mg. protein
(α^{32}P)UTP	115
(α^{32}P)UTP+CTP	114
(α^{32}P)UTP+ATP, GTP, CTP	120
(α^{32}P)UTP+PC/PCK	257
(α^{32}P)UTP+CTP, PC/PCK	160
(α^{32}P)UTP+ATP, GTP, CTP, PC/PCK	184

FIGURE 3

The effects of pH on the incorporation of (α^{32}P) UTP by an acetone dried powder from the microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles of tris-HCl buffer of the pH indicated, 5 µmoles $MgCl_2$, 0.1 µmoles of each of (α^{32}P) UTP (5×10^6 counts/min/µmole) CTP, ATP, GTP, 20 µmoles phosphocreatine, 50 µg phosphocreatine-kinase, 100 µg RNA, 20 µg bentonite and 1.5 mg of acetone dried powder in a total volume of 0.5 ml.

Incubations were performed at 37° for 20 minutes.

FIGURE 4

The time course of the incorporation of (α^{32}P) UTP by an acetone dried powder of the microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture was the same as for Figure 3 except 50 µmoles of tris-HCl pH 8.5 were used.

Incubations were performed at 37° for the times indicated.

Figure 3

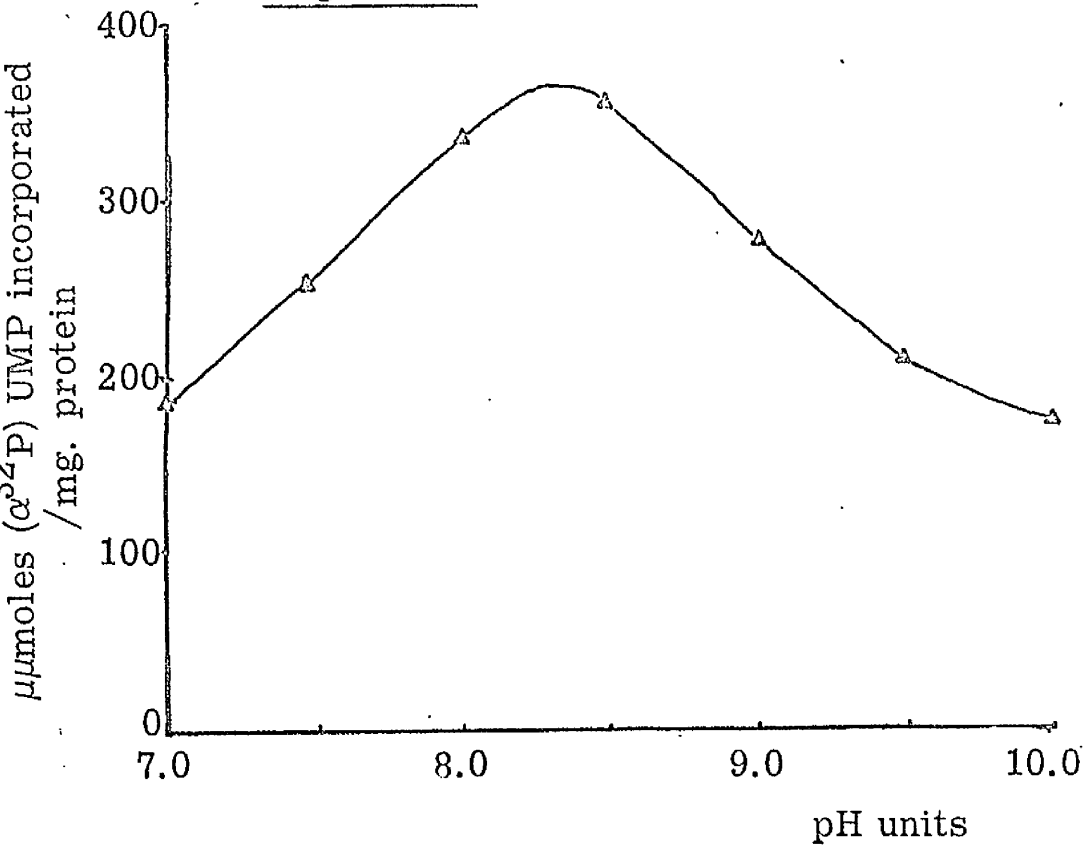


Figure 4

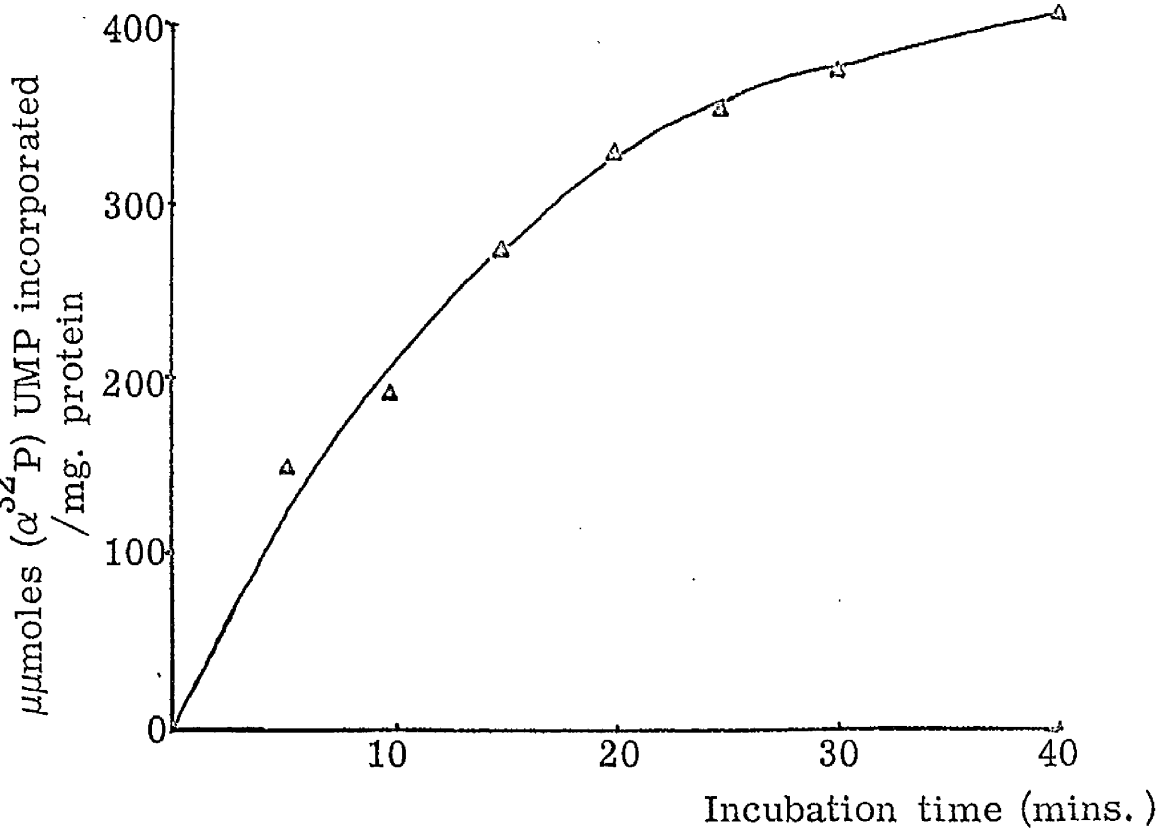


FIGURE 5

The effect of increasing concentrations of RNA and DNA on the incorporation of (α^{32}P) UTP by an acetone dried powder of the microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles tris-HCl pH 8.5, 2.0 µmoles $MgCl_2$, 0.1 µmoles of each of (α^{32}P) UTP (2.25×10^6 counts/min/µmole) CTP, ATP, GTP, 2.0 µmoles phosphocreatine, 50 µg of phosphocreatine kinase, 20 µg bentonite and 1.5 mg of acetone dried powder. RNA and DNA were added in the concentrations indicated in a total volume of 0.5 ml.

Incubations were performed at 37° for 20 minutes.

—▲— + RNA

—■— + DNA.

FIGURE 6

The effect of increasing concentrations of RNase and DNase on the incorporation of (α^{32}P) UTP by an acetone dried powder of the microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture was the same as for Figure 5. RNase and DNase were added in the concentrations indicated.

Incubations were performed at 37° for 20 minutes.

—■— + DNase

—▲— + RNase.

Figure 5

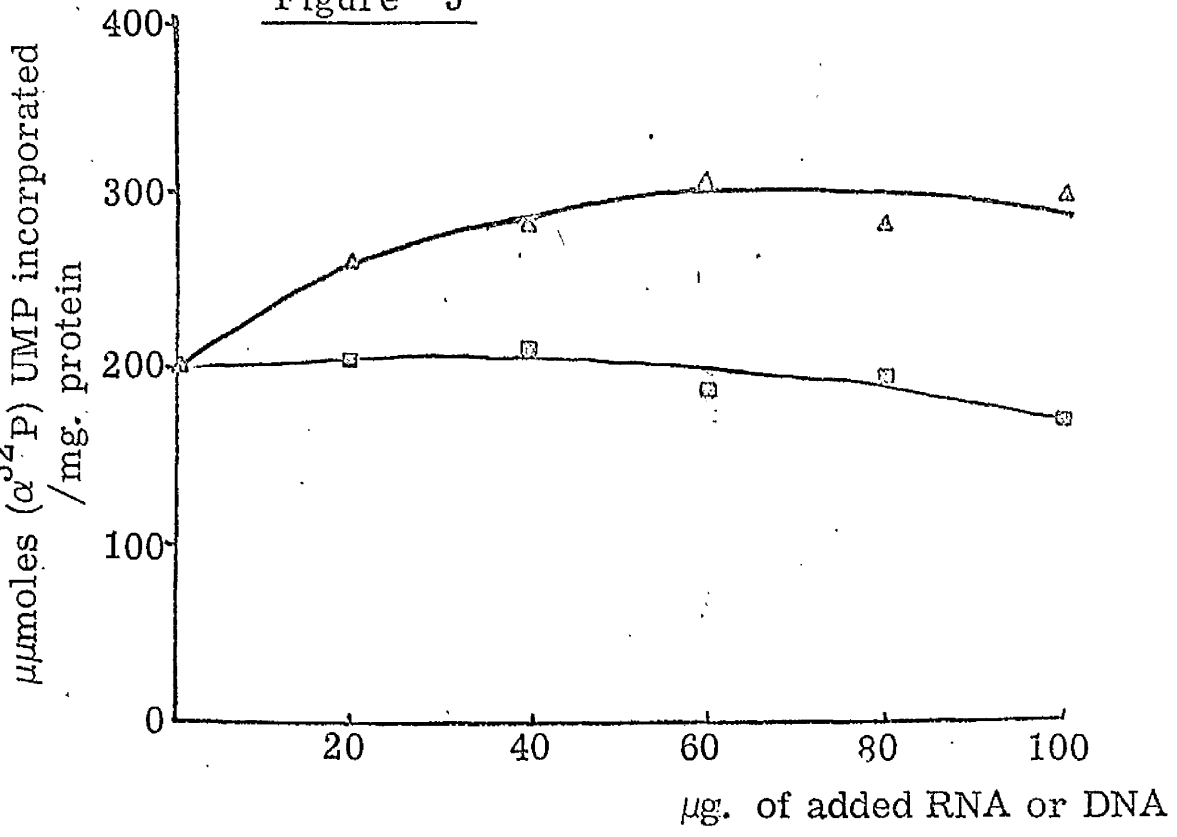
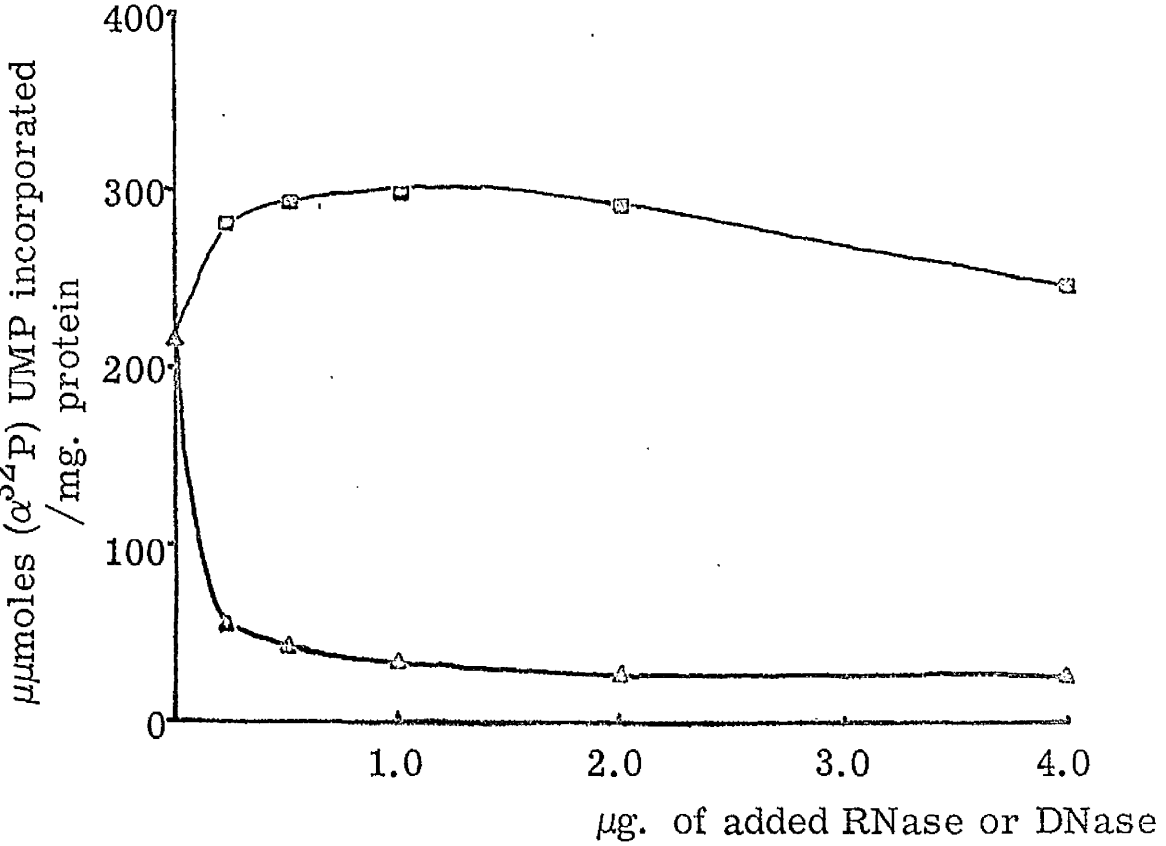


Figure 6



On the other hand, a 40 per cent stimulation is observed at a concentration of 1 µg DNase per tube which is decreased at higher concentrations. An experiment was performed in which the acetone dried powder was preincubated at 37° for increasing time intervals to allow degradation of endogenous RNA by RNase or phosphodiesterases present in the microsomes. The preparations were then assayed for the incorporation of (α^{32}P) UTP in the presence and absence of added RNA. Figure 7 shows that preincubation of the enzyme and assay in the absence of added RNA causes a gradual decrease in incorporation of (α^{32}P) UTP. The presence of added RNA gives higher levels of incorporation at all times of preincubation but it is noticeable that the absolute increase in activity is more or less constant. This loss in activity with increasing preincubation time could be due to heat inactivation of the enzyme. If it were caused by destruction of endogenous RNA primer a greater difference might have been expected between the curves after, say 5 minutes preincubation than at zero time, which was not observed.

The lack of inhibition of incorporation of (α^{32}P) UTP by increasing concentrations of actinomycin D also indicates a lack of requirement for a DNA primer (Figure 8) since the DNA dependent synthesis of RNA is highly suscept-

ible to this antibiotic. (Hurwitz et al, 1962, b).

An absolute requirement for Mg^{2+} ions is indicated by Figure 9. Incorporation of ($\alpha^{32}P$) UTP reaches a maximum as the Mg^{2+} ion concentration is increased to 2 µmoles per tube under the conditions used and remains constant at higher concentrations. Further factors affecting the incorporation of ($\alpha^{32}P$) were investigated. Figure 10 shows that versene and 2-mercaptoethanol stimulate the reaction and that this effect is additive. In further experiments these reagents were routinely included in the reaction mixture. In the presence of Mg^{2+} ions, K^{+} ions were found to inhibit the reaction slightly, while Mn^{2+} ions with the reaction mixture at pH 7.5, showed a marked inhibition of the reaction. A lower pH was used in the latter case as Mn^{2+} ions are precipitated at pH 8.5. Figure 11 shows the reaction is inhibited by spermine and putrescene, the former being the more effective inhibitor.

Time curves showing the effect of ribonucleoside 5'-triphosphates on the incorporation of ($\alpha^{32}P$) UTP in the presence and absence of phosphocreatine and phosphocreatine kinase are shown in Figure 12. With ($\alpha^{32}P$) UTP, phosphocreatine and phosphocreatine kinase only incorporation continues up to 60 minutes, removal of bentonite causes only a slight loss of activity in the later stages of the

FIGURE 7

The effect of preincubation of an acetone dried powder of the microsomal fraction of Landschutz ascites tumour cells on the incorporation of (α^{32}P) UTP assayed in the presence and absence of added RNA.

The reaction mixture contained 50 µmoles tris-HCl pH 8.5, 5 µmoles $MgCl_2$, 0.1 µmoles each of (α^{32}P) UTP (1.4×10^6 counts/min/µmole) CTP, ATP, GTP, 2.0 µmoles phosphocreatine, 50 µg phosphocreatine kinase, 20 µg bentonite and 1.5 mg of acetone dried powder. 100 µg of RNA were added where indicated in a total volume of 0.5 ml.

The incubations were performed at 37° for 20 minutes. The acetone dried powder in 0.01M tris-HCl pH 8.0 was preincubated at 37° for the times indicated before adding to the reaction mixtures.

—▲— + 100 µg RNA.

—■— No RNA added.

Figure 7

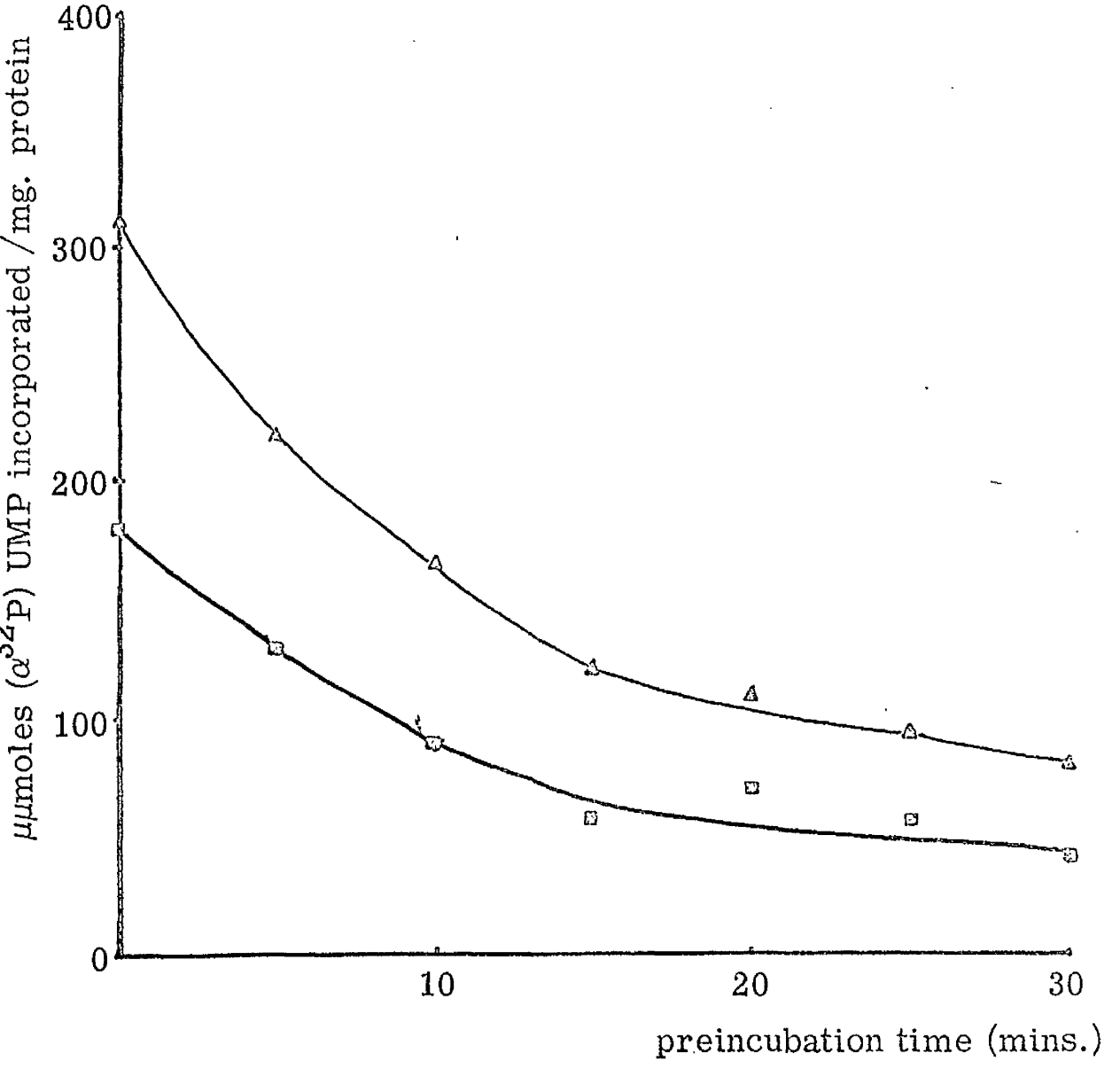

400
300
200
100
0
μμmoles (α^{32}P) UMP incorporated /mg. protein
10
20
30
preincubation time (mins.)

FIGURE 8

The effect of increasing actinomycin D concentration on the incorporation of (α^{32}P) UTP by an acetone dried powder of the microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles tris-HCl pH 8.5, 2 µmoles $MgCl_2$, 0.1 µmoles each of (α^{32}P) UTP (2.5×10^6 counts/min/µmole) CTP, ATP, GTP, 2 µmoles of phosphocreatine, 50 µg phosphocreatine kinase, 100 µg RNA, 20 µg bentonite and 1.5 mg of acetone dried powder. Actinomycin D was added as indicated in a total volume of 0.5 ml.

The incubations were performed at 37° for 20 minutes.

FIGURE 9

The effect of increasing Mg^{2+} concentration on the incorporation of (α^{32}P) UTP by an acetone dried powder of the microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture is the same as for Figure 8. The concentration of Mg^{2+} is varied as indicated.

The incubations were performed at 37° for 20 minutes.

Figure 8

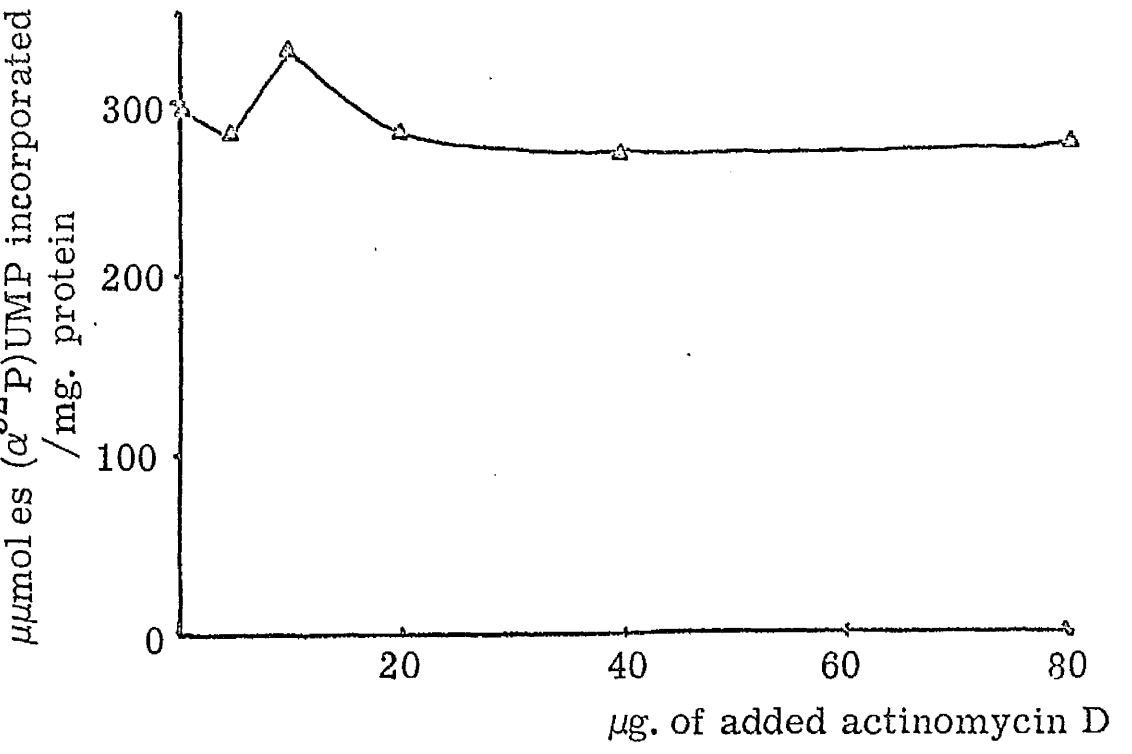


Figure 9

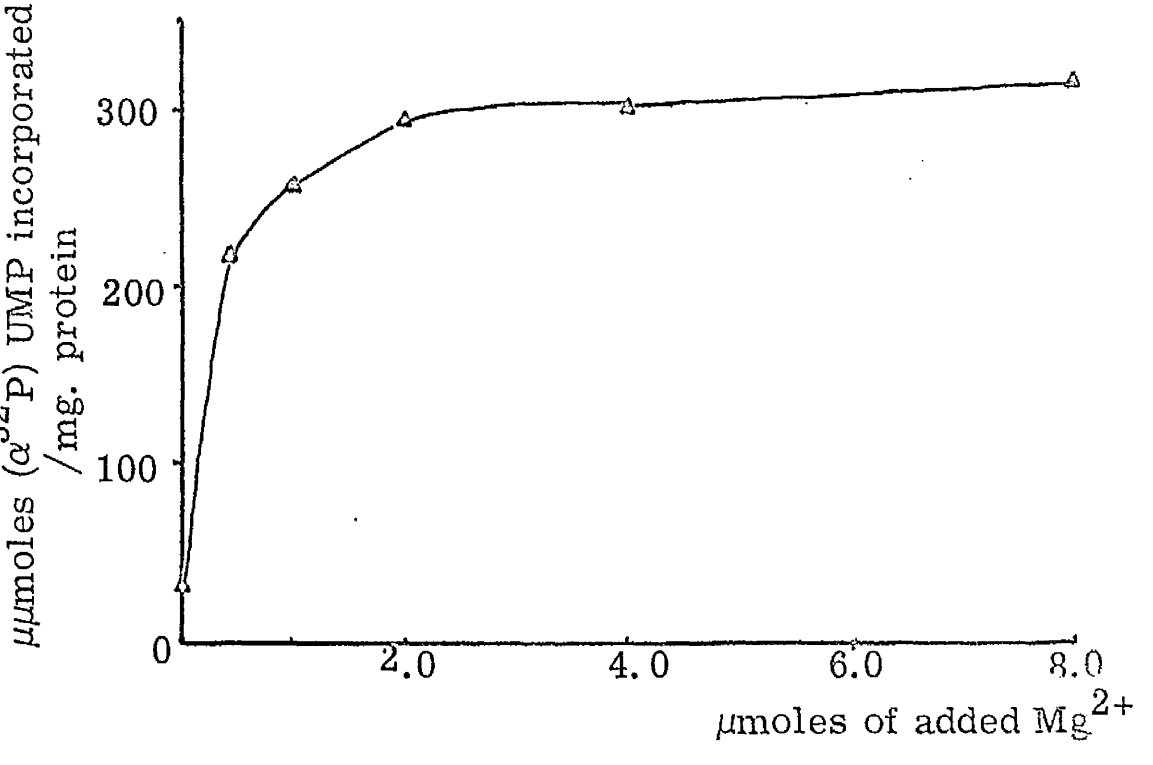


FIGURE 10

The effect of KCl, $MnCl_2$, 2-mercaptoethanol and versene on the incorporation of ($\alpha^{32}P$) UTP by an acetone dried powder of the microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles tris-HCl pH 8.5, 2.5 µmoles $MgCl_2$, 0.1 µmoles each of ($\alpha^{32}P$) UTP (1.5×10^6 counts/min/µmole) CTP, ATP, GTP, 2 µmoles phosphocreatine, 50 µg phosphocreatine-kinase, 100 µg RNA, 20 µg bentonite and 1.5 mg of acetone dried powder. KCl, $MnCl_2$, 2-mercaptoethanol and versene were added at the concentrations indicated in a total volume of 0.5 ml. Reaction mixtures containing $MnCl_2$ also contained 50 µmoles tris-HCl buffer pH 7.5 instead of pH 8.5.

The incubations were performed at 37^0 for 20 minutes.

Figure 10

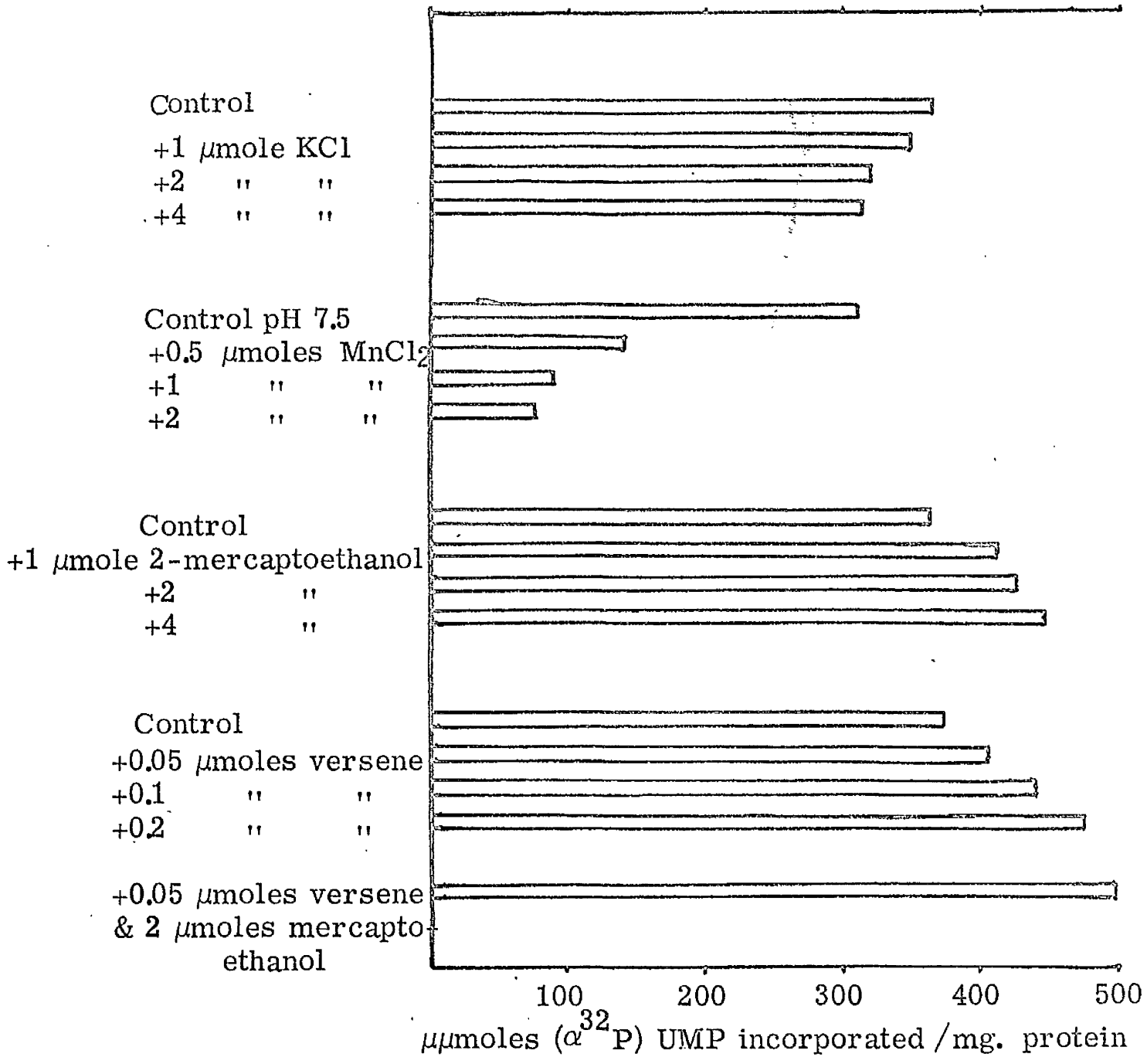

Control
+1 μmole KCl
+2 " "
+4 " "
Control pH 7.5
+0.5 μmoles $MnCl_2$
+1 " "
+2 " "
Control
+1 μmole 2-mercaptoethanol
+2 "
+4 "
Control
+0.05 μmoles versene
+0.1 " "
+0.2 " "
+0.05 μmoles versene & 2 μmoles mercapto-ethanol
100
200
300
400
500
μμmoles (α^{32}P) UMP incorporated / mg. protein

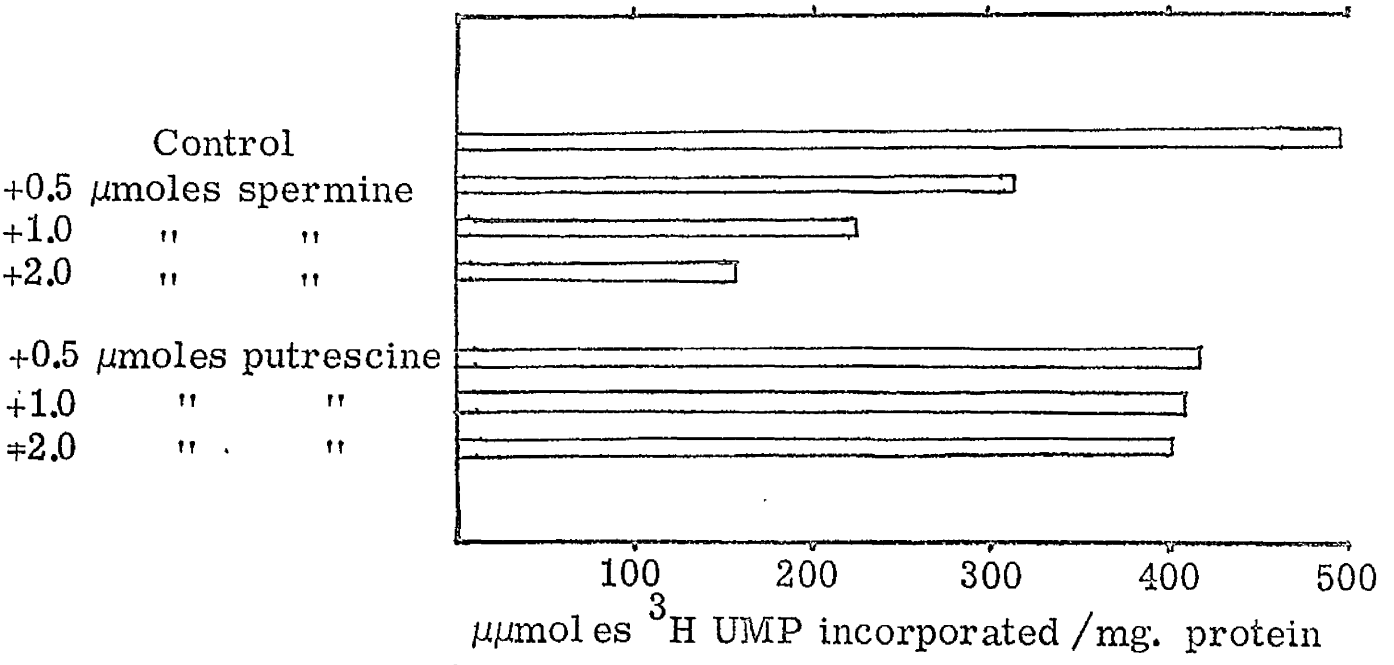
FIGURE 11

The effect of increasing concentrations of spermine and putrescine on the incorporation of ^{3}H UTP by an acetone dried powder of the microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contains 50 μmoles of tris-HCl buffer pH 8.5, 5 μmoles each of $MgCl_2$ and 2-mercapto-ethanol, 0.5 μmoles of versene, 0.1 μmoles of ^{3}H UTP, 2 μmoles phosphocreatine, 50 μg phosphocreatine kinase, 100 μg RNA and 1.5 mg of acetone dried powder. Spermine and putrescine were added in the concentrations indicated in a total volume of 0.5 ml.

Incubations were performed at 37^{o} for 20 minutes.

Figure 11


Control
+0.5 μmoles spermine
+1.0 " "
+2.0 " "
+0.5 μmoles putrescine
+1.0 " "
+2.0 " "
100
200
300
400
500
μμmoles ^{3}H UMP incorporated /mg. protein

reaction, while the presence of the ATP, GTP and CTP causes the reaction to slow down sharply after about 15 minutes. In the absence of phosphocreatine and phosphocreatine kinase there is a drastic reduction in the incorporation of (α^{32}P) UTP. Maximal incorporation occurs after 10 minutes and thereafter there is a decline in the quantity of labelled nucleotide in the polyribonucleotide product. The presence of ATP, GTP and CTP stimulate the incorporation slightly and lead to a slower decline from the maximum value. The extent to which degradation of acid soluble ribonucleotides in the reaction mixture occurs during incubation was examined by measuring the distribution of ^{32}P in UTP, UDP and UMP. The ribonucleotides were separated chromatographically and scanned in an Actigraph (see Methods, section 6). Figure 15 shows that phosphocreatine and phosphocreatine kinase largely prevent the degradation of (α^{32}P) UTP during the course of the reaction. In the absence of phosphocreatine and phosphocreatine kinase addition of ATP, GTP and CTP causes a slower degradation of (α^{32}P) UTP.

The location of ^{32}P UMP residues incorporated into the acid insoluble product was then studied. Alkaline hydrolysis releases the α^{32}P of an incorporated ribonucleoside 5'-triphosphate on to the 3'-hydroxyl of the

neighbouring nucleoside residues. In this way it is possible to detect the extent to which incorporated UMP residues are adjacent to other ribonucleosides in the polyribonucleotide product of the reaction. Thus distribution of ^{32}P (from ($\alpha^{32}P$) UTP) more or less equally between the 4 ribonucleoside monophosphates indicates simultaneous incorporation of all 4 ribonucleoside 5'-triphosphates and synthesis of a new RNA chain. Alternatively, if only UMP is predominantly labelled, formation of poly U chains is indicated. In the latter case, some ^{32}P label appearing with AMP, GMP and CMP indicates addition of poly U sequences to the ends of existing RNA chains. The distribution of ^{32}P amongst labelled ribonucleoside 2' (3')-monophosphates was examined in the products of reaction mixtures containing ($\alpha^{32}P$) UTP alone, ($\alpha^{32}P$) UTP plus GTP and ($\alpha^{32}P$) UTP plus ATP, GTP and CTP with both the microsomal fraction and the acetone dried powder prepared from it. The results are shown in Table 4. Whether the microsomal fraction or the acetone dried powder is used, little difference was detected in the distribution of ^{32}P amongst the labelled ribonucleotides in the reaction products. When ($\alpha^{32}P$) UTP alone was used the ^{32}P was largely recovered in uridine 2' (3')-monophosphate residues indicating formation of a poly U chain. Some 25 - 30 per cent of the total radioactivity was recovered in the other ribonucleoside 2' (3')-

FIGURE 12

Time course of the incorporation of (α^{32}P) UTP by a microsomal fraction of Landschutz ascites tumour cells showing the effect of bentonite and nucleoside triphosphates in the presence and absence of phosphocreatine and phosphocreatine kinase.

The reaction mixture contained 50 µmoles tris-HCl buffer pH 8.5, 5 µmoles each of $MgCl_2$ and 2-mercaptoethanol, 0.5 µmoles versene, 0.1 µmoles (α^{32}P) UTP (3.0×10^6 counts/min/µmole), 2 µmoles phosphocreatine (PC), 50 µg phosphocreatine kinase (PCK), 100 µg RNA, 20 µg bentonite and 0.76 mg of microsomal protein. Removal of bentonite, PC and PCK and addition of 0.1 µmoles each of CTP, ATP, GTP in a total volume of 0.5 ml were made where indicated.

Incubations were performed at 37° for the times indicated.

—▲— (α^{32}P) UTP + PC/PCK.
—o— (α^{32}P) UTP + PC/PCK - bentonite.
—■— (α^{32}P) UTP + PC/PCK + ATP, GTP, CTP.
—●— (α^{32}P) UTP + ATP, GTP, CTP.
—o— (α^{32}P) UTP.

Figure 12

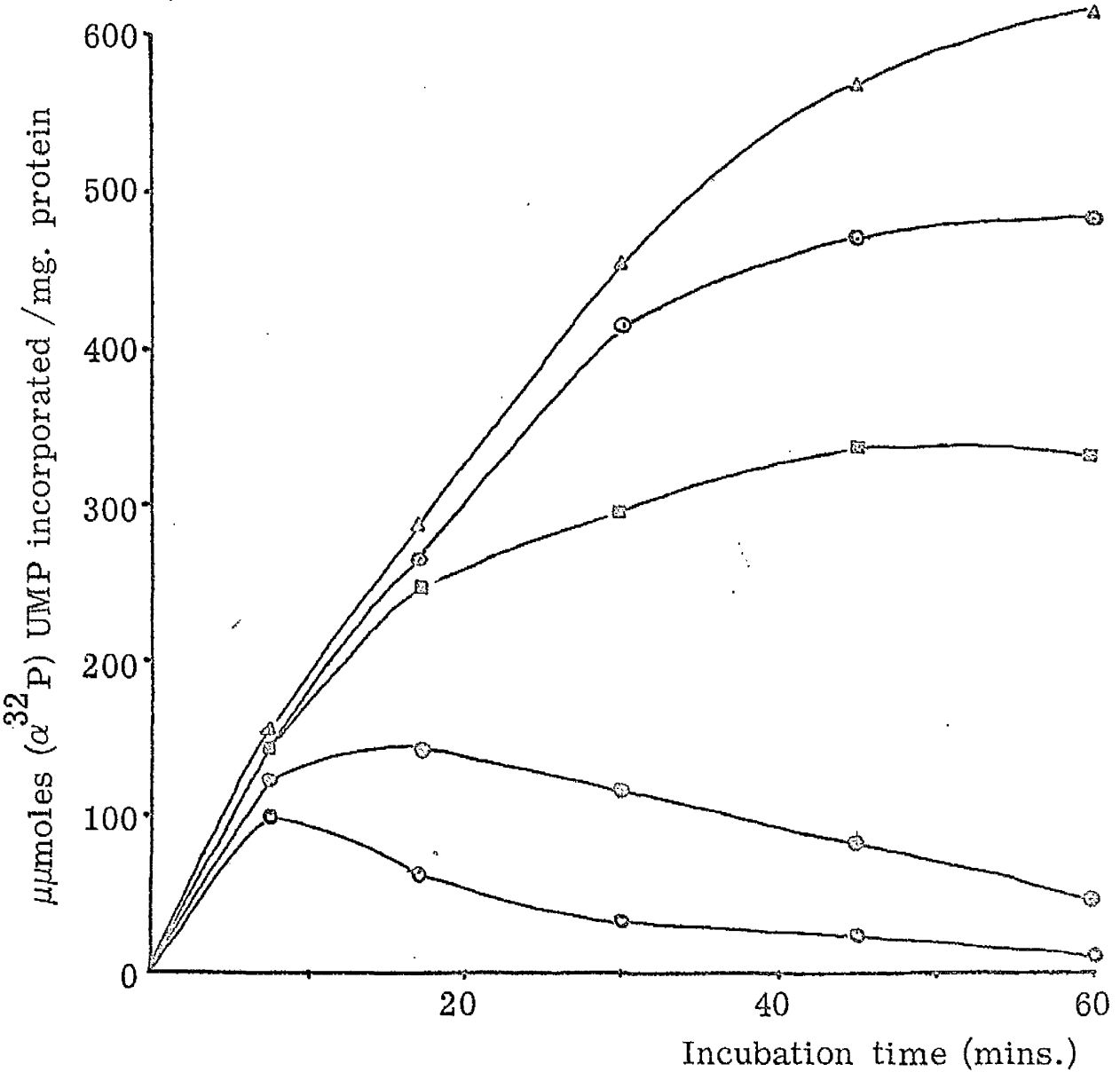

600
500
400
300
200
100
0
20
40
60
μμmoles (α^{32}P) UMP incorporated /mg. protein
Incubation time (mins.)

FIGURE 13

Percentage of (α^{32}P) UTP remaining in the reaction mixture after incubation with a microsomal fraction of Landschutz ascites tumour cells for increasing time intervals.

The reaction mixture was the same as in Figure 12. The acid soluble nucleotides were analysed as described in the Methods, section 7B.

Symbols as for Figure 12.

Figure 13

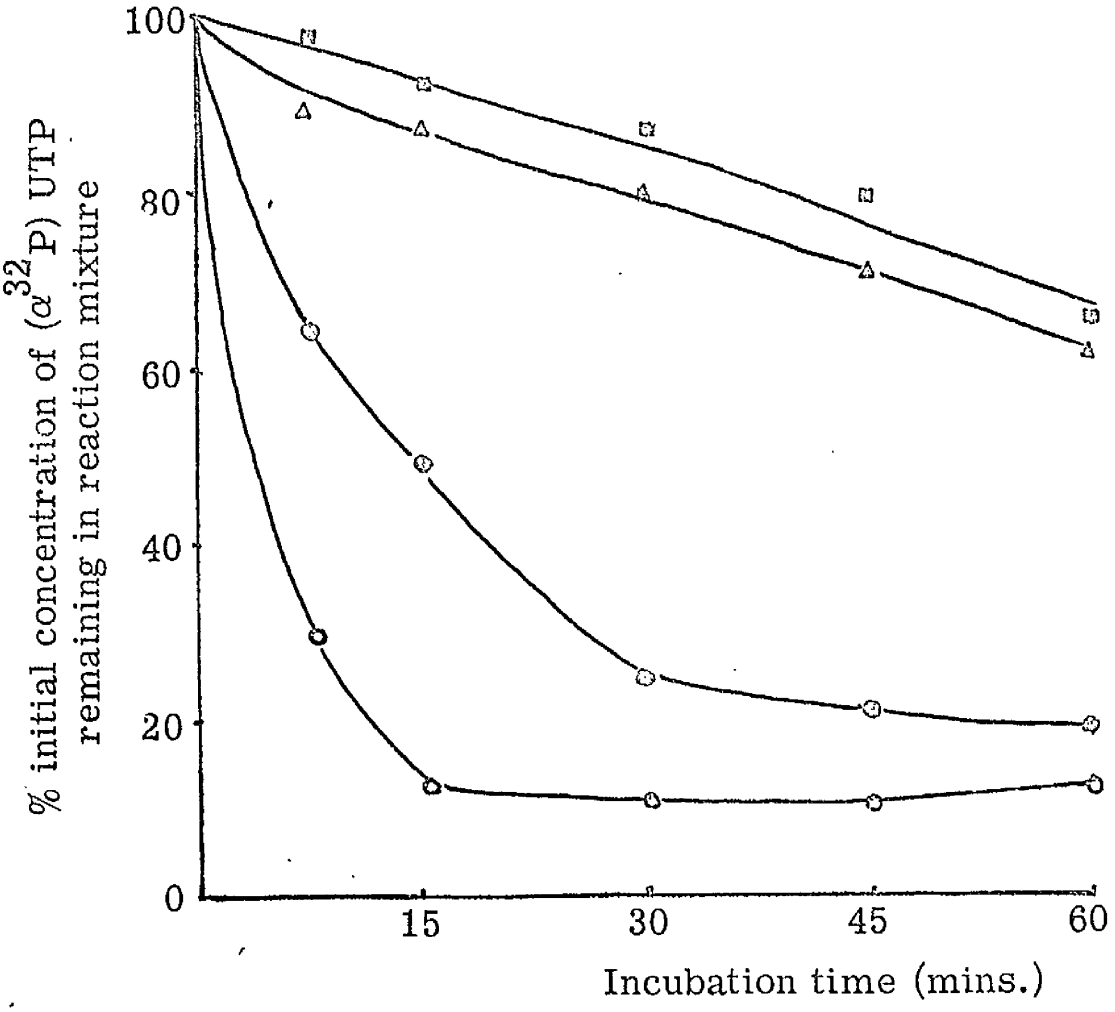

100
80
60
40
20
0
15
30
45
60
% initial concentration of (α^{32}P) UTP remaining in reaction mixture
Incubation time (mins.)

TABLE 4

The distribution of ^{32}P amongst the ribonucleoside 2'(3')-monophosphates after incorporation of ($\alpha^{32}P$) UTP by the microsomal fraction and an acetone dried powder thereof from Landschutz ascites tumour cells after alkaline hydrolysis of the product RNA.

The reaction mixture contained 50 µmoles tris-HCl buffer pH 8.5, 5 µmoles each of $MgCl_2$ and 2-mercaptoethanol, 0.5 µmoles versene, 0.1 µmoles of ($\alpha^{32}P$) UTP (10^7 counts/min/µmole), 2.0 µmoles phosphocreatine, 50 µg phosphocreatine kinase, 100 µg RNA, 20 µg bentonite and 1.2 mg protein. Where indicated 0.1 µmoles of ATP, GTP and CTP were added in a total volume of 0.5 ml.

Incubations were performed at 37° for 20 minutes.

The reaction mixtures were subjected to the procedures described in the Methods, section 7A.

Table 4

Reaction conditions	Percentage ^{32}P in each nucleotide			
	CMP	AMP	GMP	UMP
Microsomal fraction				
$(\alpha^{32}P)$UTP	3.8	8.6	10.8	76.7
$(\alpha^{32}P)$UTP+CTP	10.5	8.0	11.2	70.2
$(\alpha^{32}P)$UTP+ ATP, GTP, CTP	7.0	8.6	11.7	72.5
Acetone dried powder				
$(\alpha^{32}P)$UTP	6.9	12.9	8.8	71.2
$(\alpha^{32}P)$UTP+CTP	8.5	2.4	4.4	84.6
$(\alpha^{32}P)$UTP+ ATP, GTP, CTP	7.1	7.2	6.6	79.0

monophosphates. This probably represents at least in part lengthening of pre-existing polyribonucleotide chains and terminal labelling of the primer RNA. This pattern of labelling is not greatly changed when GTP or ATP, GTP and CTP are included in the reaction mixture. It would seem, therefore, that the major product of these reactions with the microsomal fraction and with the acetone dried powder is a polymer containing uridine residues. However, these results do not exclude the formation of small amounts of polyribonucleotide containing all 4 ribonucleotides.

In further experiments the incorporation of labelled nucleoside 5'-triphosphates were studied in the absence of any other ribonucleoside 5'-triphosphates. Bentonite was also omitted as it had no effect on the incorporation of (α^{32}P) UTP with incubation times up to 30 minutes.

3. The Incorporation of ^{3}H Ribonucleoside 5'-triphosphates of Adenine, Guanine and Cytosine and Uridine into Polyribonucleotides.

Initial investigations showed that ^{3}H ATP, GTP and CTP were separately incorporated into acid insoluble material under the same reaction conditions as (α^{32}P) UTP. Further characterisation showed that the incorporation of these ribonucleotides is influenced by the same factors and

in the same way as the incorporation of (α^{32}P) UTP. Table 5 shows the effects of RNase, DNase, actinomycin D, RNA, DNA, Mg^{2+} ions, phosphocreatine and phosphocreatine kinase and ribonucleoside 5'—triphosphates on the incorporation of ^{3}H ATP, ^{3}H GTP, ^{3}H CTP and ^{3}H UTP. The extent of the incorporation varies, being greatest for ATP, least for GTP and roughly equal for CTP and UTP. This is also shown by the time curves for the incorporation of each nucleotide (Figure 14) and the dependence of the incorporation of each on protein concentration (Figure 15). The dependence of ^{3}H ribonucleotide incorporation on protein concentration is almost linear up to 1 mg protein per reaction mixture. Usually 1.5 mg of acetone dried powder containing 0.75 - 0.88 mg of protein were added per tube and most of the results presented were performed at enzyme concentrations on the linear portion of the curve.

Although all 4 ribonucleoside 5'—triphosphates could be incorporated into polyribonucleotides, it is again striking that there was no stimulation of the uptake of any one ribonucleotide by the other 3 complementary ribonucleotides. Since this might have been related to the substrate concentration, the incorporation of ^{3}H ATP alone at increasing concentration and in the presence of GTP, CTP and UTP at similar increasing concentrations was investigated.

TABLE 5

The effect of RNase, DNase, actinomycin D, RNA, DNA, Mg^{2+}, phosphocreatine plus phosphocreatine kinase and nucleoside 5'-triphosphates on the incorporation of each of ^{3}H ATP, GTP, CTP and UTP by an acetone dried powder of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles tris-HCl buffer pH 8.5, 5 µmoles each of $MgCl_2$ and 2-mercapto-ethanol, 0.5 µmoles versene, 0.1 µmoles of ^{3}H ATP or GTP or CTP or UTP, 2.0 µmoles phosphocreatine (PC), 50 µg phosphocreatine kinase (PCK), 100 µg RNA and 1.5 mg of acetone dried powder.

Where indicated $MgCl_2$ phosphocreatine, phospho-creatine kinase and RNA were omitted and 0.25 µg RNase, 2.5 µg DNase, 20 µg actinomycin D, 100 µg DNA and 0.1 µmoles of each of the other unlabelled ribonucleoside 5'-triphosphates were added in a total volume of 0.5 ml.

Incubations were performed at 37° for 20 minutes.

Table 5

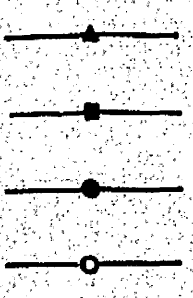
Reaction conditions	$\mu\mu$moles ^{3}H ribonucleotide incorporated /mg. protein			
	ATP	GTP	CTP	UTP
Complete system	518	97	313	204
+RNase	81	47	76	49
+DNase	539	80	300	172
-RNA	379	68	194	91
-RNA +DNA	398	53	154	80
$-Mg^{2+}$	4	0	0	0
+Actinomycin D	593	83	306	212
+3 unlabelled ribonucleotides	416	43	226	101
-PC/PCK	214	51	226	96
--PC/PCK +3 unlabelled ribonucleotides	238	29	208	73

FIGURE 14

The time course of incorporation of ^{3}H ATP, GTP, CTP and UTP by an acetone dried powder of the microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles tris-HCl buffer pH 8.5, 5 µmoles each of $MgCl_2$ and 2-mercapto-ethanol, 0.5 µmoles versene, 0.1 µmoles of ^{3}H ATP, GTP, CTP or UTP, 2.0 µmoles phosphocreatine, 50 µg phospho-creatine kinase, 100 µg RNA and 1.5 mg of acetone dried powder in a total volume of 0.5 ml.

Incubations were performed at 37° for the times indicates.



^{3}H ATP

^{3}H UTP

^{3}H CTP

^{3}H GTP.

Figure 14

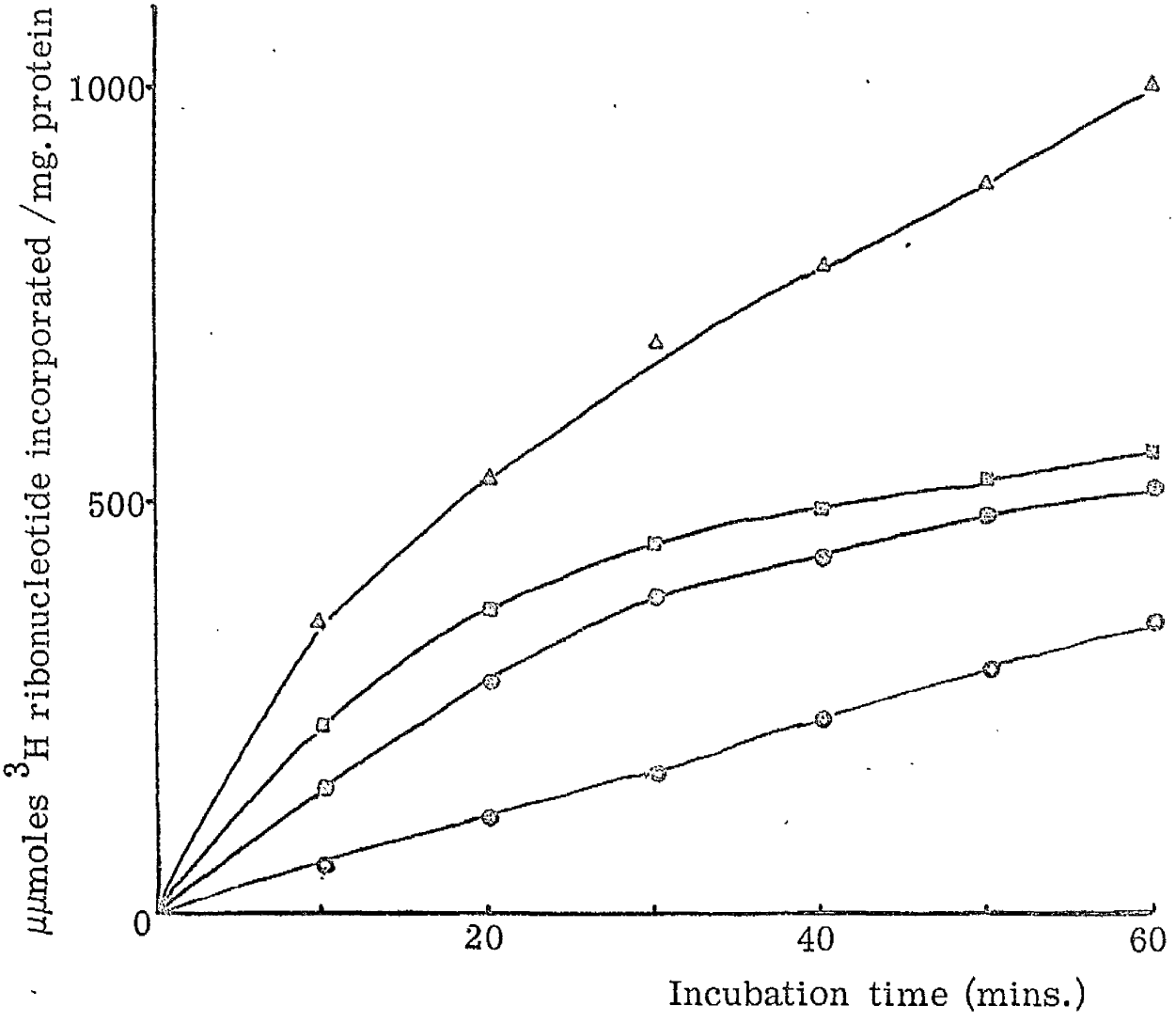

μμmoles ^{3}H ribonucleotide incorporated /mg. protein
1000
500
0
20
40
60
Incubation time (mins.)

FIGURE 15

The effect of enzyme concentration on the incorporation of ^{3}H ATP, GTP and CTP by an acetone dried powder from the microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles tris-HCl buffer pH 8.5, 5 µmoles each of $MgCl_2$ and 2-mercapto-ethanol, 0.5 µmoles versene, 0.1 µmoles of ^{3}H ATP, GTP or CTP, 2 µmoles phosphocreatine, 50 µg phosphocreatine kinase, 100 µg RNA and enzyme as indicated in a total volume of 0.5 ml.

The incubations were performed at 37° for 20 minutes.

—▲— ^{3}H ATP

—●— ^{3}H CTP

—○— ^{3}H GTP.

Figure 15

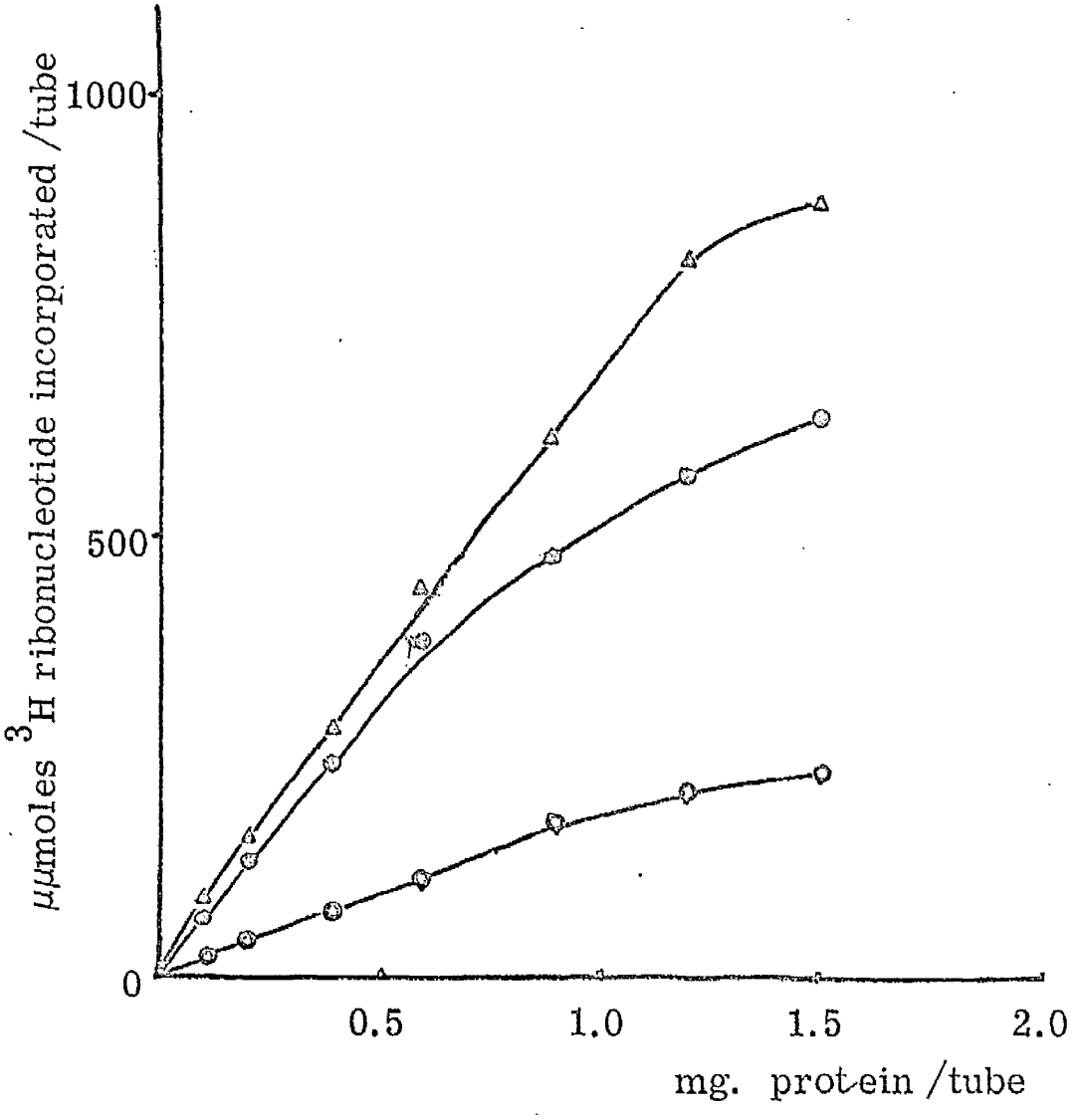

μμmoles ^{3}H ribonucleotide incorporated /tube
1000
500
0
0.5
1.0
1.5
2.0
mg. protein /tube

FIGURE 16

The effect of substrate concentration on the incorporation of ^{3}H ATP by an acetone dried powder of the microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles tris-HCl buffer pH 8.5, 5 µmoles each of $MgCl_2$ and 2-mercapto-ethanol, 0.5 µmoles versene, 2.0 µmoles phosphocreatine, 50 µg phosphocreatine kinase, 100 µg RNA and 1.5 mg of acetone dried powder. The concentration of ^{3}H ATP, either in the presence or absence of equal concentrations of GTP, CTP and UTP was varied as indicated in a total volume of 0.5 ml.

The incubations were performed at 37° for 20 minutes.

—▲— ^{3}H ATP

—■— ^{3}H ATP + GTP, CTP, UTP.

Figure 16

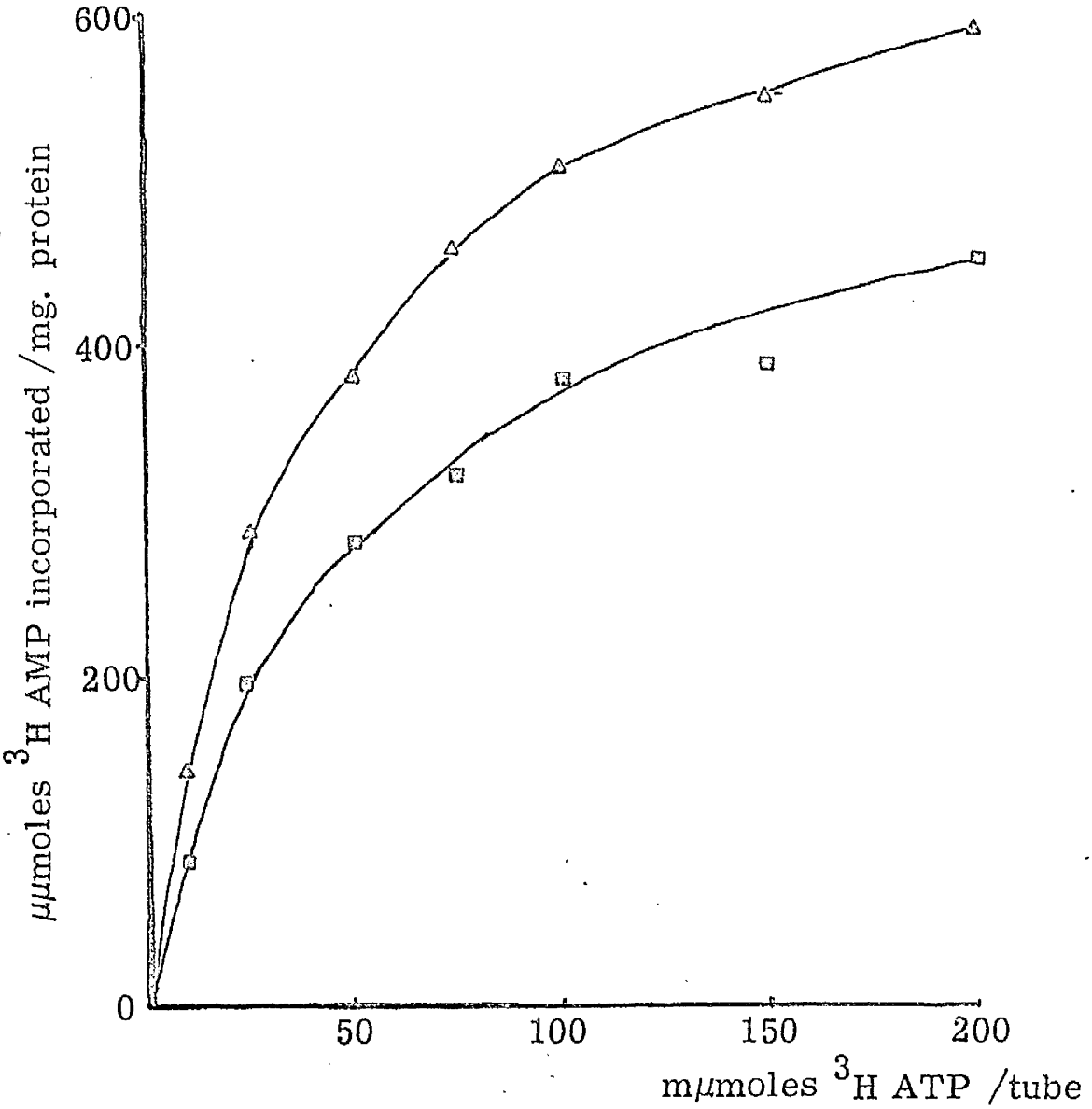

600
400
200
0
50
100
150
200
μμmoles ^{3}H AMP incorporated /mg. protein
mμmoles ^{3}H ATP /tube

FIGURE 17

The effect of pH on the incorporation of ^{3}H ATP, GTP and CTP by an acetone dried powder of the microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles tris-HCl buffer at the pH indicated, 5 µmoles each of $MgCl_2$ and 2-mercaptoethanol, 0.5 µmoles versene, 0.1 µmoles ^{3}H nucleotide, 2.0 µmoles phosphocreatine, 50 µg phosphocreatine kinase, 100 µg RNA and 1.5 mg of acetone dried powder in a total volume of 0.5 ml.

The incubations were performed at 37° for 20 minutes.

—▲— ^{3}H ATP

—●— ^{3}H CTP

—○— ^{3}H GTP.

Figure 17

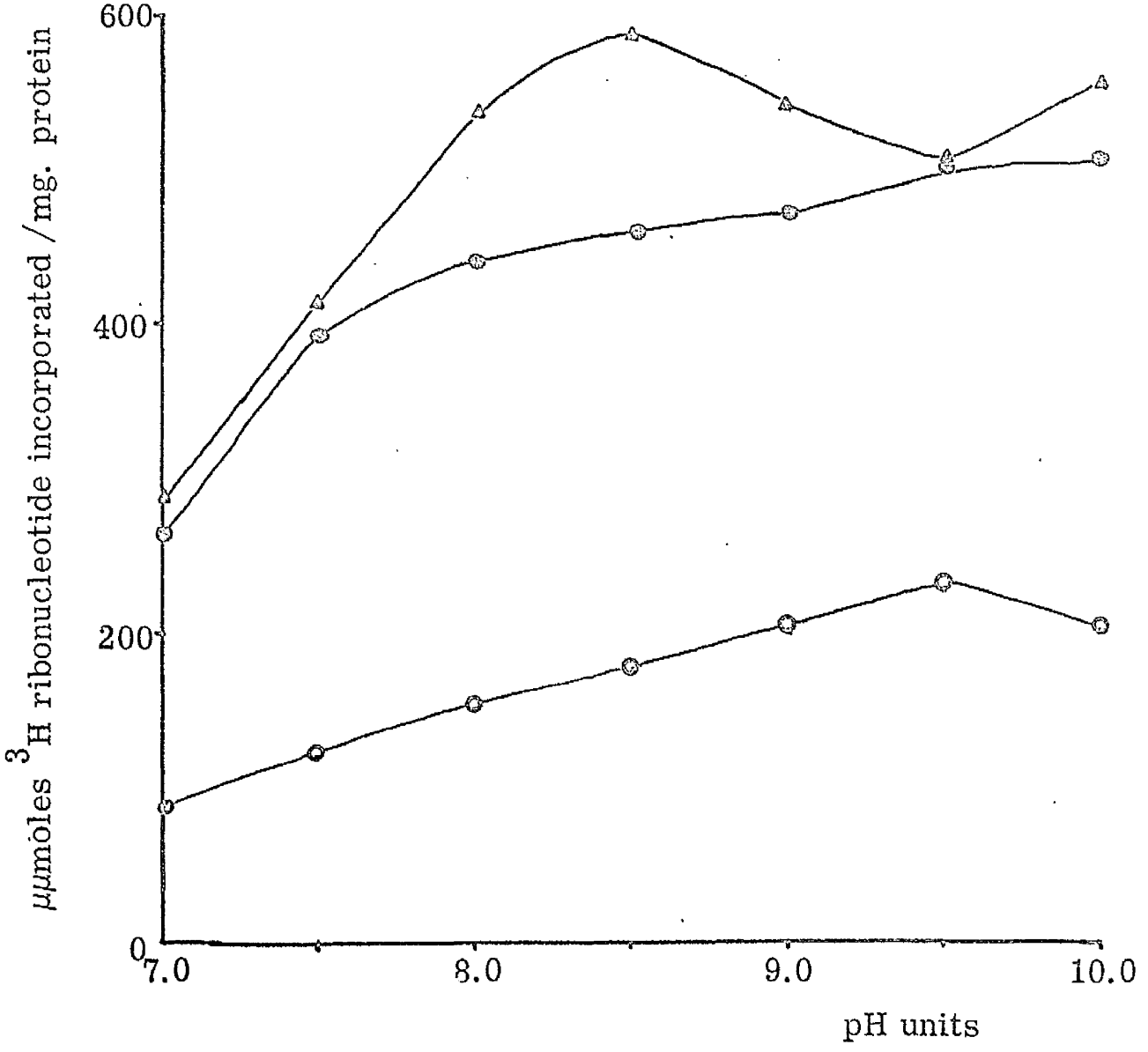

600
400
200
0
7.0
8.0
9.0
10.0
pH units
µmoles ^{3}H ribonucleotide incorporated /mg. protein

TABLE 6

The effect of phosphocreatine and phosphocreatine kinase in the presence and absence of complementary ribonucleoside 5'-triphosphates on the incorporation of ^{3}H ATP, GTP or CTP at pH 9.5 by an acetone dried powder of the microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles of tris-HCl buffer pH 9.5, 5 µmoles each of $MgCl_2$ and 2-mercapto-ethanol, 0.5 µmoles of versene, 0.1 µmoles of ^{3}H ATP, GTP or CTP, 100 µg of RNA and 1.5 mg of acetone dried powder. Additions, where indicated included 2.0 µmoles phosphocreatine, 50 µg phosphocreatine kinase and 0.1 µmoles each of the other unlabelled ribonucleoside 5'-triphosphates in a total volume of 0.5 ml.

Incubations were performed at 37° for 20 minutes.

Table 6

Reaction conditions	$\mu\mu$moles ^{3}H ribonucleotide incorporated /mg. protein		
	ATP	GTP	CTP
Complete system	181	79	213
+3 unlabelled ribonucleotides	171	67	223
+PC/PCK	427	178	307
+PC/PCK +3 unlabelled ribonucleotides	414	79	239

The results in Figure 16 show that there is no substrate concentration at which the complementary ribonucleoside 5'-triphosphates stimulate the incorporation of ^{3}H ATP; inhibition occurs over the whole range.

The effect of pH on the incorporation of ^{3}H ATP, ^{3}H GTP and ^{3}H CTP is shown in Figure 17. The incorporation of ^{3}H ATP shows a maximum at pH 8.5. The incorporation of ^{3}H GTP and ^{3}H CTP increase slowly over the range pH 7 - 9.5 but show no clear maxima. The differences in uptake between pH 8.5 and pH 9.5, 20 per cent in the case of ^{3}H GTP and 10 per cent in the case of incorporation of ^{3}H CTP were not considered large enough to warrent reinvestigation of the reaction characteristics at a higher pH. The possibility was considered that plots of incorporation against pH might be distorted at higher pH due to inhibition of phosphocreatine kinase or other ribonucleoside diphosphate kinases. Investigation showed, however, that the phosphate regenerating system stimulated the incorporation to the same extent at pH 9.5 as at pH 8.5 (see Table 6).

4. The Nature of the Homopolyribonucleotide Product.

Since considerable incorporation of ribonucleotide occurs in the absence of added RNA presumably due to priming by endogenous RNA in the microsomes it was of interest to

see whether the ribosomal RNA or other high molecular weight RNA added as a primer became labelled during the course of the reaction. Labelled RNA was extracted from reaction mixtures using the phenol technique, unincorporated nucleotides were removed by repeated precipitation and dialysis, and the RNA was analysed by sucrose density gradient centrifugation (see Methods, section 8C and 9A). The distribution of the ultraviolet absorbing material and radioactivity was measured in the fractions so obtained.

The results of such experiments are shown in Figures 18, 19 and 20 for the incorporation of ^{3}H GTP, ^{3}H UTP and ^{3}H ATP. In each case it is evident that negligible labelling of RNA occurs at zero time. Although 30S and 18S peaks of RNA are still present at the end of the reaction, they are not labelled. All the labelled material is concentrated between the 4S region and the meniscus. In the case of ^{3}H GTP incorporation, the addition of highly polymerised primer RNA (Landschutz ascites tumour whole cell RNA) shows a greater labelling in this area but still no labelling of the ribosomal RNA. In all cases RNase treatment caused a slight shift in the labelled peak nearer to the top of the gradient indicating some breakdown of the reaction product. The RNase treatment also causes disappearance of the 30S and 18S peaks

with the appearance of ultraviolet absorbing material in the region around 4S. It is also to be noted that the total extinction at 256 mμ in the incubated samples is considerably less than in the zero time controls. Assuming that the phenol extraction of RNA from the reaction mixtures is reasonably reproducible, this indicates that a proportion of the RNA present in the microsomes is degraded during the course of the reaction. There is no compensating increase in ultraviolet absorbing material in the 4S region of the gradient which suggests that such degradation is brought about by an exonuclease present in the microsomal fraction. If the degradation were caused by an endonuclease, oligonucleotide products would have been expected in the 4S region. Possibly the presence of an exonuclease is responsible for the lack of labelling of ribosomal RNA and added primer RNA.

Further investigations of the requirement for a primer were performed using ^{14}C labelled Landschutz ascites tumour cell RNA which was shown to be highly polymerised and labelled only in the purine bases (see Methods, section 8B). Assays were set up using the ^{14}C primer and the incorporation of ^{3}H ATP into polyribonucleotides was measured. RNA was extracted from the reaction mixture by the phenol method and analysed by sucrose

FIGURE 18

Sedimentation analysis in sucrose density gradients (5 - 20 per cent w/v) of ^{3}H CTP labelled RNA extracted from reaction mixtures containing a microsomal fraction from Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles tris-HCl buffer pH 8.5, 5 µmoles each of $MgCl_2$ and 2-mercapto-ethanol, 0.5 µmoles of versene, 0.1 µmoles ^{3}H CTP, 2 µmoles of phosphocreatine, 50 µg phosphocreatine kinase and 1 mg of microsomal protein in a total volume of 0.5 ml. Where indicated 100 µg ascites tumour whole cell RNA was added.

Incubations were performed at 37° for 1 hour, RNA was extracted and analysed on sucrose density gradients as described in the Methods, sections 8C and 9A.

—●— Extinction 256 mµ

—■— Radioactivity.

Figure 18

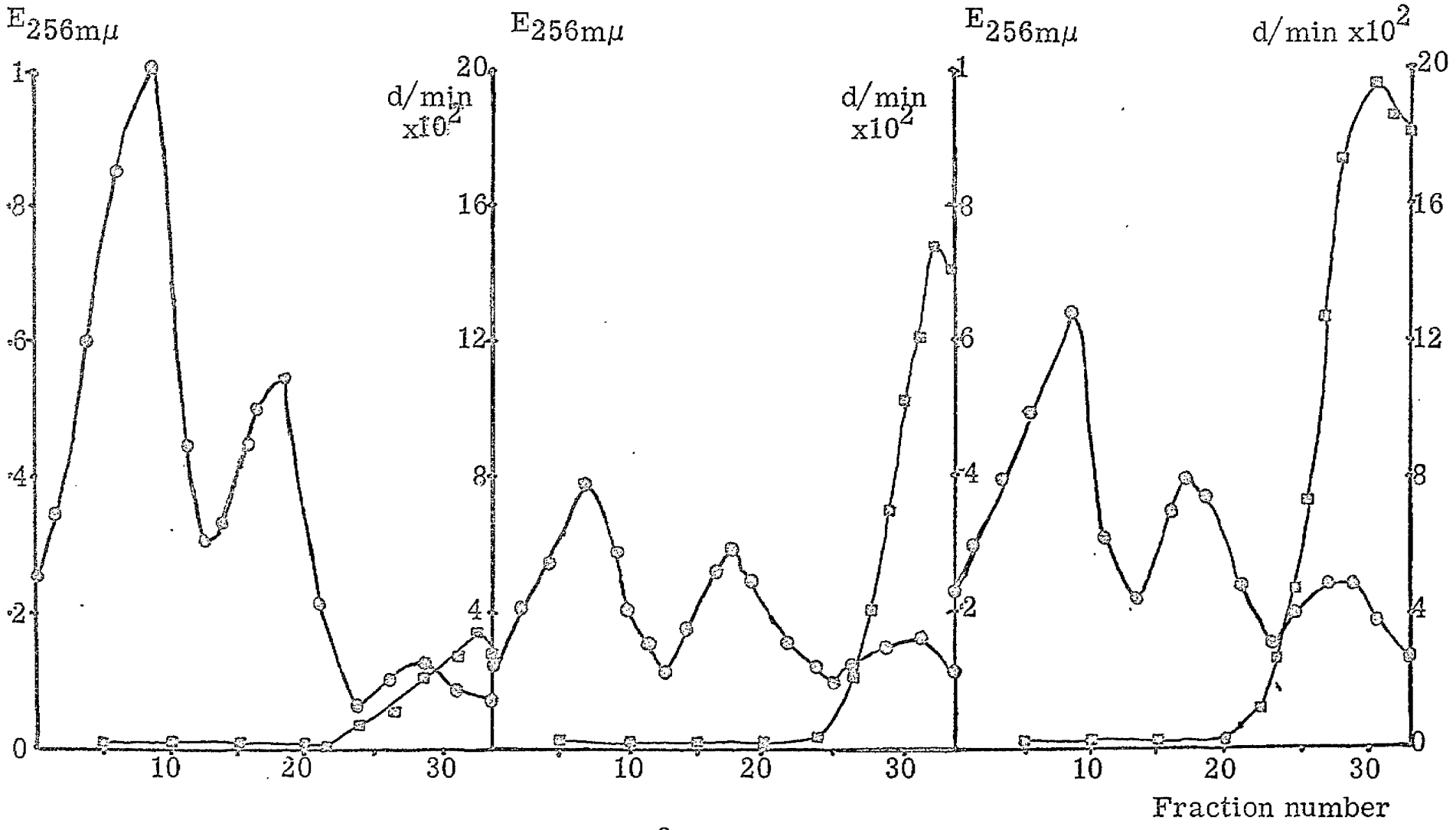

E256mμ
d/min x10²
Fraction number
A zero time
B ³H CTP
C ³H CTP + primer RNA

FIGURE 19

Sedimentation analysis in sucrose density gradients (5 - 20 per cent w/v) of ^{3}H UTP labelled RNA extracted from reaction mixtures containing a microsomal fraction from Landschutz ascites tumour cells.

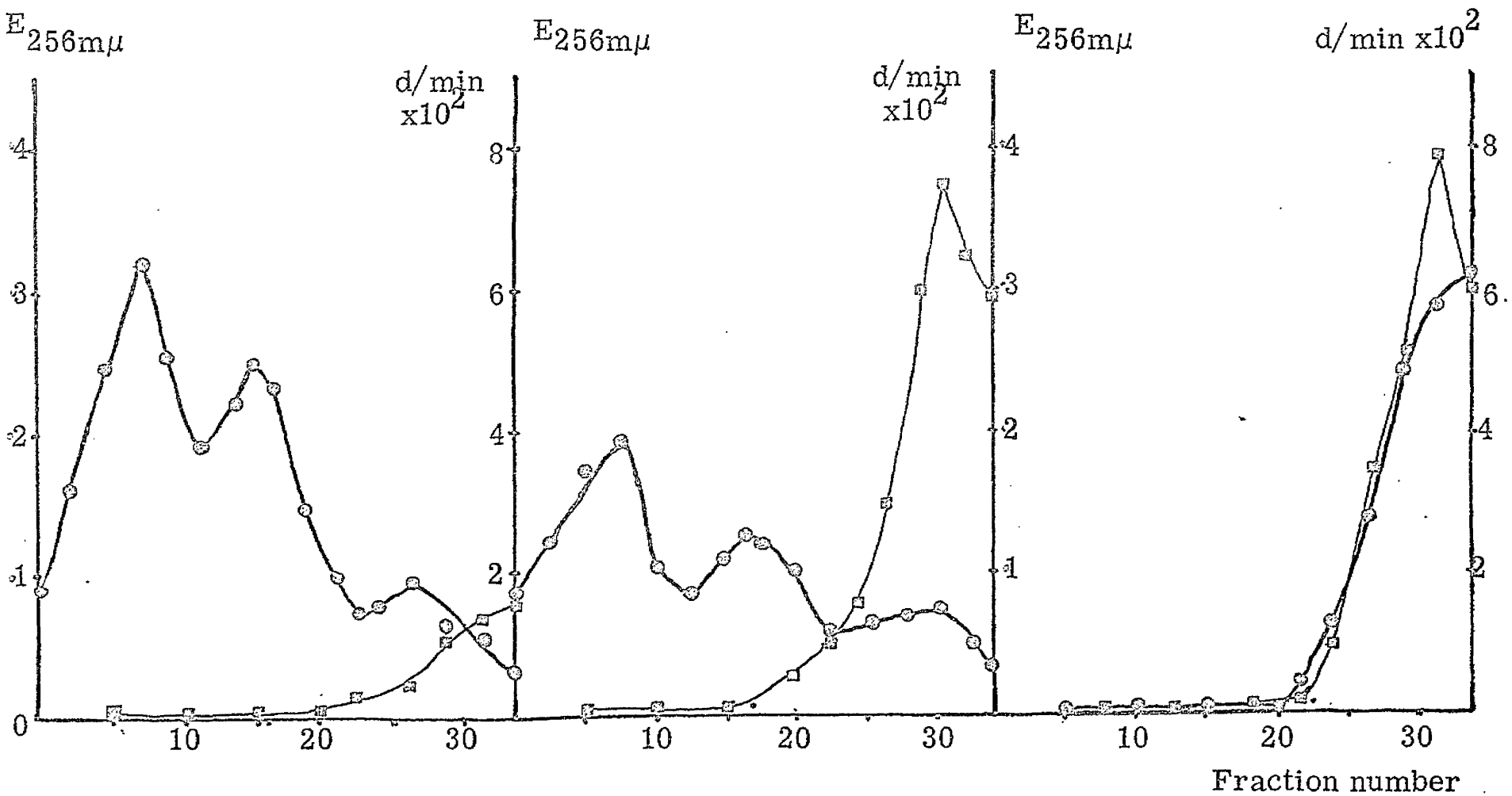
The reaction mixture and procedures were the same as described for Figure 18 except ^{3}H UTP was used and primer RNA was included.

RNA extracted from one tube (about 0.5 mgs. in 0.4 ml of buffer B) was treated with 2 µg RNase and incubated at 37° for 45 minutes before centrifugation.

—●— Extinction 256 mµ.

—■— Radioactivity.

Figure 19



A zero time

B ^{3}H UTP + primer RNA

C ^{3}H UTP + primer RNA product treated with RNase

FIGURE 20

Sedimentation analysis in sucrose density gradients (5 - 20 per cent w/v) of ^{3}H ATP labelled RNA extracted from reaction mixtures containing a microsomal fraction from Landschutz ascites tumour cells.

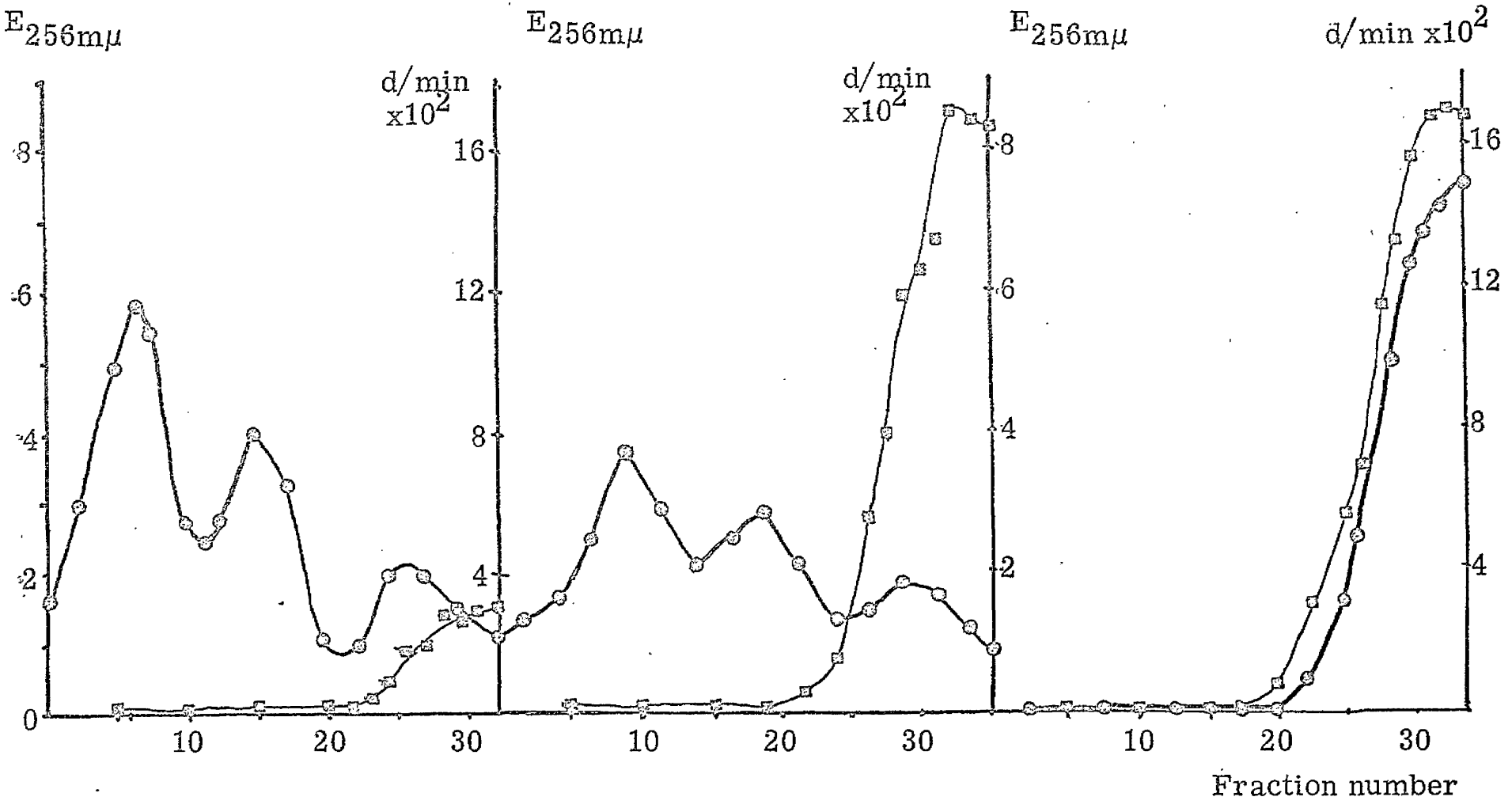
The reaction mixture and procedures were the same as described for Figure 18 except ^{3}H ATP was used and primer RNA was included.

RNA extracted from one tube (about 0.5 mgs. in 0.4 ml of buffer B) was treated with 2 µg of RNase and incubated at 37° for 45 minutes before centrifugation.

—●— Extinction 256 mµ

—●— Radioactivity.

Figure 20



A zero time

B ^{3}H ATP + primer RNA

C ^{3}H ATP + primer RNA product treated with RNase

FIGURE 21

Sedimentation analysis in sucrose density gradients (5 - 20 per cent w/v) of ^{3}H ATP labelled RNA and ^{14}C primer RNA extracted from reaction mixtures containing a microsomal fraction from Landschutz ascites tumour cells.

The reaction mixture and procedures were the same as described for Figure 18 except ^{3}H ATP was used and ^{14}C RNA (0.25×10^{6} d/min/mg) included as a primer.

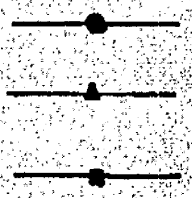
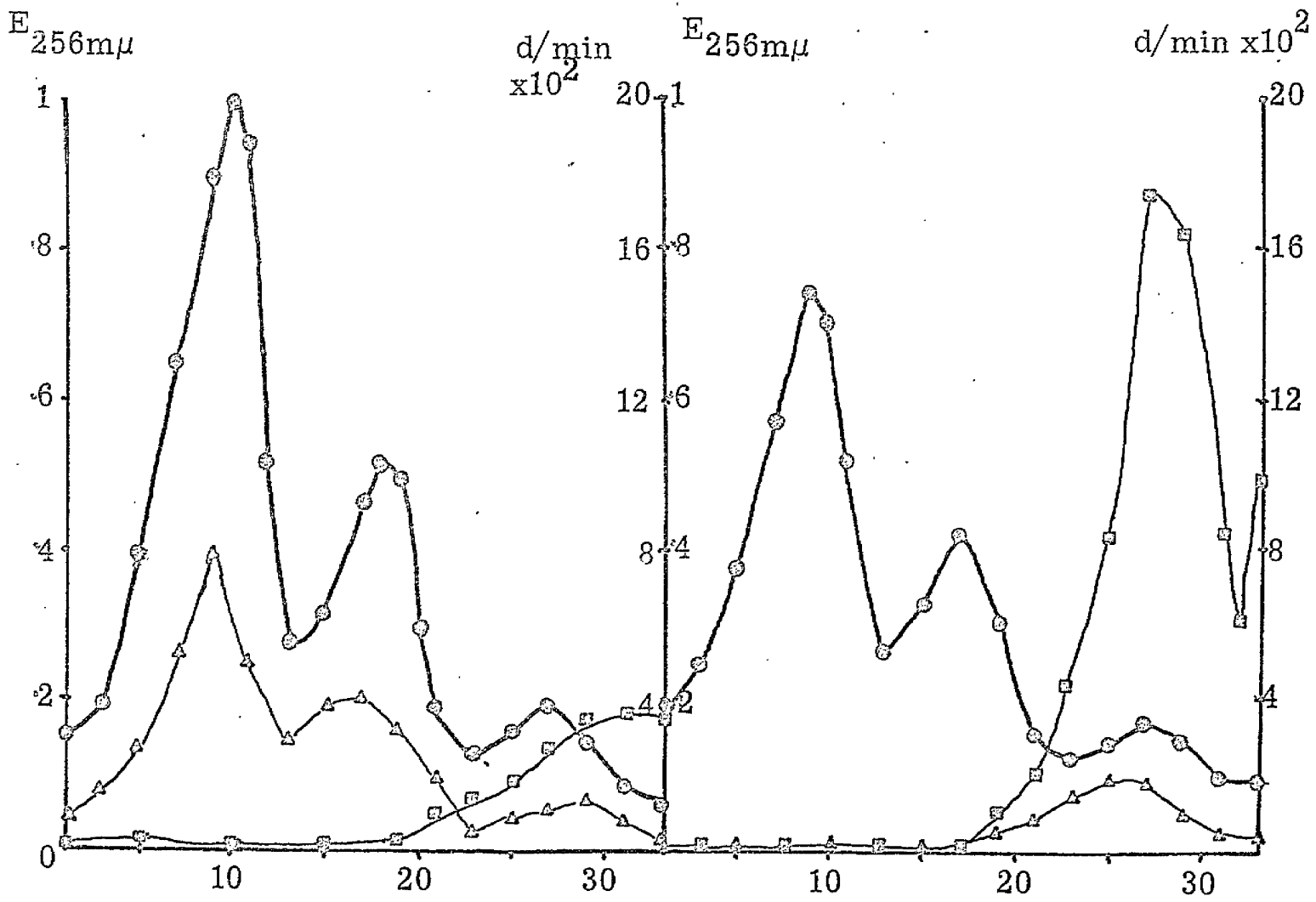


Figure 21



A zero time

B, ^{3}H ATP + ^{14}C primer RNA

density gradient centrifugation as before. Determination of ^{14}C and ^{3}H in the fractions so obtained was performed and the results are shown in Figure 21. At zero time the ribosomal peaks and 4S regions of the gradient are labelled with ^{14}C and a small amount of unincorporated ^{3}H ATP occurs between the 4S region and the meniscus. After incubation for 1 hour, a reduction in the amount of RNA in the 30S and 18S ribosomal peaks is again seen without any corresponding accumulation of oligonucleotides in the 4S region. The added ^{14}C RNA primer is however completely removed from the 30S and 18S ribosomal peaks though a small amount of ^{14}C labelling remains in the 4S region which is also labelled with ^{3}H AMP residues incorporated into poly A. The coincidence of ^{3}H and ^{14}C labels may mean that AMP residues are incorporated onto the ends of existing primer or ribosomal RNA chains. However, the presence of an exonuclease activity does not permit unambiguous interpretation of the results and AMP residues may be incorporated into separate poly A chains.

Since radioactivity was located only in the 4S region of the sucrose density gradient the priming ability of highly purified yeast sRNA was compared with the highly polymerised commercial yeast RNA used in previous experiments and with Landschutz ascites tumour RNA. As shown in

Figure 22, yeast RNA and ascites tumour RNA were found to be equally effective as primers for the incorporation of ^{3}H CTP whereas yeast sRNA was a more effective primer. On the other hand, Figure 23 shows that for the incorporation of ^{3}H UTP, sRNA had no priming effect whereas ascites tumour RNA was an effective primer and stimulated the incorporation to a greater extent than it did for ^{3}H CTP as substrate. The stimulation of ^{3}H CTP incorporation by sRNA is probably due to contamination of the microsomal fraction with the enzymes incorporating the terminal pCpCpA sequence into sRNA for which sRNA is a specific acceptor. This could explain the lack of priming ability exhibited by sRNA for the incorporation of ^{3}H UTP and indicates some specificity in the primer for this latter reaction.

The chain length of the homopolyribonucleotide sequences synthesised in the reaction was determined from the ratio of radioactivity in nucleotides and nucleosides present in alkaline hydrolysates of the product (see Methods, section 7). Table 7 shows the average chain lengths of the product obtained after the incorporation of ^{3}H ATP, ^{3}H GTP, ^{3}H CTP and ^{3}H UTP. In all cases these are less than 8 ribonucleotide residues, poly A being the shortest. When the chain length is determined after incorporation of ^{3}H UTP in the

FIGURE 22

A comparison of the effect of yeast sRNA, commercial yeast, highly polymerised RNA and ascites tumour RNA on the incorporation of ^{3}H CTP by a microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles tris-HCl buffer pH 8.5, 5 µmoles each of $MgCl_2$ and 2-mercapto-ethanol, 0.5 µmoles versene, 0.1 µmoles ^{3}H CTP, 2.0 µmoles phosphocreatine, 50 µg phosphocreatine kinase and 0.67 mg of microsomal protein. Yeast sRNA, commercial yeast RNA and ascites tumour RNA were added where indicated in a total volume of 0.5 ml.

Incubations were performed at 37° for 20 minutes.

—▲— sRNA

—■— ascites tumour RNA

—○— commercial yeast RNA.

FIGURE 23

A comparison of the effect of yeast sRNA and ascites tumour RNA on the incorporation of ^{3}H UTP by a microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture was the same as described for Figure 22 except 0.1 µmoles ^{3}H UTP were incorporated. Yeast sRNA and ascites tumour RNA were added as indicated.

Incubations were performed at 37° for 20 minutes.

Symbols as for Figure 22.

Figure 22

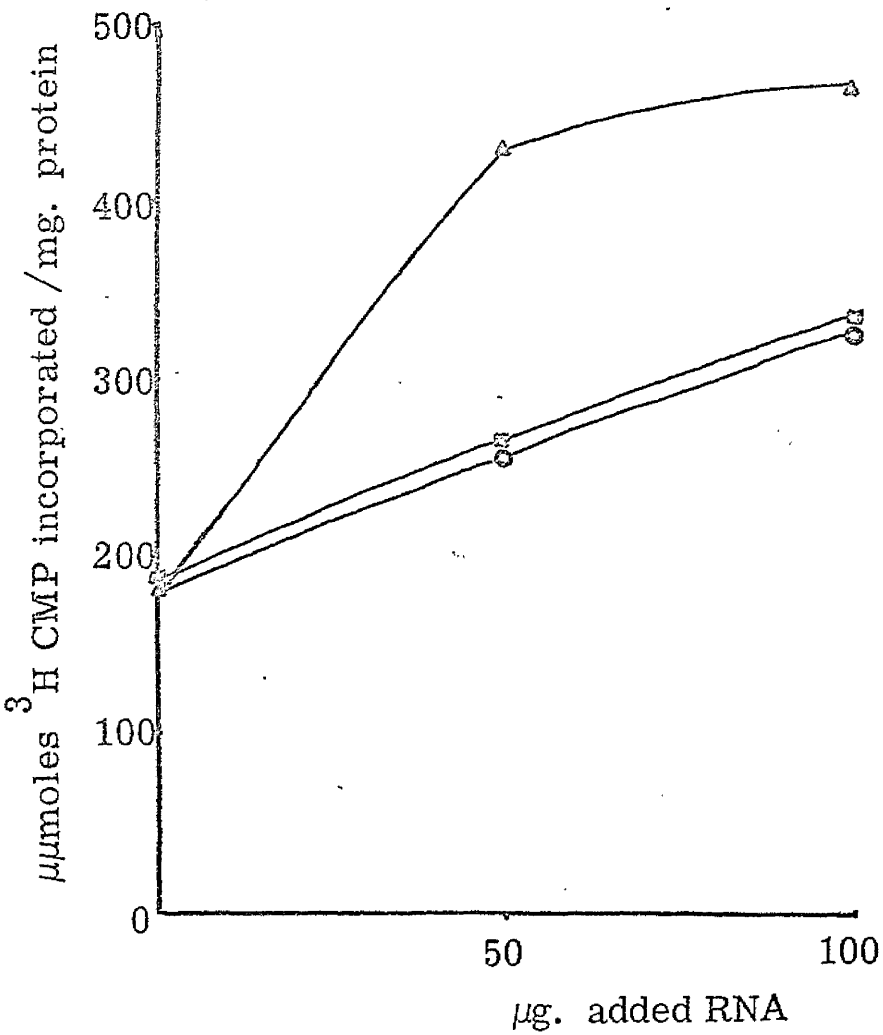


Figure 23

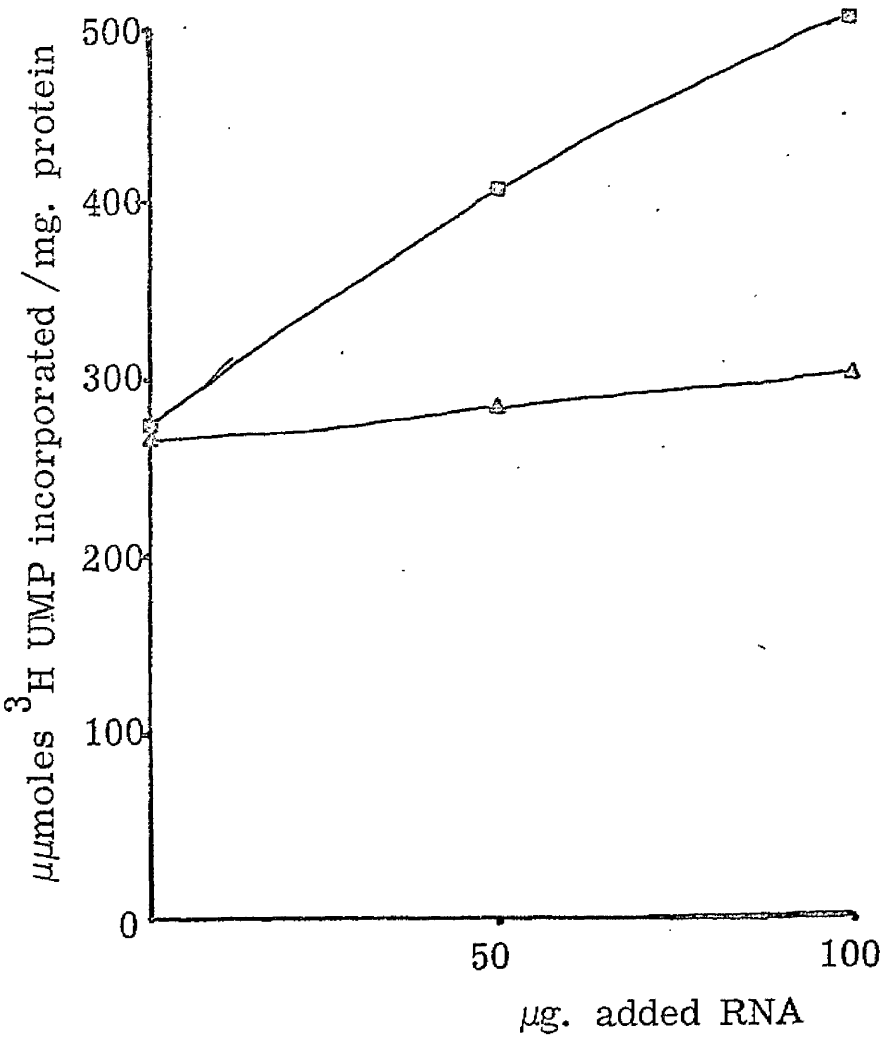


TABLE 7

The chain length of homopolyribonucleotides formed by a microsomal fraction from Landschutz ascites tumour cells.

The reaction mixtures contained 50 µmoles tris-HCl buffer pH 8.5, 5 µmoles each of $MgCl_2$ and 2-mercapto-ethanol, 0.5 µmoles versene, 2.0 µmoles phosphocreatine, 50 µg phosphocreatine kinase, 100 µg RNA and 0.8 mg microsomal protein. 0.1 µmoles of ^3H nucleotide and ATP, GTP and CTP were added where indicated in a total volume of 0.5 ml.

Incubations were performed at 37° for the times indicated, alkaline hydrolysis and separation of ribonucleotides from nucleosides was performed as described in the Methods, section 7B.

Table 7

Substrate	Chain length		
	Incubation time (minutes)		
	15	30	60
^{3}H ATP	2	2	2 or 3
^{3}H GTP	3 or 4	4 or 5	7
^{3}H CTP	2	3	3 or 4
^{3}H UTP	6 or 7	7 or 8	7 or 8
^{3}H UTP+ ATP, GTP, CTP	3 or 4	4 or 5	4 or 5

presence of ATP, GTP and CTP the incorporation is roughly halved.

5. Investigation of the Specificity of the Reaction.

Since phosphocreatine and phosphocreatine kinase stimulate the reaction and do not inhibit it, it appears unlikely polynucleotide phosphorylase is involved and ribonucleoside 5'—triphosphates and not diphosphates are the actual substrates. If the ribonucleoside 5'—triphosphates are the true precursors inorganic pyrophosphate would be expected to inhibit the reaction rather than inorganic orthophosphate. Figure 24 shows the effects of increasing concentrations of pyrophosphate and orthophosphate on the incorporation of ^{3}H UTP. Orthophosphate exerts only a very slight effect on the incorporation of ^{3}H UTP while pyrophosphate causes a 20 per cent inhibition at the maximum concentration used. The pyrophosphate was made up in $MgCl_2$ solution to prevent alteration of the Mg^{2+} ion concentration in the reaction mixture and the highest concentration used in the assay was the maximum possible that could be used without causing precipitation of pyrophosphate as the magnesium salt.

The reaction catalysed by the microsomal enzyme appears to be specific for ribonucleoside 5'—triphosphates

FIGURE 24

The effect of increasing concentrations of inorganic orthophosphate and pyrophosphate on the incorporation of ^{3}H UTP by an acetone dried powder of the microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles tris-HCl buffer pH 8.5, 5 µmoles each of $MgCl_2$ and 2-mercapto-ethanol, 0.5 µmoles of versene, 0.1 µmoles ^{3}H UTP, 2 µmoles phosphocreatine, 50 µg phosphocreatine kinase, 100 µg RNA and 1.5 mgs of acetone dried powder. Na_2HPO_4 and $Na_4P_2O_7$ made up with an equal concentration of Mg^{2+} were added in the amounts indicated in a total volume of 0.5 ml.

Incubations were performed at 37° for 20 minutes.

—▲— + orthophosphate

—■— + pyrophosphate.

Figure 24

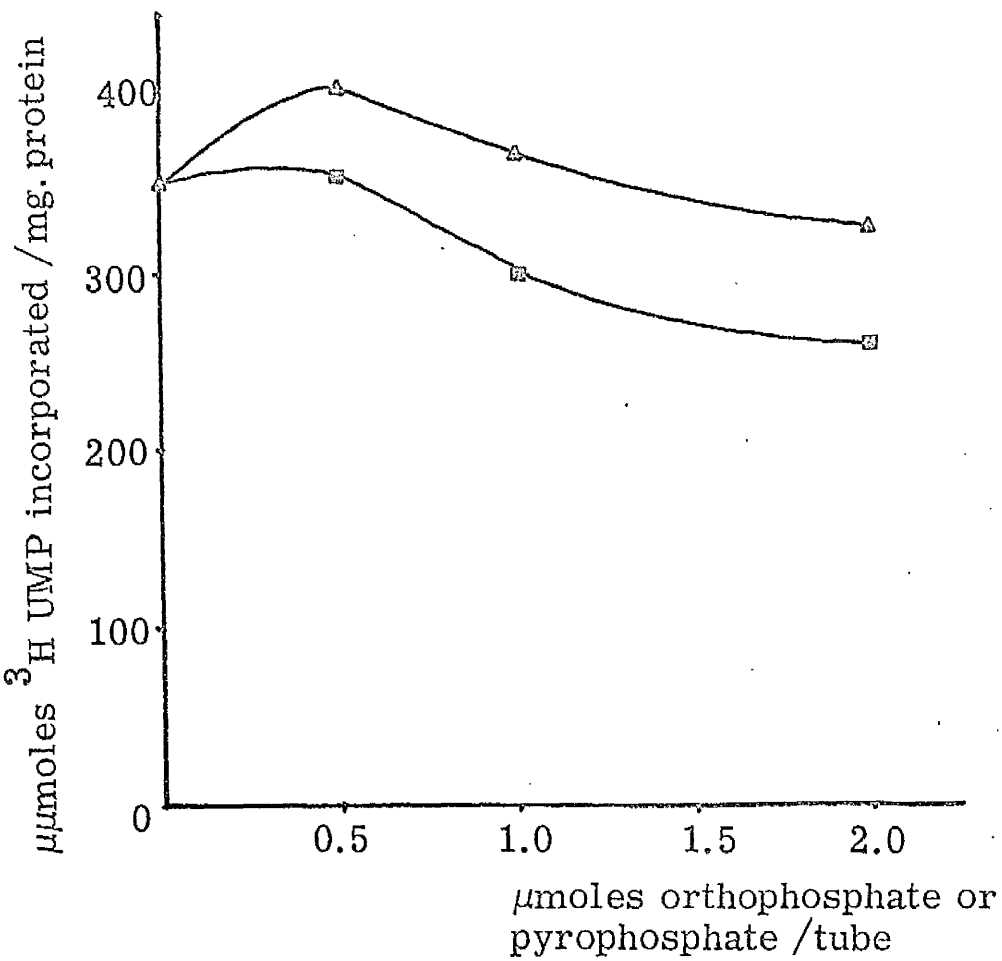

μμmoles ^{3}H UMP incorporated /mg. protein
400
300
200
100
0
0.5
1.0
1.5
2.0
μmoles orthophosphate or
pyrophosphate /tube

FIGURE 25

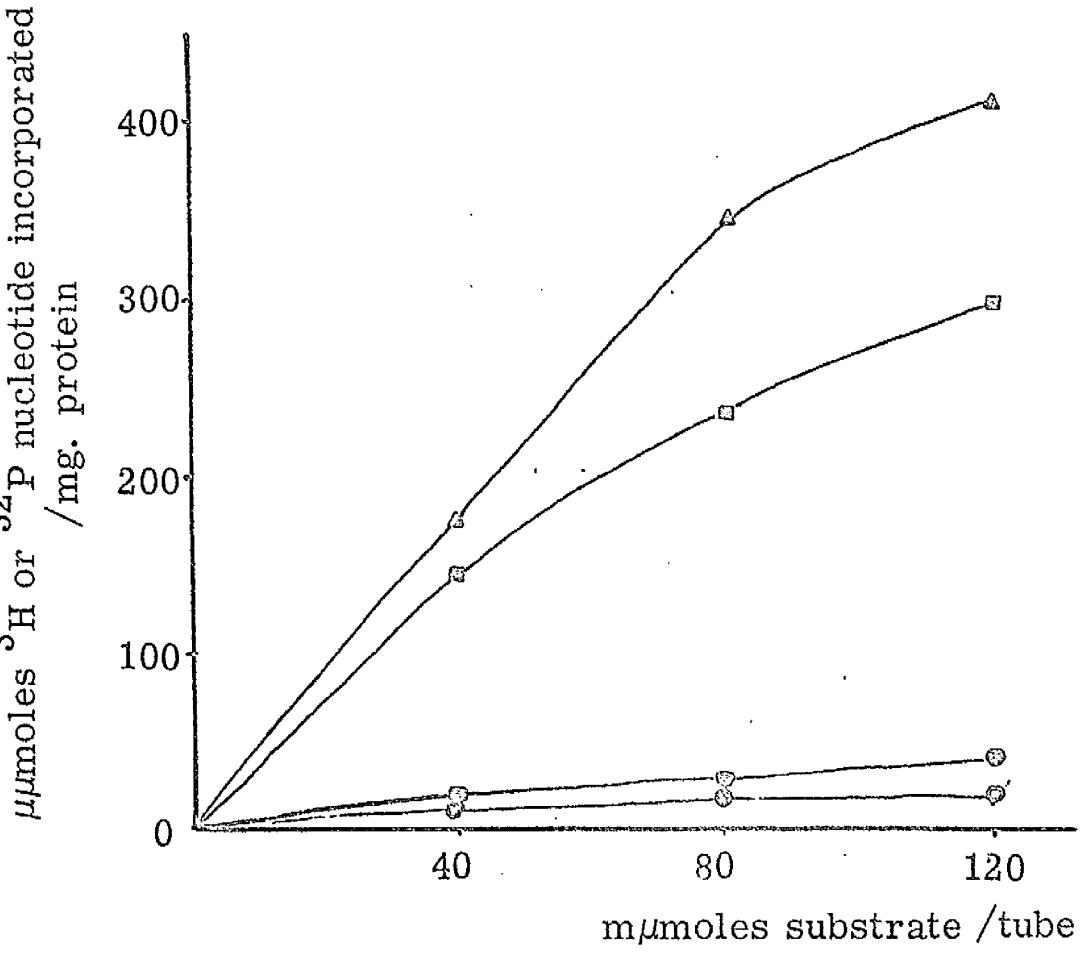
The utilisation of ribonucleoside 5'-triphosphates and deoxyribonucleoside 5'-triphosphates as substrates for polynucleotide synthesis catalysed by an acetone dried powder of the microsomal fraction of Landschutz ascites tumour cells.

The reaction mixture contains 50 µmoles tris-HCl buffer pH 8.5, 5 µmoles each of $MgCl_2$ and 2-mercapto-ethanol, 0.5 µmoles of versene, 2 µmoles of phospho-creatine, 50 µg phosphocreatine kinase, 100 µg RNA and 1.5 mg of acetone dried powder. ^{3}H ATP, UTP, dATP and (^{32}P) TTP were added in the amounts indicated in a total volume of 0.5 ml.

Incubations were performed at 37° for 20 minutes.

—▲— + ^{3}H ATP
—■— + ^{3}H UTP
—●— + ^{3}H dATP
—○— + ($\alpha^{32}P$) TTP.

Figure 25


μμmoles ^{3}H or ^{32}P nucleotide incorporated /mg. protein
400
300
200
100
0
40
80
120
mμmoles substrate /tube

since the incorporation of ^{3}H dATP and ($\alpha^{32}P$) TTP occurs to less than one twentieth the extent of ^{3}H ATP and ^{3}H UTP as shown in Figure 25.

6. Reinvestigation of the Microsomal Location of the Reaction.

Using an improved reaction mixture containing versene 2-mercaptoethanol, phosphocreatine and phosphocreatine kinase and omitting ATP, GTP, CTP and bentonite, the ability of different cell fractions to incorporate ($\alpha^{32}P$) UTP into homopolyribonucleotides was reinvestigated. Figure 26 shows the time course of incorporation by broken nuclei, mitochondria, a 105,000 x g sediment and a 105,000 x g supernatant fraction of Landschutz ascites tumour cells. Only the 105,000 x g sediment shows appreciable activity. The 105,000 x g supernatant was further fractionated by centrifugation at 150,000 x g for 2 hours giving a 150,000 x g sediment and a 150,000 x g supernatant fraction. The incorporation by these fractions is compared in Figure 27. Again the microsomal fraction shows most activity though some activity is recovered in the 150,000 x g sediment while the supernatant fractions both show low activity.

Attempts were made to subfractionate the microsomal

fraction using the sucrose iso-octane technique of Hallinan and Munro (1965), (see Methods, section 2). By this method it is possible to prepare a membrane fraction, free ribosomes and rough surfaced vesicles or membrane with bound ribosomes. A comparison of the incorporation of (α^{32}P) UTP into polyribonucleotides by these fractions and by the microsomal fraction is shown in Figure 28. The free ribosomes are most active in incorporating (α^{32}P) UTP followed in order by the untreated microsomes, the rough surfaced vesicle fraction and the membrane fraction. These differences in activity may be due to differences in the distribution of the nucleases interfering with the incorporation (see section 8) or, assuming that the reaction is catalysed by an enzyme bound to the ribosomes, to different amounts of ribosomes present in each of the fractions.

The particulate nature of the enzyme is also indicated by centrifugation of the microsomal fraction on sucrose density gradients. Figure 29 shows the distribution of the extinction at 260 mμ and 280 mμ of a microsomal fraction together with measurements of the ability to incorporate (α^{32}P) UTP. The peak of incorporation is displaced to the light side of the 80S ribosomal peak. A small sediment also appears at the bottom of the tube

and this is inactive. Attempts were made to show which ribosomal subunits, if any, were associated with the ribonucleotide incorporating activity and if possible to dissociate the enzyme from the particulate material.

Figures 29A, 30A and B show the effects of dialysis against 0.01M tris-HCl buffer pH 8.0, dialysis against 0.01M tris-HCl, 0.001M versene pH 8.0 and dialysis against 0.01M tris-HCl; 0.01M versene pH 8.0. Dialysis without versene causes partial breakdown of the 80S ribosomes to 60S and 40S ribosomes and the activity appears slightly displaced to the lighter side of the 40S ribosomes. A similar relationship between incorporating activity and the optical density curves is found when 0.001M versene is present in the dialysis medium. This would be expected to give rise to complete breakdown of the 80S ribosomes into their 60S and 40S components, though these were not resolved by the centrifugation conditions employed. When 0.01M versene is used in the dialysis medium the ribosomes appear to be disrupted and the incorporating activity remains associated with the disrupted material. Such experiments indicate that the enzymes incorporating ribonucleotides are associated with particulate material, possibly the 40S ribosomes.

Further evidence that such enzymes are bound to

FIGURE 26

The time course of the incorporation of (α^{32}P) UTP by subcellular fractions of Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles tris-HCl buffer pH 8.5, 5 µmoles each of $MgCl_2$ and 2-mercapto-ethanol, 0.5 µmoles versene, 0.1 µmoles (α^{32}P) UTP (4.5×10^6 counts/min/µmole), 2 µmoles phosphocreatine, 50 µg phosphocreatine kinase, 100 µg RNA and 0.75 mg protein in a total volume of 0.5 ml.

The incubations were performed at 37° for the times indicated.

—▲— Microsomal fraction
—○— 105,000 x g supernatant fraction
—×— Nuclei
—⊙— Mitochondria.

FIGURE 27

The time course of the incorporation of (α^{32}P) UTP by subcellular fractions of Landschutz ascites tumour cells.

The reaction mixture was as described for Figure 26. Incubations were performed at 37° for the times indicated.

—▲— Microsomal fraction
—●— 150,000 x g sediment
—○— 105,000 x g supernatant fraction
—●— 150,000 x g supernatant fraction.

Figure 26

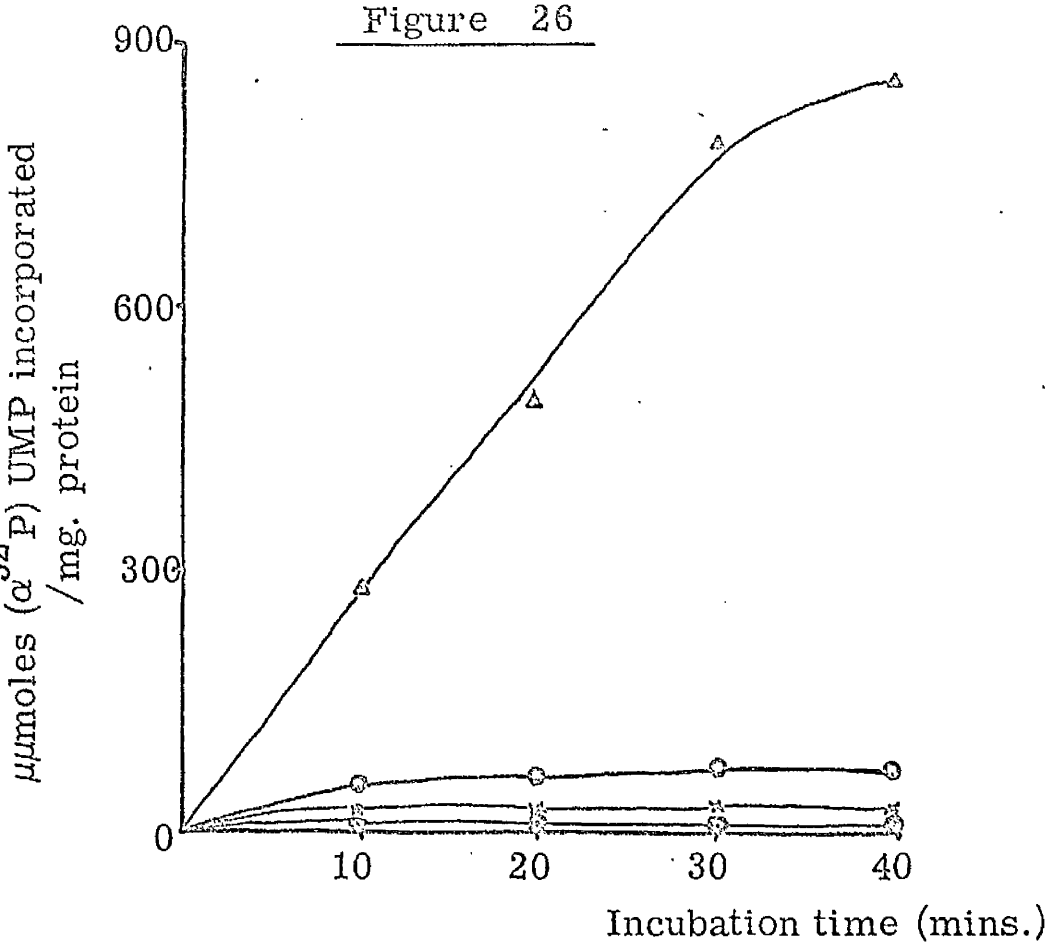


Figure 27

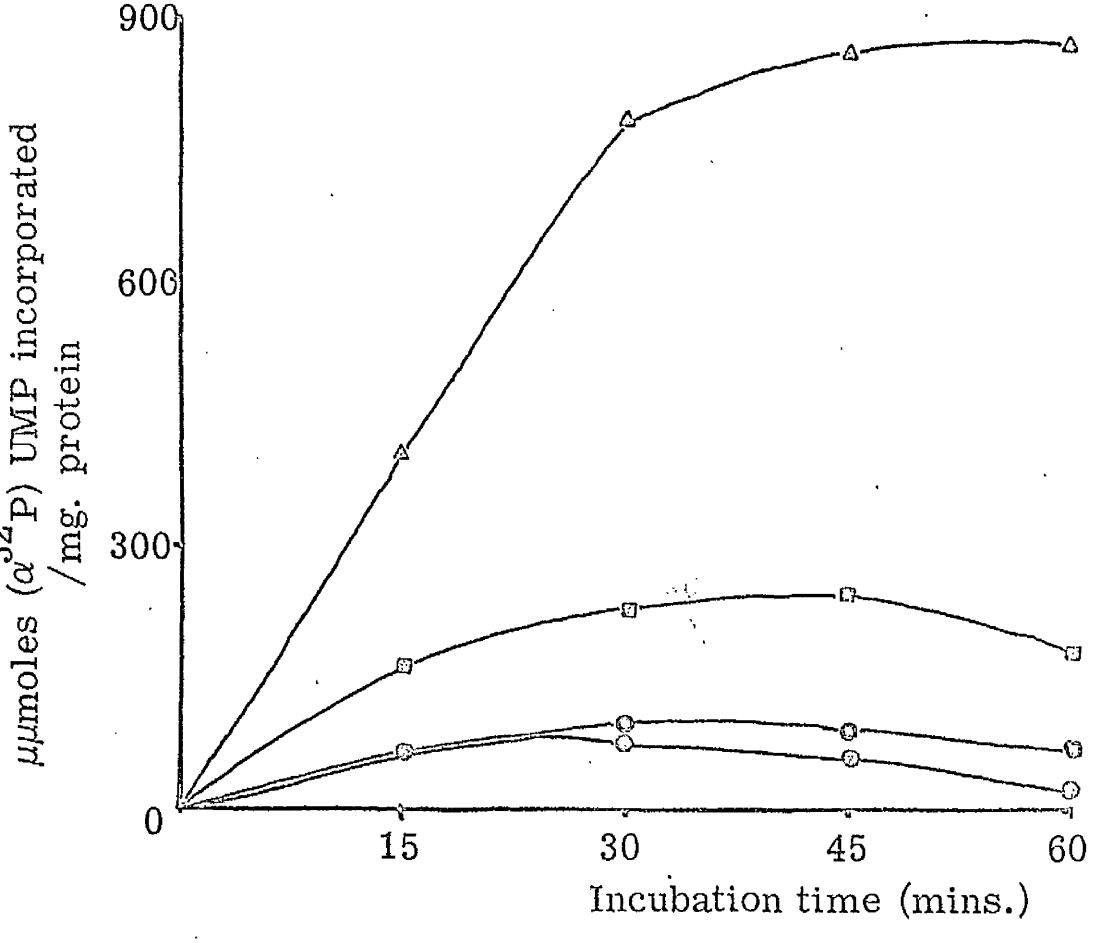


FIGURE 28

The time course of the incorporation of ^{3}H UTP into polyribonucleotides catalysed by a microsomal fraction and submicrosomal fractions (prepared by the method of Hallinan and Munro) from Landschutz ascites tumour cells.

The reaction mixture contained 50 µmoles of tris-HCl buffer pH 8.5, 5 µmoles each of $MgCl_2$ and 2-mercapto-ethanol, 0.5 µmoles of versene, 0.1 µmoles ^{3}H UTP, 2 µmoles phosphocreatine, 50 µg phosphocreatine kinase, 100 µg RNA and 0.7 mg of protein (0.3 mg in the case of the membrane fraction) in a total volume of 0.5 ml.

Incubations were performed at 37° for the times indicated.

—●— Free ribosomes

—◆— microsomal fraction

—○— 78,000 x *g* sediment

—■— membrane fraction.

Figure 28

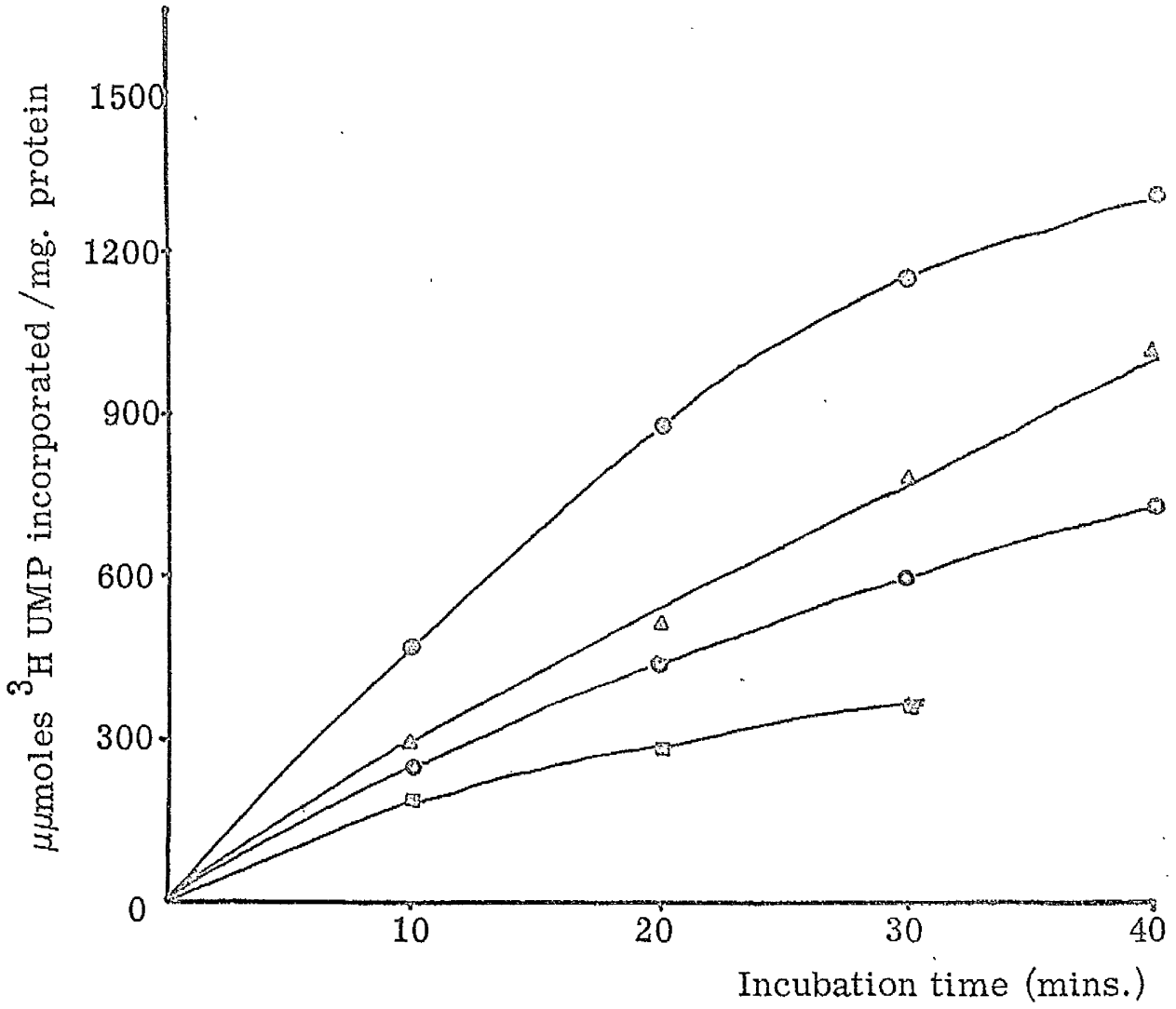

μμmoles ^{3}H UMP incorporated /mg. protein
1500
1200
900
600
300
0
10
20
30
40
Incubation time (mins.)

FIGURE 29

The incorporation of ^{3}H UTP by fractions of the microsomes of Landschutz ascites tumour cells obtained by sucrose density gradient centrifugation.

A. Untreated microsomes were centrifuged on a 5 - 30 per cent (w/v) sucrose gradient at 27,000 x g for 6 hours in a spinco SW25 rotor and fractions collected were assayed in a reaction mixture containing 50 µmoles tris-HCl pH 8.5, 5 µmoles each of $MgCl_2$ and 2-mercaptoethanol, 0.5 µmoles versene, 0.1 µmoles ^{3}H UTP, 2 µmoles phosphocreatine, 50 µg phosphocreatine kinase, 100 µg RNA and 0.3 ml of each microsomal fraction from the gradient in a total volume of 0.5 ml.

Incubations were performed at 37° for 20 minutes.

B. The microsomal fraction was dialysed against 0.01M tris-HCl buffer pH 8.0, at 2 - 4° for 12 hours before centrifugation and assay as in A above.

—●— Extinction at 260 mµ

—○— Extinction at 280 mµ

—▲— Radioactivity.

Figure 29

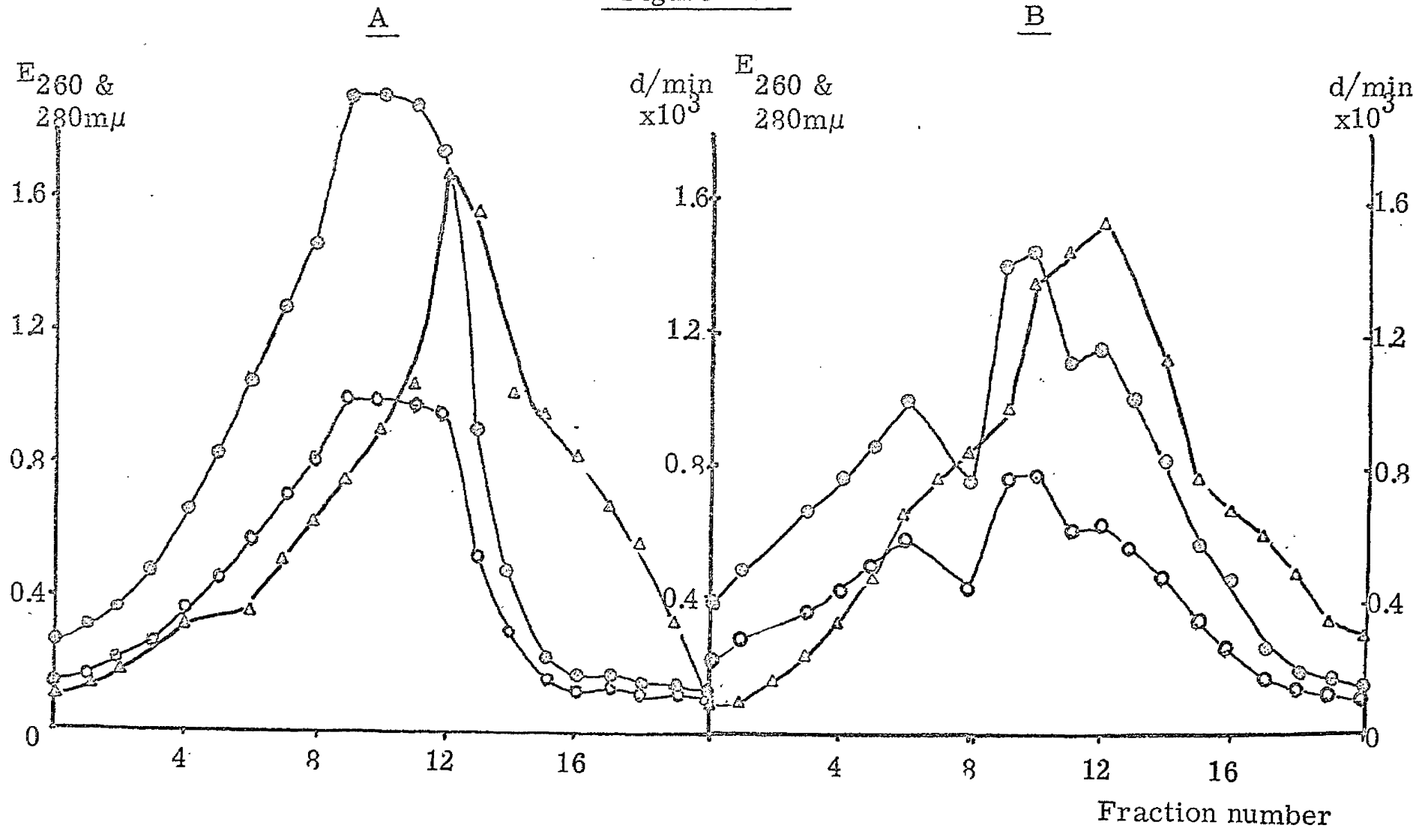

A
B
E_{260} & 280mμ
d/min x10^3
E_{260} & 280mμ
d/min x10^3
1.6
1.2
0.8
0.4
0
4
8
12
16
Fraction number

FIGURE 30

The incorporation of ^{3}H UTP by fractions of the microsomes from Landschutz ascites tumour cells obtained by sucrose density gradient centrifugation.

A The microsomal fraction was dialysed against 0.01 M tris-HCl, 0.001 M versene pH 8.0 at 2 - 4° for 12 hours before centrifugation and assay as described in Figure 29A.

B The microsomal fraction was dialysed against 0.01 M tris-HCl 0.01M versene pH 8.0 at 2 - 4° for 12 hours before centrifugation and assay as described in Figure 29A.

—●— Extinction at 260 mμ

—○— Extinction at 280 mμ

—▲— Radioactivity.

Figure 30

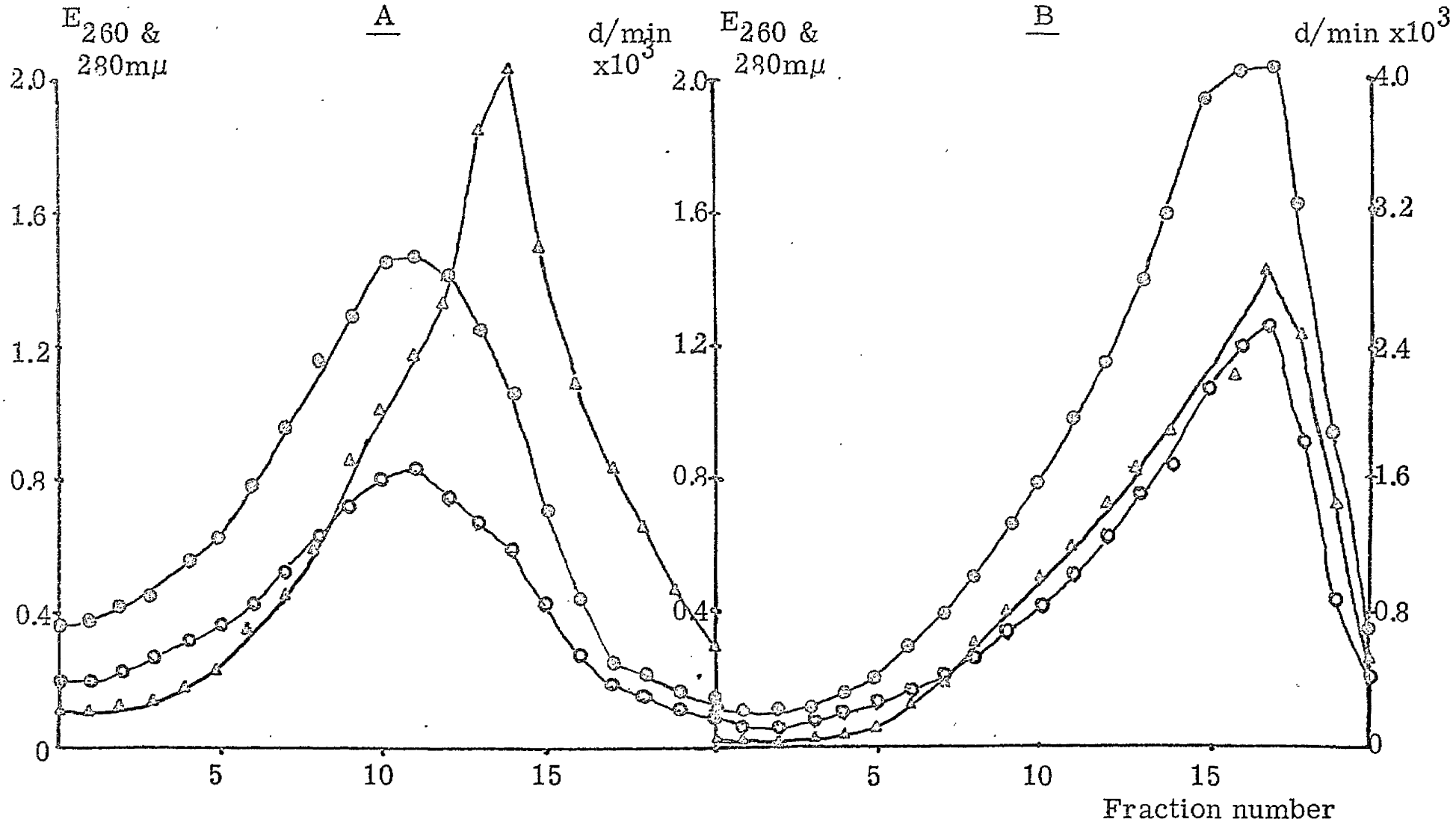

A
B
E_{260} & 280mμ
d/min x10^3
E_{260} & 280mμ
d/min x10^3
2.0
1.6
1.2
0.8
0.4
0
4.0
3.2
2.4
1.6
0.8
0
5
10
15
5
10
15
Fraction number

FIGURE 31

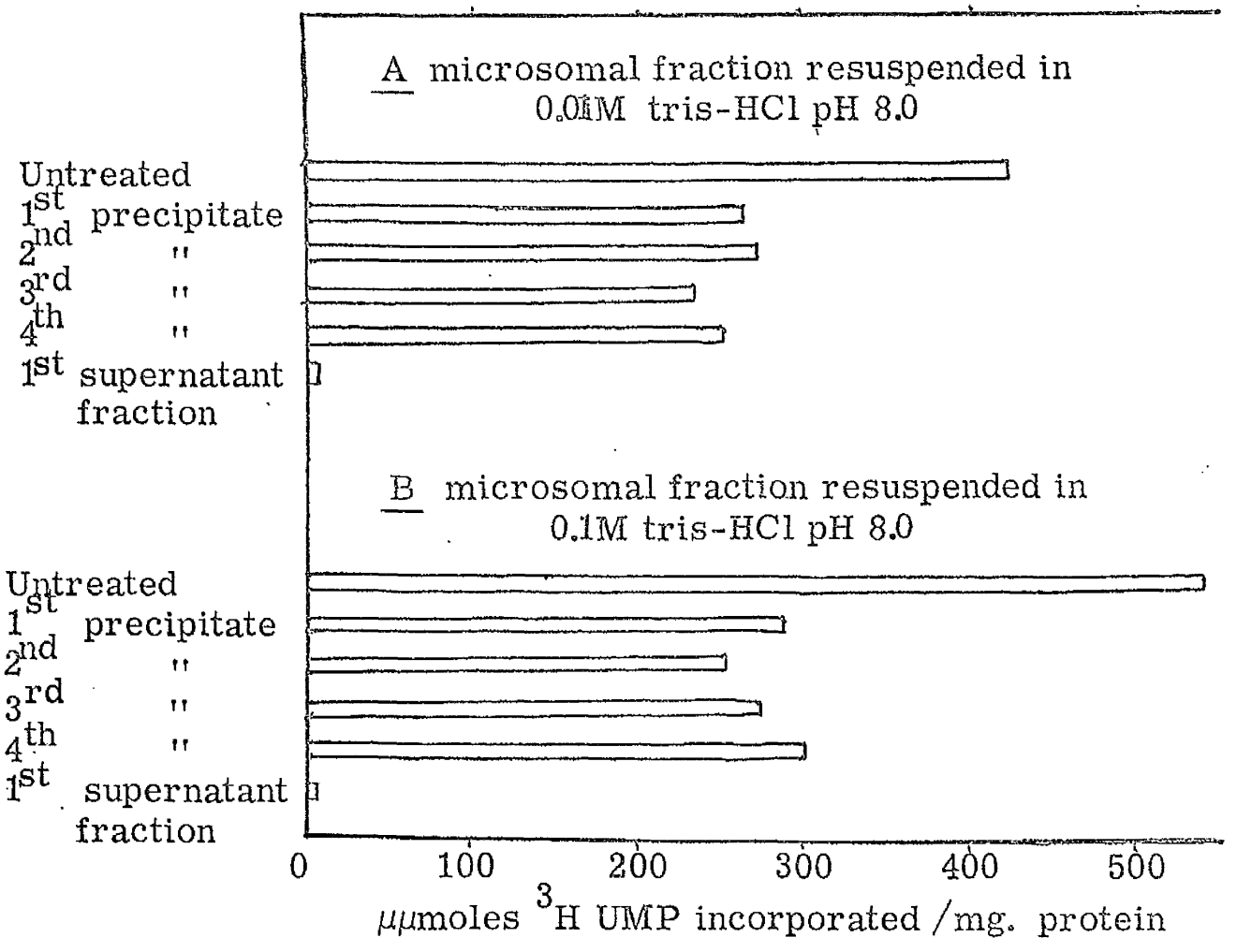
The effect on the incorporation of ^{3}H UTP by a microsomal fraction of Landschutz ascites tumour cells of repeated precipitation and resuspension of the microsomes.

The microsomal fraction at a protein concentration of 2.5 mg/ml was treated with 0.25 ml of 1 per cent (w/v) streptomycin sulphate/ml. The precipitate was resuspended in the original volume of buffer, either 0.1 M tris-HCl pH 8.0 or 0.01 M tris-HCl pH 8.0. Streptomycin sulphate and 0.1 M tris-HCl were removed by dialysis against 0.01 M tris-HCl pH 8.0 for 8 hours.

The reaction mixture contained 50 µmoles tris-HCl buffer pH 8.5, 5 µmoles each of $MgCl_2$ and 2-mercapto-ethanol. 0.5 µmoles versene, 0.1 µmoles ^{3}H UTP, 2 µmoles phosphocreatine, 50 µg phosphocreatine kinase, 100 µg RNA and 0.66 mg protein in a total volume of 0.5 ml.

Incubations were performed at 37° for 20 minutes.

Figure 31


A microsomal fraction resuspended in 0.01M tris-HCl pH 8.0
Untreated
1st precipitate
2nd "
3rd "
4th "
1st supernatant fraction
B microsomal fraction resuspended in 0.1M tris-HCl pH 8.0
Untreated
1st precipitate
2nd "
3rd "
4th "
1st supernatant fraction
0
100
200
300
400
500
μμmoles 3H UMP incorporated /mg. protein

particulate material is shown in Figure 31. Repeated washing of the microsomal fraction by precipitation with streptomycin sulphate and resuspension in buffer of 2 different ionic strengths failed to remove any incorporating activity from the microsomes, though there was an initial drop in activity no incorporating activity could be detected in the first supernatant fraction.

7. The Existence of Homopolyribonucleotides *in vivo*.

The foregoing results demonstrate the ability of a cytoplasmic enzyme to form limited amounts of relatively small homopolyribonucleotides which may be attached to the end of RNA chains. Attempts were made to identify homopolyribonucleotide sequences formed *in vivo* utilising the fact that any poly A sequences would be resistant to the action of pancreatic RNase. ^{14}C RNA was prepared (see Methods, section 8B), applied to a column of methylated serum albumin on kieselguhr and separated into sRNA and ribosomal RNA by gradient elution with saline phosphate buffer. The radioactivity and optical density profile was compared with the same amount of RNA treated at 37° for 45 minutes with 2 µg of pancreatic RNase which was similarly analysed on a column of methylated serum albumin on kieselguhr (see Methods, section 12). From the results shown in Figure 32

FIGURE 32

Analysis on a methylated serum albumin column of ^{14}C RNA prepared from Landschutz ascites tumour cells before and after treatment with RNase.

The preparation of MAK columns and the elution of RNA is given in the Methods, Section 12.

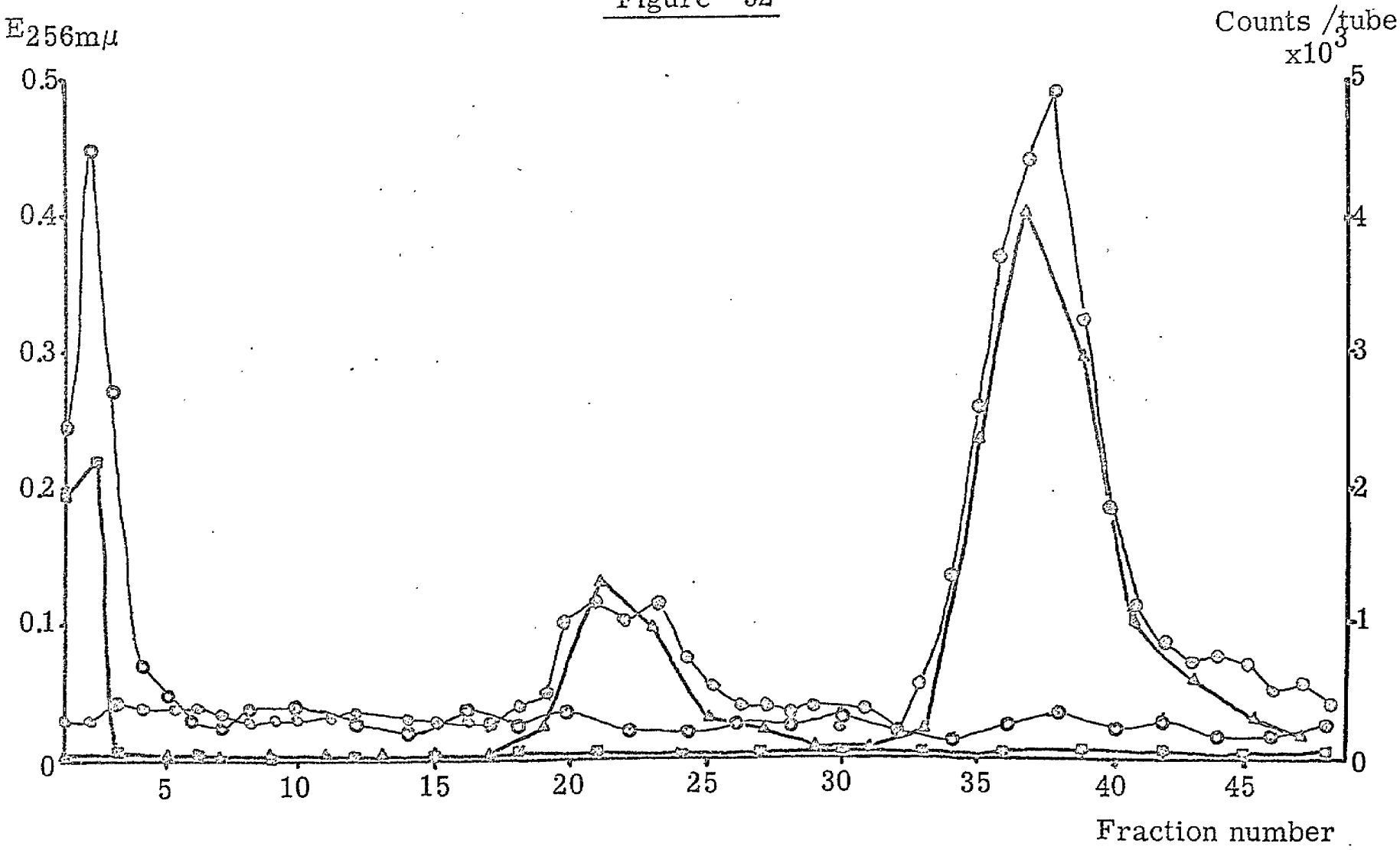
—●— Extinction of RNA

—○— Extinction of RNase treated sample

—▲— Radioactivity of RNA

—■— Radioactivity of RNase treated sample.

Figure 32


$E_{256m\mu}$
0.5
0.4
0.3
0.2
0.1
0
Counts /tube
x10^3
5
4
3
2
1
0
5
10
15
20
25
30
35
40
45
Fraction number

it appears that there are no RNase resistant sequences of poly A either in the 4S or ribosomal RNA regions. Any short sequences in the degradation products would be difficult to separate from naturally occurring poly A sequences in ribosomal RNA. This experiment excludes the in vivo formation of large poly A molecules though it is still possible that short sequences exist in the free state or attached to the ends of RNA chains.

8. Nuclease Activity of the Microsomal Fraction.

Nuclease activity was determined under the same conditions as those employed for the measurement of ribonucleotide incorporation into acid insoluble material except the ribonucleoside 5'-triphosphates were omitted (see Methods, section 13). Figure 33 shows the time course of nuclease activity. Considerable hydrolysis of added RNA occurs which is not inhibited by bentonite and in the absence of added RNA hydrolysis of endogenous microsomal RNA can occur though at a much slower rate than exogenous RNA. Figure 34 shows that p-nitrophenyl thymidine 5'-phosphate and p-nitrophenyl thymidine 5'-phosphate are effective substrates for the nuclease activity indicating that both 5' and 3' phosphodiesterase activity is present. Lack of inhibition by bentonite may mean that no endonuclease

FIGURE 33

Nuclease activity of a microsomal fraction of Landschutz ascites tumour cells with RNA as substrate.

The reaction mixture contained 50 μmoles tris-HCl buffer pH 8.5, 5 μmoles each of $MgCl_2$ and 2-mercapto-ethanol, 0.5 μmoles of versene, 2 μmoles phosphocreatine, 50 μg phosphocreatine kinase and 0.75 mg microsomal protein.

Where indicated 100 μg RNA and 20 μg bentonite were added in a total volume of 0.5 ml.

Incubations were performed at 37° for the times indicated.

—●— + RNA and bentonite
—■— + RNA
—○— No RNA added.

FIGURE 34

Nuclease activity of a microsomal fraction of Landschutz ascites tumour cells with p-nitrophenyl thymidine 3'-phosphate and p-nitrophenyl thymidine 5'-phosphate as substrates.

The reaction mixture was as above except 2.0 μmoles each of either p-nitrophenyl thymidine 5' or 3'-phosphate were added as substrate.

Incubations were performed at 37° for the times indicated.

—●— + p-nitrophenyl thymidine 5'-phosphate
—○— + p-nitrophenyl thymidine 3'-phosphate.

Figure 33

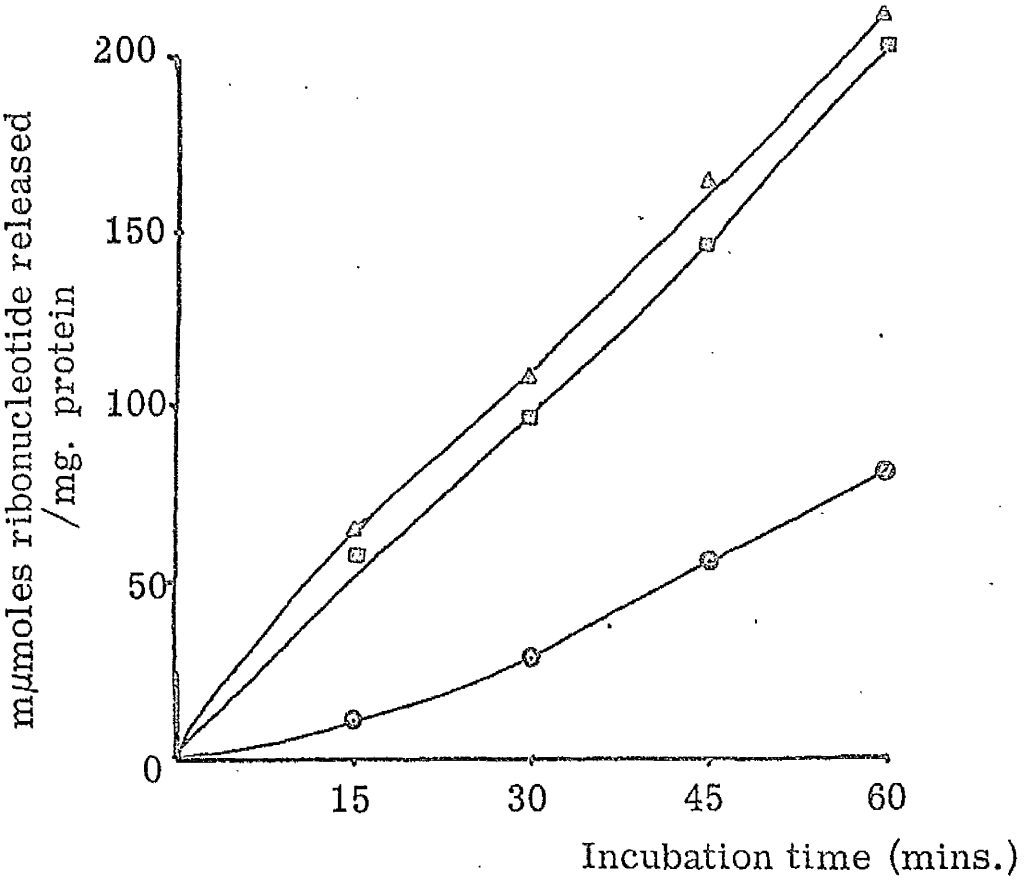
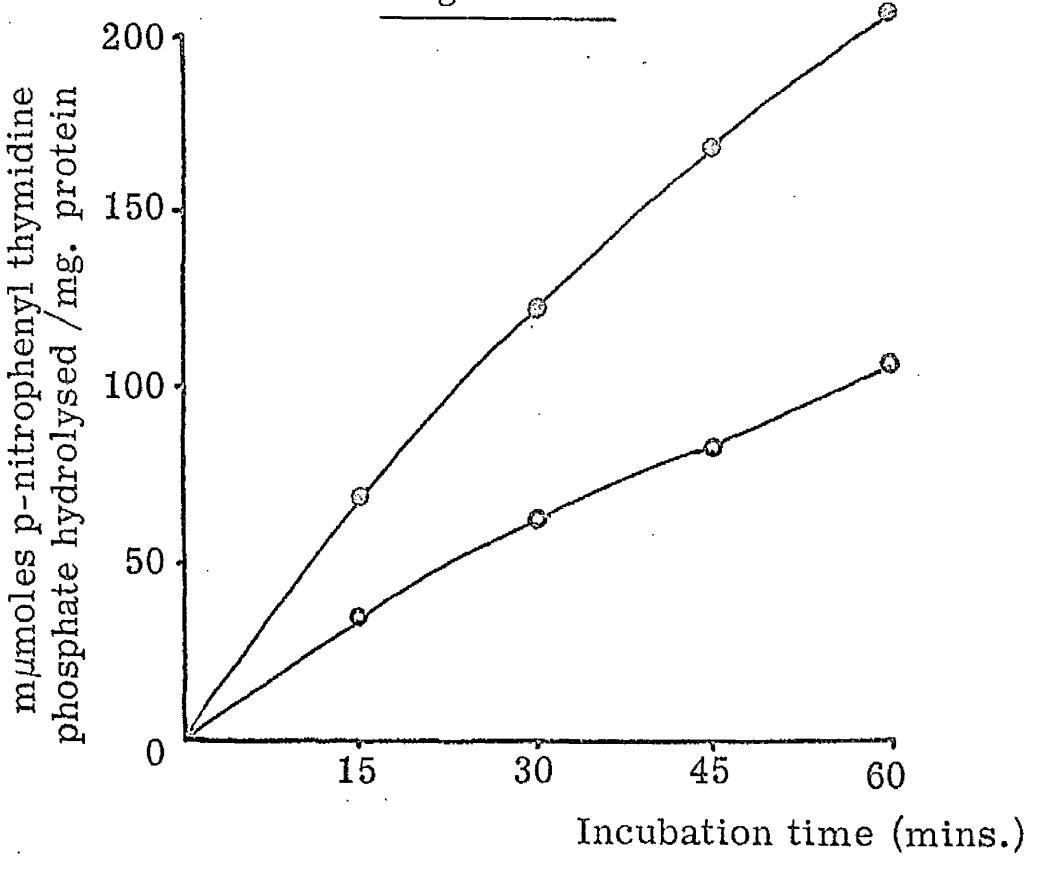


Figure 34



activity is present though bentonite may only be an effective inhibitor of soluble nucleases and not those that are particle bound. The results with p-nitrophenyl thymidine phosphates as substrates are in agreement with those of Razzell (1961a), who has identified a phosphodiesterase activity in the microsomes from rat liver and kidney. It is apparent from Figures 33 and 34 that the rate of breakdown of endogenous and exogenous RNA by these nucleases is very much higher than the maximum rate observed for ribonucleotide incorporation (Figure 12 and 14). The existence of such nuclease activity under the assay conditions employed for the incorporation reaction probably explains the low level of incorporation, the short chain lengths synthesised and the lack of labelling of either primer RNA or ribosomal RNA seen on sucrose density gradient analysis of the RNA extracted from reaction mixtures.

D I S C U S S I O N

DISCUSSION

1. The Characteristics of the Microsomal Enzyme.

A. The nature of the polyribonucleotides synthesised.

Initial experiments were designed to detect the incorporation of (α^{32}P) UTP into polyribonucleotides. The incorporation of (α^{32}P) UTP was at first observed to be stimulated by the presence of ATP, GTP and CTP. This effect was however abolished by an ATP regenerating system added to the reaction mixtures and in these circumstances the presence of ATP, GTP and CTP slightly inhibited the utilisation of (α^{32}P) UTP. These results could be related to the extent to which degradation of (α^{32}P) UTP to UDP and UMP occurred during incubation of the reaction mixtures. Whilst the addition of ATP, GTP and CTP to the reaction mixtures to some extent prevented the degradation of (α^{32}P) UTP this was much more effectively prevented by an ATP regenerating system (see Figure 13).

Alkaline hydrolysis of the reaction products after the incorporation of (α^{32}P) UTP followed by measurements of the distribution of radioactivity amongst the ribonucleoside 2'(3')-monophosphates shows that UMP residues

are mainly incorporated adjacent to UMP residues, whilst 25 - 30 per cent of the label appears in AMP, GMP and CMP. This pattern of labelling is largely unchanged when (α^{32}P) UTP alone, (α^{32}P) UTP plus CTP or (α^{32}P) UTP plus ATP, GTP and CTP are present in the reaction mixture. From such an experiment the following tentative conclusions may be drawn as to the nature of the product and the function of the primer. (1) Incorporation of UMP residues onto the ends of existing RNA chains may occur on which a further poly U sequence is synthesised. This occurs whether UTP alone or UTP plus ATP, GTP and CTP are present in the reaction mixture. (2) Separate poly U chains may be synthesised. Since the four ribonucleoside 5'-triphosphates have been shown to be incorporated under the same conditions (see Table 5) separate homopolyribonucleotide chains for each substrate may be formed. To account for the labelling of AMP, GMP and CMP residues when (α^{32}P) UTP, ATP, GTP and CTP are present in the reaction mixture, such residues would have to be incorporated into the poly U sequence. (3) Alternatively, some heteropolyribonucleotide chain synthesis may take place whilst most of the labelling in UMP residues is due to formation of poly U synthesised by a separate enzyme or perhaps a subunit of RNA polymerase. However,

in the case when (α^{32}P) UTP alone is present in the reaction mixture it would then be necessary to postulate the presence of sufficient endogenous ATP, GTP and CTP in the microsomal fraction to account for the observed labelling of AMP, GMP and CMP residues. (This also applies in (2) above.) (4) A combination of the above possibilities may occur.

The chain lengths of homopolyribonucleotide synthesised on the incorporation of ^{3}H ribonucleoside 5'-triphosphates were calculated from the ratio of labelled ribonucleosides to ribonucleoside 2'(3')-monophosphates. The results, (see Table 7) show that the poly U chain length is roughly halved when ATP, GTP and CTP are added to the reaction mixture. This tends to eliminate possibility (2) discussed above.

It is known that the highly purified bacterial DNA primed RNA polymerases exhibit homopolyribonucleotide synthesis at a higher substrate concentration than is required for synthesis of heteropolyribonucleotides. (Fox and Weiss, 1964). In one experiment (see Figure 16) the incorporation of ^{3}H ATP alone at increasing concentrations and in the presence of GTP, CTP and UTP at similar increasing concentrations was investigated. GTP, CTP and UTP inhibited the incorporation of ^{3}H ATP

at all concentrations tested. This experiment tends to eliminate possibility (3) discussed above.

The microsomal enzyme shows lack of stimulation of incorporation of one labelled ribonucleoside 5'-triphosphate by the presence of the other three and in the case of the incorporation of ^{3}H ATP lack of stimulation at any substrate concentration. Such considerations together with the short chain lengths of product found to be synthesised and the lack of randomisation of label from ($\alpha^{32}P$) UTP in the presence of ATP, GTP and CTP make the possibility of heteropolyribonucleotide synthesis by the microsomal fraction extremely unlikely. From the experiment on the distribution of radioactivity amongst the ribonucleoside 2'(3')-monophosphates it is reasonable to conclude UMP sequences are being incorporated into existing RNA chains.

Since the microsomal fraction contains 40 - 50 per cent of endogenous RNA no absolute requirement for RNA or any other polyribonucleotide as primer could be directly demonstrated. However, stimulation of the incorporation of ($\alpha^{32}P$) UTP by RNA and not by DNA, inhibition by RNase and not by DNase or actinomycin D indicates RNA rather than DNA can act as a primer. To obtain information about the role of RNA as a primer in

the incorporation of ribonucleoside 5'-triphosphates labelled RNA was extracted from the reaction mixtures and analysed by sucrose density gradient centrifugation. The results (Figures 18, 19, 20) show no labelling of primer or ribosomal RNA occurs. The position of labelling of RNA in the gradient indicates synthesis of oligoribonucleotides or labelling of sRNA. Yeast sRNA has been shown to stimulate the incorporation of ^{3}H CTP but not ^{3}H UTP. This indicates some contribution to the labelling of the extracted RNA due to synthesis of the pCpCpA terminal sequence of sRNA when ^{3}H ATP and ^{3}H CTP are present in the reaction mixtures. It seems unlikely, however, the incorporation is entirely due to incorporation into sRNA. Determination of the chain lengths of the homopolyribonucleotide products of the reaction shows that short sequences are synthesised. This is in agreement with the position of labelled material seen in sucrose density gradients and suggests synthesis of separate oligoribonucleotide chains. Such an interpretation disagrees with that suggested by the distribution of ^{32}P amongst the ribonucleoside 2'(3')-monophosphates obtained by alkaline hydrolysis of the product RNA which indicates attachment of ribonucleoside monophosphate residues to primer RNA chains. The latter conclusion

is supported by the results shown in Figure 21. The coincidence of the remaining ^{14}C primer RNA and ^{3}H AMP residues incorporated in RNA extracted from reaction mixtures containing ^{14}C highly polymerised ascites RNA and ^{3}H ATP indicate labelling of the ^{14}C primer RNA. However, the remaining ^{14}C RNA could be sRNA, possibly labelled with ^{3}H AMP residues or small oligonucleotides undegraded by nucleases and not necessarily labelled with ^{3}H AMP residues.

The RNA extracted from the incubated reaction mixtures shows no increase in ultraviolet absorbing material in the 4S region on analysis by sucrose density gradient centrifugation. The amount of RNA extracted from the reaction mixtures is considerably less in the incubated samples (Figures 18 and 19) than in zero time controls. Such observations indicate little or no degradation of the RNA by an endonuclease present in the microsomal fraction. The loss of RNA in the incubated reaction mixtures can be accounted for by the presence of a phosphodiesterase in the microsomal fraction. 3' and 5' phosphodiesterases have been shown to be present by direct assay with p-nitrophenyl thymidine 3'-phosphate and p-nitrophenyl thymidine 5'-phosphate as substrates.

If there is terminal labelling of RNA it might be

expected that the ribosomal RNA peaks should be labelled, especially in the case when no primer RNA is added. The ribosomal protein presumably protects ribosomal RNA from degradation by the phosphodiesterases. Assuming that the incorporation of ribonucleoside 5'-monophosphate residues occurs on the ends of existing RNA chains it seems reasonable to suppose that association of the incorporating enzyme with the ends of the primer RNA chain would prevent simultaneous attack by a 5' phosphodiesterase. In order to account for the results obtained a 3' phosphodiesterase, working from the other end of the same chain would have to remove at least 98 per cent of the chain. This is unlikely as the ribosomal RNA will be associated with protein and in any case is not completely degraded after incubation for one hour in the reaction mixture. However, the amount of degradation by the 5' phosphodiesterase may considerably exceed the amount of synthesis by the incorporating enzyme and may be able efficiently to remove newly synthesised sequences from the ribosomal RNA chains. This would account for lack of labelling of the 30S and 18S ribosomal RNA peaks but not for the appearance of radioactivity between the 4S region and the meniscus of the sucrose density gradients. The phosphodiesterase may have limited

endonucleolytic activity in the absence of a separate endonuclease and may remove the newly synthesised sequence with a few residues of the primer RNA chain, though there is no evidence for this alternative. Analysis of RNA extracted from reaction mixtures by sucrose density gradient centrifugation indicates synthesis of separate oligonucleotide chains. On the other hand, the distribution of ^{32}P amongst the ribonucleoside 2'(3')-monophosphates obtained on alkaline hydrolysis of the product RNA indicates incorporation of UMP residues onto existing RNA chains.

In view of the short chain lengths of homopolyribonucleotides formed in the reaction it is possible that treatment of the reaction mixtures with acid does not bring about complete precipitation of the oligoribonucleotides from the supernatant fraction. Complete precipitation would be expected, however, if the nucleotide incorporated is attached to existing RNA chains in the reaction mixture. Details of the minimum chain lengths of oligoribonucleotides insoluble in acid are not known. Chains of 5 - 10 residues may be expected to be insoluble in trichloroacetic acid depending on their ribonucleoside composition. The presence of protein and RNA in the reaction mixtures may be expected to coprecipitate

short oligonucleotides though there may not be sufficient coprecipitating material to absorb oligoribonucleotides completely. It is possible therefore that only a proportion of the product may be measured and that the actual amount of polyribonucleotide synthesis is greater than that observed.

B. The Intracellular Location of the Enzyme.

Fractionation of disrupted cell homogenates by differential centrifugation suggests that the incorporation of ribonucleoside 5'-triphosphates is almost entirely located in the microsomal fraction. Virtually no activity could be detected in broken nuclei, the mitochondria and the 105,000 x g supernatant fraction. Nuclease activity and reaction conditions designed to detect a RNA dependent RNA polymerase presumably prevents the detection of the DNA dependent RNA polymerase known to be present in the nucleus. (Mason and Smellie, 1965). Nucleases may also prevent detection of ribonucleotide incorporation into polyribonucleotides in the 105,000 x g supernatant fraction especially as further centrifugation of this fraction at 150,000 x g produces a sediment which is about one third as active as the microsomal fraction. This also indicates the particulate nature of the incorporating enzyme which is supported by experiments

involving centrifugation of the microsomal fraction in sucrose density gradients and precipitation of the microsomal fraction with streptomycin sulphate, (see Results, section 6). It might be expected that the mitochondrial fraction would show some incorporation of ribonucleotides into polyribonucleotides as a DNA dependent RNA polymerase has been identified in the mitochondrial fraction of N. crassa. (Luck and Reich, 1964). No reaction was detected in initial experiments with a mitochondrial fraction from ascites tumour cells, possibly because of their ATPase activity or lack of permeability of the ribonucleoside 5'—triphosphates to the mitochondrial membranes. The reaction conditions employed are not, however, appropriate for the detection of a DNA primed RNA polymerase which in any case may not be present in the mitochondria from Landschutz ascites tumour cells.

Although the microsomal fraction appears to be the only cytoplasmic subfraction incorporating ribonucleotides into polyribonucleotides, there is a possibility that disruption of the ascites cells in five volumes of dilute buffer solution causes a redistribution of protein between the cell sap and various particulate components of the cell. This may also be complicated by leakage

of components out of the nucleus due to a change in the environment of the nuclear membrane and any contribution from broken nuclei or nuclear components of cells undergoing mitosis. Nuclear contamination of the cytoplasm by such effects is likely to be small. Nevertheless, experiments on the location of enzymes involved in incorporating ribonucleotides into polyribonucleotides are at the best a description of their location in cell homogenates.

2. Comparison of the Microsomal Enzyme with Other Enzymes of Polynucleotide Biosynthesis.

The microsomal system appears to require ribonucleoside 5'-triphosphates as substrates like all other systems synthesising polyribonucleotides except polynucleotide phosphorylase which requires ribonucleoside 5'-diphosphates. Presumably phosphodiester bond formation proceeds by nucleophilic attack of the 3'-hydroxyl of a nucleoside monophosphate residue at the growing end of a newly synthesised chain on the phosphate group of an incoming ribonucleoside 5'-triphosphate with the elimination of a pyrophosphate group. Such a reaction mechanism has been postulated for phosphodiester bond formation catalysed by the DNA

polymerase (Kornberg,1959) for which there is evidence that free 3'-hydroxyl groups are required. By analogy, phosphodiester bond formation catalysed by DNA primed RNA polymerases, RNA primed RNA polymerases and homopolyribonucleotide forming systems may proceed by a similar mechanism, though there is no direct evidence for this.

The microsomal enzyme shows some similarities to polyribonucleotide biosynthesis by DNA dependent RNA polymerases or RNA dependent RNA polymerases. The former have been identified in mammalian tissues (Ro and Busch, 1964; Eason and Smellie, 1965) and purified from bacterial sources (Krakow and Ochoa, 1963; Nakamoto, Fox and Weiss, 1964), while the latter have been partially purified from cells infected with RNA viruses. (Haruna et al, 1963; Weissmann et al, 1964). The incorporation of the four ribonucleoside 5'-triphosphates by these enzymes requires the presence of Mg^{2+} or Mn^{2+} ions in the reaction mixture. Usually a mixture of Mg^{2+} and Mn^{2+} or Mn^{2+} ions alone are used for assay of the DNA dependent RNA polymerase, though some RNA dependent RNA polymerases are inhibited by Mn^{2+} and specifically require Mg^{2+} ions. (Cline, Eason and Smellie, 1963; Baltimore and Franklin, 1963). In general, the pH optima are variable, probably being influenced by the degree of contamination by

interfering enzymes but are usually about pH 7.5 for purified enzymes. The DNA dependent RNA polymerase is inhibited by DNase, actinomycin D and RNase, while the viral RNA dependent RNA polymerases are inhibited by RNase and not by DNase or actinomycin D. Polyamines, particularly spermine or spermidine stimulate the incorporation of the four ribonucleoside 5'-triphosphates into RNA with both types of enzyme.

Normally the DNA dependent RNA polymerase shows a requirement for double stranded DNA which is a better primer than heated DNA. Polyribonucleotides will also act as primers for the enzymes purified from M. lysodeiktieus and A. vinelandii, the complementary base or bases to the primer being incorporated. (Nakamoto and Weiss, 1962; Krakow and Ochoa, 1963). There is no evidence that the viral RNA dependent RNA polymerases can utilise DNA as a template. Single stranded RNA is first converted to a double stranded form with the purified RNA dependent RNA polymerases which then acts as a template for the production of single stranded RNA. (Weissmann et al, 1964). Simultaneous incorporation of the four ribonucleoside 5'-triphosphates is required for heteropolyribonucleotide synthesis and omission of any one of these reduces the incorporation to virtually zero. (Furth et al, 1962;

Haruna et al, 1963). Alkaline hydrolysis of the product RNA shows ^{32}P from ($\alpha^{32}P$) ribonucleoside 5'-triphosphate to be distributed roughly equally amongst the ribonucleoside 2'(3')-monophosphates reflecting the base composition of the DNA or RNA used as a template. (Hurwitz et al, 1962a). In these respects the DNA dependent RNA polymerases differ markedly from the microsomal enzyme which, although it incorporates the four ribonucleoside 5'-triphosphates, only appears to catalyse the synthesis of limited homopolyribonucleotide sequences and not the net synthesis of RNA. Hence the microsomal enzyme is better compared with other homopolyribonucleotide forming systems.

A system from homogenates of chick embryos incorporates ATP into poly A. (Chung, Mahler and Enrione, 1960). An enzyme having similar activity has been partially purified from a particle free preparation of chorio-allantoic membranes of eleven day old chick embryos. (Venkataraman and Mahler, 1963). Poly A sequences 8 - 10 residues long are produced by this enzyme. An enzyme synthesising poly A has been purified from extracts of calf thymus nuclei and appears specifically to require a poly A primer. (Edmonds and Abrams, 1960, 1962). The incorporation of GTP into poly G sequences has been

shown to be catalysed by a separate enzyme obtained from the same source. (Abrams, Edmonds and Biswas, 1962). A more highly purified enzyme forming poly A sequences has been obtained from E. coli. (August, Ortiz and Hurwitz, 1962). In this case separate chains of poly A containing 100 - 200 AMP residues appear to be synthesised. Incorporation of UMP residues into existing polyribonucleotide chains has been detected in the nuclei of Landschutz ascites tumour cells (Smellie, 1962) and nuclear ribosomes from the same source catalyse the incorporation of AMP and UMP residues into ribosomal RNA. (Burdon, 1963a).

Enzymes incorporating UMP residues into poly U sequences have been found in the microsomal fraction of pigeon liver. (Straus and Goldwasser, 1961). An enzyme fraction has been partially purified from a pH5 precipitate of a supernatant fraction obtained by centrifugation of rat liver homogenates at 105,000 x g for one hour. The partially purified enzyme incorporates UMP residues into poly U in the presence of Mg^{2+} ions and AMP residues into poly A in the presence of Mn^{2+} ions. (Klemperer, 1963a, b). The 105,000 x g supernatant fractions used by Klemperer (1963a) and by Burdon and Smellie (1962) from Landschutz ascites tumour cells

in experiments on the incorporation of (α^{32}P) UTP into polyribonucleotides may both contain considerable amounts of microsomal material. The fraction from ascites tumour cells gave an incorporation in terms of mμ moles of UTP per mg protein comparable to the present microsomal enzyme even in the absence of an ATP regenerating system. Possibly centrifugation at 105,000 x g for one hour removed the bulk of the ATPase and phosphodiesterase activity.

The enzymes described above are usually specific for the incorporation of single ribonucleoside 5'—triphosphates into existing primer RNA chains. In general, longer homopolyribonucleotide sequences appear to be synthesised as the enzymes are more highly purified. Variable inhibition of incorporation of the specific ribonucleoside 5'—triphosphate occurs in the presence of the other complementary ribonucleoside 5'—triphosphates. Mg^{2+} ions are usually required and in some cases spermidine inhibits the incorporation. (Strauss and Goldwasser, 1961). There is little specificity for the primer RNA though the extent of incorporation often varies considerably with synthetic polyribonucleotides and RNA from different sources. Oligoribonucleotides with free 3'-hydroxyl groups can act as primers for the incorporation

of AMP residues by a rat liver enzyme. Tri and tetra-oligonucleotides are the most effective primers. (Klemperer, 1964). This is in contrast to the specific requirement for sRNA by an enzyme purified from the cell sap and catalysing the formation of the pCpCpA terminal sequence of these molecules. (Hecht, Zamecnik, Stephenson and Scott, 1958; Preiss, Dieckmann and Berg, 1961).

The microsomal system differs from systems of the above type in that the four ribonucleoside 5'-triphosphates are independently incorporated into homopolyribonucleotide products, though of short chain length. It is possible that the oligoribonucleotide chains which are formed are separate from the primer or template. Other crude systems may also incorporate the four ribonucleoside 5'-triphosphates but such an activity may be lost with partially purified enzymes which show a specific substrate requirement for a single ribonucleoside 5'-triphosphate. The RNA requirement of the microsomal system differentiates it from systems synthesising homopolyribonucleotide requiring DNA as a primer. The highly purified bacterial DNA dependent RNA polymerases catalyse the formation of separate homopolyribonucleotide chains at a higher substrate concentration than required for replicative synthesis of a DNA or RNA template. This reaction is

primed by RNA and heated DNA is a better primer than native DNA. Mn^{2+} ions are required and the reaction is inhibited by the other complementary ribonucleoside 5'-triphosphates and spermidine. (Fox and Weiss, 1964; Stevens, 1964). Mammalian systems catalysing the DNA dependent incorporation of AMP and UMP residues into homopolyribonucleotides have been described by Chambon et al (1963) in hen liver and by Burdon (1963b) in nuclei from Landschutz ascites tumour cells. Chains of about 100 residues are formed with both systems which are probably separate from the primer DNA. Heated DNA is a more effective primer than native DNA for the incorporation of UTP and this reaction is also inhibited by the presence of complementary ribonucleoside 5'-triphosphates. (Burdon, 1963b). All four ribonucleoside 5'-triphosphates are incorporated onto the ends of DNA chains by an enzyme described by Krakow et al (1961). One ribonucleoside residue is added to the ends of DNA chains.

3. Reactions Interfering with the Microsomal Incorporation of Ribonucleoside 5'-Triphosphates.

Enzymes present in the microsomal fraction that could interfere with the incorporation of ribonucleoside

5'-triphosphates are of two types, (a) other DNA or RNA polymerases, (b) enzymes acting on the substrates or products of the reaction. In the former group DNA polymerase is known to be a soluble enzyme which occurs in the cytoplasm (Keir, Smellie and Siebert, 1962) and it could conceivably be absorbed to the microsomal fraction. The specificity of substrate and template requirements for this enzyme indicate it is not affecting the incorporation of ribonucleoside 5'-triphosphates by the microsomal system. The DNA dependent RNA polymerase is probably not present as this enzyme is more likely to be entirely located in the nucleus. Lack of priming by DNA and lack of inhibition by DNase and actinomycin D indicate that it is not contributing to the microsomal incorporation. An RNA dependent RNA polymerase of the viral type can be eliminated as this enzyme requires the presence of four ribonucleoside 5'-triphosphates to replicate an RNA template whereas the microsomal enzyme synthesises homopolyribonucleotide sequences. Furthermore, a comparison of ribonucleotide incorporation in uninfected and infected cells shows the RNA dependent RNA polymerase activity to appear predominantly in a large particle fraction of cells infected with an RNA virus. (Eason, Cline and Smellie, 1963; Baltimore and

Franklin, 1963). Polynucleotide phosphorylase has never been conclusively demonstrated in mammalian cells. In any case stimulation of ribonucleoside 5'—triphosphate incorporation by an ATP regenerating system, lack of inhibition by inorganic orthophosphate and a slight inhibition by inorganic pyrophosphate indicate ribonucleoside 5'—triphosphates and not diphosphates are the true substrates for the microsomal system. Some contamination of the microsomal preparation with the sRNA terminal sequence synthesising enzyme is indicated by stimulation of the incorporation of ^{3}H CTP by sRNA compared with the lack of stimulation of the incorporation of ^{3}H UTP. Presumably sRNA would also stimulate the incorporation of ^{3}H ATP by the microsomal system. The presence of such an enzyme activity associated with the microsomal system should not seriously affect the results of the incorporation of ^{3}H ATP or ^{3}H CTP as only trace amounts of sRNA are present in the microsomal preparations and the primer RNA used in routine assays.

Amongst the enzymes acting on the substrates or products of the reaction ATPase (EC 3.6.1.3) is certainly present in the microsomal fraction. ($\alpha^{32}P$) UTP present in the reaction mixture was shown to be considerably degraded during incubation unless an ATP regenerating

system was included in the reaction mixture. Enzymes may also be present which degrade ribonucleoside residues but these presumably act on the ribonucleoside or the ribonucleoside 5'-monophosphate as would enzymes catalysing conversions between each of the purine or pyrimidine nucleosides. In the presence of an ATP regenerating system the effects of such enzymes would be expected to be negligible. Examination of the release of acid soluble nucleotides in the absence of added substrates shows considerable hydrolysis of ribosomal RNA and added primer RNA. Lack of degradation of ribosomal RNA extracted from reaction mixtures incubated for one hour and analysed by sucrose density centrifugation suggests the presence of an exonuclease in the microsomal system. Direct assay with p-nitrophenyl thymidine 3'-phosphate and with p-nitrophenyl thymidine 5'-phosphate as substrates confirms the presence of both 3' and 5' phosphodiesterases. Such an activity may seriously limit the incorporation of ribonucleotides observed, account for the short chain lengths in the product and the lack of labelling of ribosomal RNA assuming incorporation of ribonucleotides into existing RNA chains as has already been discussed in section 1A.

4. Significance of the Microsomal Incorporation of Ribonucleotides into Homopolyribonucleotides.

If homopolyribonucleotide sequences are synthesised *in vivo* they are likely to be of a limited nature. The experiment involving RNAse treatment of ^{14}C RNA prepared *in vivo* in Landschutz ascites tumour cells excludes the possibility that large sequences of poly A exist *in vivo*. Such an experiment does not exclude limited homopolyribonucleotide sequence formation either of new chains or involving extension of existing RNA chains.

The microsomal location of the synthetic activity suggests an involvement in protein synthesis. Slight differences between the sedimentation coefficients of newly formed ribosomes and mature ribosomes have been detected. (Kono, Otaka and Osawa, 1964). Such differences could perhaps be accounted for by limited synthesis of homopolyribonucleotide sequences onto the ends of existing ribosomal RNA chains by the microsomal enzyme detected *in vitro*. Such sequences may be necessary to promote adequate binding of messenger RNA or sRNA molecules to the ribosomes making them functional in protein synthesis.

It is known that the secondary structure of polyribonucleotides determines its affinity for the ribosomes

(Okamota and Takamora, 1963a, b) which in *in vitro* systems affects the ability of a given polyribonucleotide to code for various amino acids. (Singer, Jones and Nirenberg, 1963). Limited homopolyribonucleotide synthesis onto the ends of natural messenger RNA molecules may be necessary to reduce the helical content of the ends of the chain and thus facilitate interaction of the messenger RNA with a ribosome. Such terminal sequences may interfere with the coding properties of the messenger RNA and the sequence would have to be such that it does not code for any amino acid. However, poly U, poly A and poly C code for phenylalanine, lysine and proline respectively. Only poly G appears not to correspond to a coding triplet and this on its own has considerable helical secondary structure though limited sequences at the ends of messenger RNA molecules may not. There is no evidence from *in vitro* systems that any incorporation of ribonucleoside triphosphates is required for protein synthesis except in crude systems. GTP, as far as is at present known appears to be involved in the formation of peptide bonds on the ribosomal surface and not in any limited poly G synthesis at the ends of messenger RNA molecules. The above suggestions also tend to be excluded as no labelling of ribosomal

or primer RNA could be detected by sucrose density gradient centrifugation in RNA extracted from reaction mixtures following incorporation of ribonucleotide 5'-triphosphates.

If limited homopolyribonucleotide synthesis of new chains occurs *in vivo*, rather than being involved in protein synthesis these may be a product of the enzymic activity of newly synthesised subunits of a DNA dependent RNA polymerase each of which is responsible for catalysing the formation of phosphodiester bonds between specific ribonucleoside 5'-triphosphates and the 3'-hydroxyl of the growing point of the RNA chain. Even though the subunit nature of *E. coli* DNA dependent RNA polymerase has been demonstrated (Fuchs *et al*, 1964) the individual enzymic activities of the subunits would be expected to be primed by DNA. This is not the case with the ascites tumour cell microsomal enzyme. An alternative possibility is that the activity is due to subunits of a viral RNA dependent RNA polymerase. This could be due to latent virus infection or partially inhibited viral replication or as a result of the induction of the ascites tumour by a virus which is incorporated into the cell genome and replicated with it. Partial expression of the viral genome may produce a RNA dependent RNA polymerase

modified in such a way that the subunits do not interact to give an RNA polymerase of the correct configuration. There is evidence that virus infection can induce neoplasia in normal cells and in some types of neoplasm the transformed cells continue to release virus particles while in others no further virus particles can be detected (see Dulbecco, 1963, for a review).

The remaining possibility is that the incorporation of ribonucleotides into polyribonucleotides is due to a non-enzymic reaction catalysed in a non-specific way by the ribosomal nucleoprotein or to a synthetic reaction catalysed by nucleases. Dialysable oligonucleotides from DNA digests are repolymerised to material having sedimentation coefficients up to 4S in the presence of Ecteola-cellulose. (Bendich, Rosenkrans and Fitt, 1961). Incubation at room temperature for seven days was required. The time course of incorporation of ribonucleoside 5'-triphosphates would argue against such a mechanism for the ascites tumour microsomal system.

Pancreatic ribonuclease and takadiastase have been found to catalyse the synthesis of oligoribonucleotides from ribonucleoside 2'(3')-monophosphates. (Heppel, Whitfeld and Markham, 1955; Sato-Asano and Egami, 1958). Polymer formation is favoured by low

temperatures (2^{o}) and high substrate concentrations.

There is a possibility that the incorporation of ribonucleotides into homopolyribonucleotides catalysed by the microsomal fraction is due to reversal of phosphodiesterase activity also found to be associated with this fraction. Similar phosphodiesterases appear in the microsomal fraction of rat liver and kidney. Drastic procedures are required to separate the enzyme from the microsomal fraction of hog kidney indicating that it is firmly bound to particulate material. (Razzell, 1961a, b). Under conditions where spleen phosphodiesterase is capable of bringing about almost complete hydrolysis of p-nitrophenyl thymidine 5'-phosphate it is also capable of synthesising di, tri and tetra-thymidine oligonucleotides. (Razzell and Khorana, 1961). The phosphodiesterase activity in the microsomal fraction from ascites tumour cells appears to be at least 100 times greater than the maximum activity of the fraction incorporating ribonucleoside 5'-triphosphates into homopolyribonucleotides. The substrate concentrations are not, however, comparable, approximately saturating concentrations of substrate appear to have been used for measuring the incorporation of ribonucleotides (see Figure 16) but twenty times the amount of p-nitrophenyl

thymidine 5'-phosphate was used for measurement of 5' phosphodiesterase activity.

Reversal of phosphodiesterase activity would account for synthesis of new oligonucleotide chains as is suggested by analysis of RNA extracted from the reaction mixture by sucrose density gradient centrifugation and determination of the chain lengths of the products. If it is assumed that ribonucleoside 5'-monophosphates from the hydrolysis of RNA by the 5'- phosphodiesterase can initiate the reverse reaction this would explain stimulation of ribonucleoside 5'-triphosphate incorporation by RNA and appearance of ^{32}P label after ($\alpha^{32}P$) UTP incorporation amongst the complementary ribonucleoside 2'(3')-monophosphates obtained on alkaline hydrolysis of RNA products in the reaction mixtures. It is then necessary to postulate that the reverse reaction only synthesises phosphodiester bonds at a significant rate between the same ribonucleoside 5'-triphosphates as substrates. RNA hydrolysis can however occur between all sixteen possible dinucleotide pairs though individual rates of hydrolysis of such pairs may vary considerably. Furthermore, the incorporation of TMP residues into poly T occurs to less than one twentieth the extent of the incorporation of UMP residues into poly U even though

p-nitrophenyl thymidine 5'-phosphate is readily hydrolysed by the microsomal fraction. Such considerations indicate a reversal of 5' phosphodiesterase activity is not involved in the synthetic reaction. This is also indicated by a requirement for ribonucleoside 5'-triphosphates for the incorporation reaction whereas ribonucleoside 5'-monophosphates would be required for reversal of the hydrolytic reaction. There is evidence that snake venom phosphodiesterase shows ATP pyrophosphatase activity hydrolysing ATP to AMP and pyrophosphate but not hydrolysing ADP. (Doman, 1959; Razzell and Khorana, 1959). In this way sufficient ribonucleoside monophosphates may be provided in proximity to the active site of the phosphodiesterase to cause reversal of the hydrolytic reaction. Conceivably the synthetic reaction may not be a true reversal of the hydrolytic reaction and may require ribonucleoside 5'-triphosphates as substrates. The elimination of pyrophosphate by nucleophilic attack of the 3'-hydroxyl of one ribonucleotide on the α-phosphate group of another ribonucleoside 5'-triphosphate may occur more readily than the elimination of water between two molecules of ribonucleoside 5'-monophosphate.

There is no definite evidence that the synthetic

reaction is catalysed by the phosphodiesterase though such a possibility cannot be entirely ruled out. The enzyme catalysing the incorporation or ribonucleoside 5'—triphosphates into homopolyribonucleotides is therefore separate from the 5' phosphodiesterase, though the latter enzyme may be expected to reduce the chain lengths of the homopolyribonucleotides synthesised and possibly account for the lack of labelling of ribosomal or primer RNA.

5. Conclusions.

Attempts made with *in vitro* systems to show RNA synthesis, i.e. transcription of the base sequences in a template RNA molecule involving simultaneous incorporation of the four ribonucleoside 5'—triphosphates catalysed by cytoplasmic fractions proved negative. This is in agreement with *in vivo* experiments on RNA synthesis in which evidence from several types of experiment:- the kinetics of nucleotide incorporation, followed autoradiographically or by cell fractionation, "pulse chase" experiments in the presence and absence of actinomycin D, nuclear transplantation and removal experiments both surgically, and as observed naturally in erythroblast maturation, all indicate lack of

cytoplasmic RNA synthesis. Thus it would appear that all cellular RNA, messenger, ribosomal and sRNA is synthesised in the nucleus on a DNA template. Hence the ribosomes would not appear to be self replicating particles occurring in the cytoplasm and the possibility is eliminated that transcription of DNA templates produces only a limited number of copies of RNA, which are then further replicated in the cytoplasm to give an adequate rate of protein synthesis. A possible exception to this scheme is that mitochondria may be organelles which can self-replicate independently of nuclear division in the cytoplasm. There is evidence that mitochondria contain DNA which could specify sufficient messenger RNA for the synthesis of all mitochondrial proteins.

However, the results of the present experiments and other investigators do show that cytoplasmic fractions are capable of limited homopolyribonucleotide synthesis *in vitro* besides catalysing the synthesis of terminal sRNA sequences. There is no obvious requirement for such a system to function *in vivo* nor have homopolyribonucleotides existing either in the free state or attached to existing RNA chains been detected *in vivo*. The possibility is discussed that the incorporation of ribonucleotides *in vitro* is due to reversal of phosphod-

iesterase activity which may be precluded *in vivo* by different ionic conditions and substrate concentrations.

S U M M A R Y

SUMMARY

1. Experiments were performed *in vitro* to detect enzymes catalysing the net synthesis of RNA in the cytoplasm of Landschutz ascites tumour cells. Enzyme fractions were obtained by differential centrifugation of disrupted cell homogenates. The incorporation of (α^{32}P) UTP into acid insoluble material in a system containing RNA, bentonite ATP, GTP and CTP was found to occur mainly in the microsomal fraction. An acetone powder of the microsomes was prepared and its properties further investigated.

2. ATP, GTP and CTP were shown to stimulate (α^{32}P) UTP incorporation but this effect was abolished by an ATP regenerating system.

3. The incorporation of (α^{32}P) UTP showed a pH optimum of 8.5, required RNA but not DNA as a primer, was inhibited by RNase but not DNase or actinomycin D and showed an absolute requirement for Mg^{2+} ions.

4. Alkaline hydrolysis of the reaction products after (α^{32}P) UTP incorporation and determination of the distribution of radioactivity amongst the ribonucleoside 2'(3')-monophosphates showed UMP to be mainly labelled. This was so whether (α^{32}P) UTP, (α^{32}P) UTP plus CTP or (α^{32}P) UTP plus ATP, GTP and CTP were included in the

reaction mixtures. Hence homopolyribonucleotide synthesis was indicated.

5. Time curves were obtained for the incorporation of (α^{32}P) UTP alone and (α^{32}P) UTP plus ATP, GTP and CTP in the presence and absence of an ATP regenerating system. The stimulation of the incorporation by the ATP regenerating system could be related to the extent of (α^{32}P) UTP degradation.

6. Versene and 2-mercaptoethanol were found to stimula the incorporation of (α^{32}P) UTP. These reagents were henceforth routinely included in the reaction mixture. Mn^{2+} ions, spermine and putrescine inhibited the incorporation of (α^{32}P) UTP and KCl slightly inhibited the incorporation.

7. Homopolyribonucleotide synthesis was further investigated using ^{3}H ribonucleoside 5'-triphosphates. ^{3}H ATP, ^{3}H GTP and ^{3}H CTP were incorporated under essentially the same conditions as (α^{32}P) UTP.

8. The incorporation of ^{3}H ATP at increasing substrate concentrations was inhibited by equal concentrations of GTP, CTP and UTP over the range of concentrations tested.

9. Determination of the average chain lengths of the homopolyribonucleotides showed the main products were oligoribonucleotides of short chain length.

10. Attempts were made to show the role of primer RNA in homopolyribonucleotide synthesis. Extraction of RNA from reaction mixtures and analysis by sucrose density gradient centrifugation showed labelled material only between the meniscus and 4S region of the gradient and no labelling of the ribosomal RNA peaks.

11. Comparison of the priming ability of sRNA, commercial yeast RNA and ascites tumour whole cell RNA showed the latter two to be equally effective primers for the incorporation of ^{3}H UTP whereas sRNA was not a primer. sRNA, however, stimulated the incorporation of ^{3}H UTP.

12. The synthetic reaction catalysed by the microsomal fraction is specific for ribonucleoside 5'-triphosphates. ^{3}H dATP and ($\alpha^{32}P$) TTP were incorporated to less than one twentieth of the extent of the corresponding ribonucleoside 5' triphosphates. A requirement for triphosphates as the substrates is shown by inhibition of the incorporation of ($\alpha^{32}P$) UTP by inorganic pyrophosphate ions and not by orthophosphate ions and stimulation of the incorporation of ribonucleoside 5'-triphosphates by an ATP regenerating system.

13. The microsomal nature of the incorporating enzyme was reinvestigated. Enzyme fractions obtained by differential centrifugation of disrupted cell homogenates

were assayed under the reaction conditions used to detect homopolyribonucleotide synthesis. The microsomal fraction was again shown to be the most active. Centrifugation of the microsomes on sucrose density gradients and repeated washing by streptomycin precipitation demonstrated the particulate nature of the incorporating enzyme.

14. Attempts were made to demonstrate homopolyribonucleotide formation *in vivo*. ^{14}C RNA prepared from ascites tumour cells, treated with RNase and analysed on MAK columns showed no RNase resistant sequences with sedimentation coefficients of 4S or greater.

15. 3' and 5' phosphodiesterases were detected in the microsomal fraction. These enzymes are likely to affect the results and interpretation of the incorporation reaction.

16. It is concluded that no RNA primed RNA synthesis occurs in the cytoplasm though an enzyme catalysing the synthesis of homopolyribonucleotides appears to be present. The significance of such a reaction is discussed.

B I B L I O G R A P H Y

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