STUDIES ON THE SYNTHESIS OF RIBONUCLEOTIDE HOMOPOLYMERS

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ABBREVIATIONS

A	Adenine
G	Guanine
C	Cytosine
U	Uracil
AMP, GMP, CMP, UMP	5′- phosphates of the above nucleosides
2′-NTP, 3′-NTP	2′-, 3′- phosphates of the nucleosides
ADP, GDP, CDP, UDP	5′- (pyro) diphosphate of adenosine etc.
ATP, GTP, CTP, UTP	5′- (pyro) triphosphate of adenosine etc.
Pi, PPi	inorganic orthophosphate and pyrophosphate
pX, pXpY, pXpYpZ	mono-, di- and tri-nucleotides terminating in free 2′(3′) hydroxyl groups
pCpCpA	terminal trinucleotide sequence of s-RNA
poly N	3′→5′ polymer of ribonucleotide
RNA	ribonucleic acid or ribonucleate
DNA	deoxyribonucleic acid or deoxyribonucleate
mRNA; rRNA; tRNA	messenger RNA; ribosomal RNA; transfer RNA
Aminoacyl-tRNA	t-RNA carrying aminoacyl residues
RNase	ribonuclease
DNase	deoxyribonuclease
(^{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
(^{32}P) CEP	2-cyanoethyl phosphate labelled with a radioactive phosphorus atom.

tris	2-amino-2-hydroxymethylpropane -1,3-diol.
tris-HCl buffer	tris adjusted to the required pH with concentrated HCl
EDTA	Ethylenediamine tetraacetic acid
p-Nitrophenyl-pT	p-Nitrophenyl deoxythymidine 5′-phosphate

C O N T E N T S

Experimental (cont'd.) Page

INTRODUCTION

1. Structure of the Nucleic Acids

1. DNA

DNA exists mainly in the cell nucleus in the form of a deoxyribonucleoprotein complex. Careful extraction leads to preparations with high molecular weights in the range $10^6 - 10^9$.

Complete hydrolysis of DNA yields the purine bases adenine and guanine, the pyrimidine bases cytosine and thymine, a sugar component and phosphoric acid. 5-Hydroxymethyl-cytosine replaces cytosine in the T even coliphages (Wyatt and Cohen, 1950).

In 1929 Levene and Mori identified the sugar obtained from thymus DNA as a deoxyribose which was subsequently demonstrated to be 2-deoxy-D-ribose by optical rotation studies. The sugar from a variety of DNAs have since been shown to be chromatographically identical. In the T even coliphages glucose occurs in glycosidic linkage with the hydroxymethyl group of 5-hydroxymethyl-cytosine (Lehman and Pratt, 1960).

Partial hydrolysis of the molecule yields the purine 9-β-D-2′ deoxyribofuranosides and the corresponding pyrimidine 1- or 3-β-D-2′ deoxyribofuranosides. Deoxyribonucleoside 3′ or 5′ monophosphates can also be derived from DNA depending on the method of hydrolysis. Electrometric titration and analytical studies on the intact molecule are consistent with the model shown in Figure 1 of a polynucleotide chain with the sugar residues joined by phosphodiester linkages.

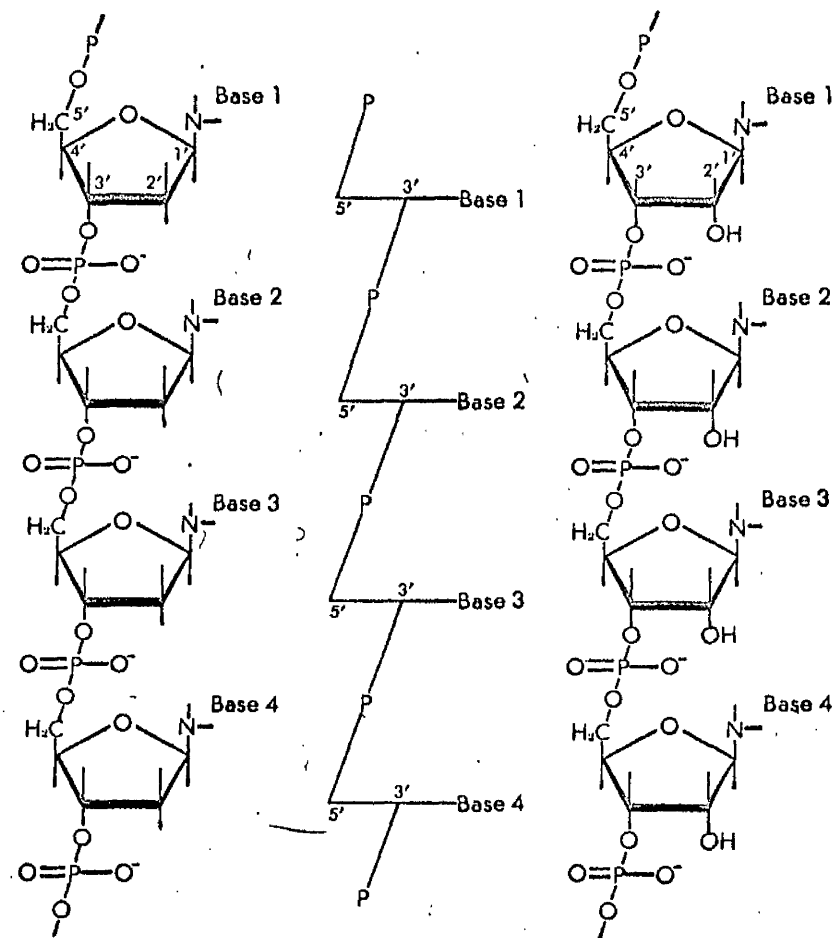
Chargaff was the first to describe the regularities in the base composition of DNA. The amounts of adenine and thymine

F I G U R E 1

The structure of DNA and RNA polynucleotides.

(From "The biosynthesis of Macromolecules" by V.M. Ingram, 1965, W.A. Benjamin, Inc., p.13)

Figure 1.



DNA polynucleotide

Schematic form of DNA and RNA polynucleotides

RNA polynucleotide

and of guanine and cytosine are equal in DNA from a variety of sources. The importance of this observation was revealed when it became clear that the molecule consists of two polynucleotide chains held together by hydrogen bonding between the strands. Watson and Crick (1953 a and b). were the first to suggest the double helical nature of DNA on the basis of the X-ray analysis performed by Wilkins and his colleagues (1953). The Watson and Crick model was confirmed and modified slightly following more detailed X-ray analysis (see Wilkins (1956) for a review) and is now generally accepted.

The constituent polynucleotide strands are antiparallel and wound round a common axis to form a right handed double helix with a narrow groove and a wide groove running along the length of the molecule. There are ten bases per turn of the helix and the chains are held together by hydrogen bonding between the adenine on one strand and thymine on the other, and by the similar base pairing of cytosine and guanine. Forces caused by stacking of the base-pairs also contribute toward the stability of the helix.

As a result the sequence of bases on one strand automatically determines the sequence of bases on the opposite, complementary, strand. The significance of the self-directing nature of this structure in relation to the problem of DNA replication did not escape Watson and Crick (1959b).

X-ray analysis on intact nucleohistone, bacterio-phage and trout sperm indicated that DNA exists in the helical configuration <u>in situ</u> in the living cell (Wilkins, Stokes and Wilson, 1953; Franklin and Gosling, 1953).

Single stranded non helical DNA has been isolated from the coliphages X174, S and S13 (Davidson, 1965). The DNA from phage ϕX 174 was surprisingly found to be in the form of an unbroken circle (Sinsheimer, 1959; Fairs and Sinsheimer, 1962 a, b and c). Double stranded circular DNA has now been observed from phage T2 (Thomas and MacHattie, 1964), phage Lambda (Fox and Messelson, 1963), polyoma virus (Dulbecco and Vogt, 1963), E. Coli (Cairns, 1963), and boar sperm (Hotta and Bassel, 1965).

2. RNA

RNA occurs in all cells, both in the nucleus and the cytoplasm, and is found in particularly large amounts in tissues active in protein synthesis.

Bulk RNA extracted from the whole cell can readily be fractionated into various subclasses by a variety of techniques. About 80% of this is found in the small cytoplasmic particles called ribosomes. Ribosomes are composed of two ribonucleo - protein sub-particles which sediment at about 30S and 50S respectively. The RNA from the small subunit has a molecular weight in the range $0.4 - 0.7 \times 10^6$ and sediments at 16 - 18S, while that from the large subunit has a molecular weight in the range $1.0 - 1.5 \times 10^6$, and sediments at 23 - 30S, (Spirin, 1963). 15% is composed of soluble RNA (s-RNA) which has a molecular weight of about 25,000 and sediments at about 4S. The remaining 5% is a heterogeneous population of RNAs of varying sizes depending on the cell type. High molecular weight RNA can be isolated from certain RNA containing viruses. The functions of the various sub-

classes of RNA will be discussed in section 2.

As in DNA the components and the structure of RNA have been deduced by studies of chemical and enzymatic degradation products. The bases found in RNA are mainly the purines adenine and guanosine and the pyrimidines cytosine and uracil. Methylated bases, particularly 5-methylcytosine and 6-methyl amino purines, are particularly abundant in s-RNA, and, to a lesser extent in ribosomal RNA (Dunn, 1959).

The sugar in RNA from a wide variety of sources has been shown to be D ribose and it is now widely assumed that this is the sugar in all RNAs.

The sugar linkage in RNA nucleotides is identical to that in the corresponding nucleotides from DNA. 5- -D-ribofuranosyl uracil (pseudouridine) can be isolated from both s-RNA and ribosomal RNA, and indeed can comprise up to 25% of the uridine nucleotides in s-RNA (Davidson, 1965).

Evidence from electrometric titrations and analysis of degradation products suggests the polynucleotide structure of Figure 1, where D-ribose replaces D-deoxyribose. The consensus of opinion is against a branching structure (Cohn, 1955).

The hydrodynamic properties of RNA chains in solution, where, under conditions of varying ionic strength, they behave like flexible molecules which can make a smooth transition from a lightly coiled to an extended state, is in sharp contradiction to the rigid, helical structure of DNA (Spirin, 1963). In 1955 Chargaff reported a lack of constant pairing of adenine and uracil,

and of cytosine and guanine, in the base composition of various RNAs. Since RNA from a variety of sources behaves as a single polynucleotide chain on digestion with ribonuclease, the original hypothesis, that RNA resembles DNA in secondary structure, was in some doubt.

However, on mixing the synthetic copolymers poly A and poly U, a complex is achieved which exhibits properties compatible with Watson-Crick type of double helix, with base pairing between adenosine and uracil (Warner, 1957; Rich and Davies, 1956). As with DNA, when the temperature, or pH, is raised, the ordered structure of the molecule is disrupted, with a concomitant sharp rise in the absorbance at 260 mu, (hyperchromic effect).

When solutions of naturally occurring RNA are treated in a like manner, similar but less pronounced results are obtained, suggesting the presence of restricted helical regions. Since RNA chains are single unbroken molecules (Spirin, 1963), it follows that the chains must fold back on themselves to form double helical regions. X-ray analysis has confirmed the presence of helical regions in RNA from a variety of sources and in s-RNA the extent of helix formation may be extensive. The extent of helix formation is virtually 100% in the RNA from reovirus and wound tumour virus, and in these cases the RNA is double stranded (Gomatos and Tamm, 1963).

The RNA of TMV virus is held in a helical conformation *in situ* by hydrogen bonding with its protein coat. On extraction, however, it loses this helical structure and assumes the properties of a random coil (Knight, 1963). As yet there is little information

available as to the precise conformation of cellular RNA in situ.

Great excitement has been caused by the work of Holley and his colleagues, who have succeeded in working out the nucleotide sequence of specific alanine transfer RNA, (Holley, Apgar, Everett, Madison, Marquisee, Merrill, Penswick and Zamir, 1965) This enormous stride forward should make it possible, in the near future, to determine the precise 3 dimensional configuration of the molecule.

2. The Biological Function of the Nucleic Acids

1. DNA

The genetic importance of DNA has been suspected ever since it was found to be localised in the cell nucleus, and there is now an impressive body of evidence to support the view that DNA is the carrier of genetic information. The most important confirmatory studies have been those employing bacteria, in which DNA was identified as the transforming principle and the mechanisms of phage infection and transduction, and the process of sexual reproduction in bacteria, were elucidated. Szybalski has carried out successful transformation experiments with human cells in tissue culture, (Szybalski and Szybalski, 1962) although the significance of studies using live ducklings is under considerable doubt (Benoit, Leroy, Vendrely and Vendrely, 1957 and 1960). DNA is also metabolically stable and the DNA content per set of chromosomes is a constant in any given species - properties to be expected in the genetic material.

Ideally the genetic substance should: 1) duplicate exact copies of itself and pass them on to the progeny, and 2) pass on its information to the cell. All living cells are possessed of an ingenious mechanism whereby 1) is achieved.

The classical experiments of Meselson and Stahl suggested that following cell division in Esch. Coli each daughter cell contained one of the original strands of the parental DNA double helix, and a newly synthesised strand of DNA, (Meselson and Stahl, 1958). The studies of Bollum, Keir, Kornberg and others (see

Smellie, 1965, for a review) have demonstrated that the enzyme DNA polymerase or DNA nucleotidyltransferase, which can be extracted from both bacterial and animal cells, can carry out the synthesis of new DNA *in vitro*. Using single-stranded regions of the DNA as templates, the enzyme catalyses the condensation of deoxyribonucleoside triphosphates to form new strands of DNA, releasing stoichometric amounts of inorganic pyro phosphate. During synthesis, template DNA directs the sequence of nucleotides in the new strands by the formation of base pairs, so that the new strands are complementary to the old. New double helices are formed containing one old, and one new strand of DNA. Thus, there is available a mechanism for the formation of two identical daughter helices from the parental DNA.

It should be emphasised that there is much about the replication of DNA which is as yet ill understood; in particular the control of initiation of synthesis, the role of histones in animal cells during synthesis, the mechanism of strand separation, the protection of the DNA during replication and the problem of replicating two antiparallel chains at the same time and in the same direction.

2.2. The Genetic Code

The distinctive characteristics of different cells can be attributed to a large degree, if not entirely, to the different proteins which they contain. The manner in which the sequence of nucleotides in DNA determines the sequence of amino acids in cellular proteins is now well understood and well documented.

Only a brief outline will be attempted here. Crick and his colleagues (Crick, Barnett, Brenner, and Watts-Tobin, 1961) considered the codon (the unit of nucleotides determining one amino acid) to be a sequence of three nucleotides in DNA, and unequivocal proof of the triplet theory has been obtained (Stacholin, Wittstein, Oura, and Noll, 1964).

2.3. RNA and protein synthesis

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Since the cytochemical studies of Brachet and Caspersson it had been suspected that RNA might be involved in protein synthesis (Brachet, 1950, and Caspersson 1942 and 1950). However, with the development of cell free systems it became apparent that protein synthesis actually occurs on the ribosomes, either free, or when attached to membranes, as in the microsomes (for a review see Brachet, 1959). Brenner, Jacob and Meselson (1961) first introduced the concept that DNA might be the template for a "messenger" RNA (m-RNA) which could complex with the ribosomes, and direct the synthesis of specific polypeptides. This was supported by the observation in 1961 that added poly U directed the specific incorporation of phenylalanine into polypeptide material in a cell free system from Esch. coli, (Nirenberg and Matthaei, 1961). The concept of m-RNA received further support when it was realised that in both animal and microbial cells protein synthesis occurs in vivo on polysomes - clusters of ribosomes attached to a single strand of RNA (Rich, Warner and Goodman, 1963; Gilbert, 1963).

Early work quickly established the need for s-RNA, or more

accurately transfer RNA (t-RNA), in cell free protein synthesis. The 3′ terminal sequence is always C.C.A. in this group of molecules. Following activation by ATP (Hoagland, Keller and Zamecnick, 1956) an amino acid is esterified to the 2′ or 3′ hydroxyl of the terminal ribose of a t-RNA molecule by a specific activating enzyme (Davidson, 1965). It is considered that there are specific transfer RNA molecules for each amino acid, and each specific activating enzyme. The amino acyl RNA is then transferred to the ribosome where it becomes attached to the 50S subparticle (Campbell, 1965).

The "adaptor" hypothesis for t-RNA was first suggested by Crick in 1958. This hypothesis suggests that each t-RNA molecule has a trinucleotide sequence corresponding to the codon for its amino acid. This triplet becomes aligned to a complementary sequence, the "anticodon", in the messenger RNA, which is considered to be attached to the 30S ribosomal sub-particle (Campbell, 1965). Peptide bond formation can then take place, and the process repeated until a polypeptide is assembled, the amino acid sequence of which has been specified by the m-RNA.

As well as the two main ribosomal RNAs a smaller component which sediments at about 5S, has been extracted from the ribosomes of E. Coli, (Rosset and Monier, 1963; Rosset, Monier and Julien, 1964) mammalian cells (Galibert, Larson, Lelong and Boiron, 1965 and 1966), and from the aquatic fungus Blastocladiella emersonii, (Comb and Katz, 1964). The base composition of this RNA is similar to, but significantly different from that of s-RNA, and it contains no

methylated bases. In Esch.coli there appears to be one to two molecules of 5 s RNA per ribosome, localized on the 50s subunit, (Zehavi-Willner and Comb, 1966; Rosset et al, 1964). The precise function of the three ribosomal RNAs is not at all clear at the present time.

2.4. The genetic function of RNA

In a number of microbial, plant and animal virus which contain RNA as the nucleic acid component, it has been shown that the genetic information necessary for virus reproduction is carried by the RNA.

3. In vivo Synthesis of RNA

3.(1) Cellular site of RNA synthesis.

When animal cells are exposed to radioactive precursors of RNA and the cells fractionated, radioactivity first appears in the RNA of the nucleus, and then, after a lag, in the RNA of the cytoplasm. (Marshak 1948; Jeener and Szafarz, 1950; Scallio, McIndoe and Davidson, 1955;) Autoradiographic methods have also been used with a variety of cells and have confirmed the earlier results. The length of the lag period appears to be related to the length of the cell cycle. If cells are pulsed with radioactive RNA precursors and then incubated in non-radioactive medium, incorporation into nuclear RNA soon stops while label may continue to accumulate in cytoplasmic RNA (Feinendigen, Bond, Shreeve and Painter, 1960; Perry 1962; Watts 1964a)

The interpretation of such experiments has generally been that RNA synthesised in the nucleus is transferred to the cytoplasm. However, certain considerations must be borne in mind when analysing such results. The dynamics of the low molecular weight precursor pools in animal cells are not well understood. Feinendigen, Bond, and Hughes (1961) have obtained evidence for the compartmentalisation of such pools in HeLa cells, but the situation is obviously complex. Watts (1964a) has demonstrated that the rate of incorporation of labelled RNA precursors into the RNA of rapidly dividing HeLa cells is dependent on the exogenous concentration. Under suitable conditions incorporation into cytoplasmic RNA was linear, with no observable lag. Once

label has entered the pools it becomes extremely difficult to deplete them of radioactivity, even by exposing the cells to media containing an excess of unlabelled precursors, (Prescott, 1962; Sisken and Kinosita, 1961). In some experiments label continued to be incorporated into total RNA for some time after the "chase" in unlabelled medium had begun (Harris, 1959; Feinendigen, Shreeve and Painter, 1960; Perry, Errera, Hell and Durwald, 1961; Harris, Fisher, Rodgers, Spencer and Watts, 1963; Sisken and Kinosita, 1961).

A rapid turnover of a substantial portion of the rapidly labelled nuclear RNA has been demonstrated in dividing animal cells (Feinendigen et al, 1962; Harris and Watts, 1963; Harris, Fisher, Spencer and Watts, 1963; Attardi, Parnas, Hwang, and Attardi, 1966) and not all of this RNA is necessarily transferred to the cytoplasm, (Harris et al, 1963; Paul and Struthers, 1963; Attardi et al, 1966). In addition, the intensive work of recent years has served to demonstrate the highly heterogeneous nature of the RNA in animal cells, and the differences in the rates of synthesis and turnover of its sub-classes.

In view of these considerations it is clear that exact kinetic analysis of the experiments described above is very difficult. On the basis of such results alone it would be impossible to rule out breakdown of labelled nuclear RNA into the acid soluble pools, with subsequent reincopporation in the cytoplasm. There is, however, strong confirmatory evidence for

the nuclear origin of all cellular RNA.

When the nucleus of an amoeba which has been heavily labelled with P^{32}-phosphate is introduced to an unlabelled recipient cell, most of the radioactivity is gradually shifted to the cytoplasm of the recipient (Goldstein & Plaut, 1955). The label appeared to be in a form continually sensitive to ribonuclease, and no significant movement of radioactivity into the nucleus of the recipient could be detected. In a subsequent article Goldstein (1963) extended this approach and concluded that the acid soluble pool of the nucleus did not contribute directly to cytoplasmic RNA, but was first incorporated into nuclear RNA.

These experiments are complementary to studies in which the capacity of enucleated fragments of cells to synthesise RNA is measured.

Microsurgery on Amoeba proteus or Acetabularia yields nucleated and enucleated fragments of the cells. On exposure to radioactive precursors of RNA, nucleated fragments show heavy incorporation into nuclear RNA. Incorporation into enucleate fragments is variable but readily observable (Plaut and Rusted, 1957; Naora, and Brachet, 1960; Schweiger and Bremer, 1961). In the case of A. proteus at least this incorporation was not due to contamination by bacteria (Cummins, and Plaut, 1964).

However, Rabinovitch, and Plaut (1962 a & b) described the existence of large numbers of rickettsial-like endosymbionts, which contain DNA, in the cytoplasm of amoeba, and these may be

the source of the extranuclear RNA synthesis. Actinomycin D (see Section 4 (i)) does not block such synthesis entirely, but the permeability of the DNA-containing particles to the drug is not known (Cummins and Plaut, 1964). According to Naora et al (1960), a large proportion of the cytoplasmic RNA in Acetabularia is associated with the chloroplasts, and most of the residual synthesis in enucleate fragments also appears to be in this fraction. Baltus, E. and Brachet, J. (1963) have demonstrated the presence of DNA in the chloroplasts of Acetabularia. Since chloroplasts from broad bean leaves, which also contain DNA, are capable of supporting DNA-dependant RNA synthesis in vitro (Kirk, 1963 and 1964), it is reasonable to suppose that residual RNA synthesis in Acetabularia is also dependant on chloroplast DNA.

More clear cut results have been obtained employing cells which contain no protoplasts or identifiable endosymbiants. After enucleation in HeLa cells (Goldstein, Micou and Crooker, 1960), Amoeba (Prescott, 1960) and Tetrahymena (Prescott, 1962) all fail to incorporate detectable amounts of radioactive nucleosides into RNA.

During prophase and telophase in animal cells, the chromosomes, which remain dispersed throughout interphase, become condensed, until at mitosis they take the form of discrete, separate bodies. During the period of chromosome condensation RNA synthesis gradually decreases, until at mitosis it has virtually ceased (Prescott, 1964). The dependance of RNA

synthesis on the dispersed state of the chromosomes provides presumptive evidence for the nuclear site of RNA synthesis.

Several lines of study in recent years have provided strong evidence that most cellular RNA is synthesised on a DNA template (see section 4). This being so, it would not be unreasonable to expect RNA and DNA synthesis to be mutually exclusive, and indeed, a decrease in RNA synthesis during DNA replication has been observed in a number of cells (Taylor, 1958; Sisken, 1959). In the ciliated protozoon Euplotes, DNA synthesis occurs in waves, and can be detected at any one time in a narrow region of the macronucleus (Gall, 1959). Autoradiographic studies revealed that RNA synthesis continues unabated in most of the macronucleus, but is completely absent in the region of DNA replication. (Prescott and Kimball, 1961).

This large body of evidence indicates that the major portion of cellular RNA is synthesis in the nucleus, and must be transferred to the cytoplasm. It might be mentioned at this point that in the case of virus infected cells RNA transcription can occur in the cytoplasm.

The *in vivo* synthesis of the known sub-classes of RNA will now be considered.

3. (ii) Ribosomal RNA

The bulk of cellular RNA is found as the two high molecular weight molecules found in the ribosomes (r-RNA). This RNA is metabolically stable and exhibits very little turnover in rapidly dividing cells. Ribosomes exist mainly in the cytoplasm but ribonucleoprotein particles similar to ribosomes can be extracted

from the nucleus (Tso and Sato, 1959; Rendi, 1960; Wang, 1961; Pogo and Pogo, Litton, Allfrey and Mirsky, 1962; Burdon, Wykes and Wilkie, 1963; Flamm and Birnstiel, 1964).

The importance of the nucleolus in the formation of a major fraction of cytoplasmic RNA was suggested by the experiments of Perry and his colleagues (Perry, 1960; Perry, Hell, and Errera, 1961). Irradiation of the nucleoli of HeLa cells with a microbeam of ultraviolet light abolished virtually all of the incorporation of labelled precursors into nucleolar RNA, the subsequent accumulation of label into cytoplasmic RNA being reduced by 70%. The synthesis of new RNA in the extranucleolar region of the nuclei was not immediately affected.

The presence of ribosome like particles, which contain RNA with sedimentation characteristics identical to cytoplasmic RNA, has been demonstrated in the nucleoli of pea seedlings, (Birnstiel, Chipchase, and Hyde, 1963). The particles were not found in the pure chromatin fraction. Hybridisation studies indicated that this RNA was identical in nucleotide sequence to cytoplasmic RNA (Chipchase, and Birnstiel, 1963).

Elegant experimental techniques involving microdissection of nuclei and subsequent microanalysis of the constituents, enabled Edström et al to demonstrate that the base composition of nucleolar RNA closely resembles that of cytoplasmic RNA., (Edström, 1960; Edström, Grampp, and Scher, 1961; Edström and Gall, 1963).

Following partial hepatectomy both the amount and turnover rate of the RNA of the regenerating liver tissue increases. The

nucleoli enlarge and contain increased amounts of ribonucleoprotein-like particles (Busch, Byvoet and Smetana, 1963). The base composition of the rapidly synthesized RNA shifts so as to become similar to that of r-RNA, and this is believed to be due to an increase in the proportion of RNA synthesized in the nucleolus, (Muramatsu and Busch, 1965). Evidence has also been presented that liver nucleoli synthesize the preribosomal RNA to be discussed below (Steele, Okamura and Busch, 1965).

The complete absence of the synthesis of r-RNA in nucleolate mutants of Xenopus laevis is most striking, (Brown and Gurdon, 1964) and Ritossa and Spiegelman have provided direct evidence that the DNA coding for r-RNA is localized in the nucleolar organizer region in Drosophila melanogaster (Ritossa and Spiegelman, 1965).

When cells are exposed to radioactive precursors of RNA and the RNA extracted from the cytoplasm and analyzed by sucrose density gradient centrifugation, the label begins to appear after a short lag in the two r-RNAs, which sediment at about 28S and 18S, and also in the 4S RNA. Disregarding the 4S RNA for the moment it would appear that the rate of r-RNA labelling depends on the rate of cell division, being greater in rapidly dividing HeLa cells or L cells, than in liver, or for example, than the immature duck erythrocyte. The need to synthesize ribosomal RNA may also be related to the need to provide continuous supplies of m-RNA to the cytoplasm.

In all cells studied so far, label appears earlier in the

18S than the 28S r-RNA (Hiatt, 1962; Girard, Penman and Darnell, 1964; Rake and Graham, 1964; Meoh and Vassalli, 1965; Girard, Latham, Penman and Darnell, 1965; Attardi, Parnas, Kwang and Attardi, 1966).

However as discussed above incorporation is first observed in nuclear RNA. Both in dividing and non-dividing cells, pulse labelling experiments have shown the occurrence of a rapidly labelled RNA in the form of molecular species greater than 28S (up to about 50S)(Scherrer and Darnell, 1962; Perry, 1962; Scherrer, Latham and Darnell, 1963; Georgiev, Samarina, Lezman, Smirnov and Severtsov, 1963; Brown and Gurdon, 1964; Rake and Graham, 1964; Yoshikawa-Fukada, Fukada and Kawade, 1965; Meoh and Vassalli, 1965; Penman, 1966).

In many of these studies two rapidly labelled components are observed sedimenting at 45-50S and 40 - 45S respectively. Scherrer et al have proposed that the rapidly labelled high molecular weight material in HeLa cells is the precursor of r-RNA (Scherrer and Darnell, 1962; Scherrer, Latham and Darnell, 1963). After a brief exposure to radioactive RNA precursors the 45S RNA became highly labelled. In the presence of actinomycin D, which blocked further synthesis, the label shifted in 20 min to 35S RNA, with some label appearing in 18S RNA. By 60 min both 28S and 18S RNA became labelled, and at 240 min. virtually all the radioactivity was found in r-RNA. This result was confirmed in L cells by Penwick (1964). In addition the base composition of rapidly labelled 35S - 45S RNA has been reported

to be similar to r-RNA in HeLa cells,(Scherrer, Latham and Darnell, 1963) ascites tumour cells, (Georgiev et al, 1963 and 1964) rat liver cells, (Hiatt, 1964; Steele, Okamura and Busch, 1965; Muramatsu and Busch, 1964) spleen and lymph cells, (Mach and Vassali, 1965) and mouse sarcoma cells, (Kempf and Mandel, 1966).

Rake and Graham (1964) identified the two rapidly labelled nuclear RNAs in L cells as sedimenting at 40S and 50S. The 50S material became labelled more quickly than the 40S; after a lag radioactivity appeared in 18S RNA, and then 28S. On the basis of complicated kinetic analysis of their results the authors concluded that the delay in the entry of label into cytoplasmic RNA was occasioned by two sequential precursors, and that the 50S and 40S RNA met the requirements for such precursors.

Recently Penman, (1966) has analysed the nuclear and cytoplasmic events occurring during r-RNA in the HeLa cell system in greater detail. Using a method which ensured minimal contamination of nuclei with cytoplasm, the cells were fractionated and the nuclear and cytoplasmic RNA analysed, after exposure to ^{14}C-uridine. After 10 min only the nuclei contained radioactive RNA, which appeared in the sucrose gradient as polydisperse material with a sharp peak at 45S. At 30 min. two nuclear peaks at 35S and 45S were apparent, and 18S cytoplasmic RNA became heavily labelled. After 60 min. the 18S RNA continued to accumulate label, and radioactivity had begun to enter 28S RNA. If actinomycin was administered at 10 min both

radioactivity and optically opaque material had disappeared from the 45S region. A radioactive peak at about 30S remained. Label continued to accumulate in 16S RNA but even after two hours was never found in 28S RNA. Penman maintains that 45S nuclear RNA breaks down to 35S nuclear RNA and 18S cytoplasmic RNA. The 35S RNA is presumed to give rise to 28S cytoplasmic RNA.

However, closer scrutiny of Penman's results, suggests that the situation is more complex. In the nuclei a large peak of optically dense material is observed at 30S as distinct from 28S cytoplasmic RNA. No 16S RNA is observed in the nuclear preparation used. The 30S RNA appears to have a slower rate of turnover than the rapidly labelled 35 - 45S material. The function of this RNA is not known. In the presence of actinomycin D, the rapidly labelled 45S RNA is rapidly degraded, and radioactivity appears immediately in the 30S region. No labelled 35S RNA ever appears.

Therefore, on the basis of these results, the possibility that 45S and 35S RNA are synthesised independently cannot be excluded.

Furthermore, Yoshikawa-Fukada et al (1965) have reported that in L cells in culture, only 40S RNA has a r-RNA-like base composition; that of 50S RNA resembled L cell DNA. Hybridisation experiments with r-RNA and rapidly labelled 40S and 50S L cell RNA, suggested that 40S RNA was more like ribosomal RNA than 50S RNA, (Perry, Srinivasan, and Kelley, 1964). Muramatsu et al (1966) have analysed the rapidly labelled nucleolar and extranucleolar RNA

from rat liver nuclei. The nucleotide composition of nucleolar 45S and 35S RNA was found to be similar to 28S nucleolar RNA. No nucleolar 18S RNA was found, suggesting that the 28S and 18S RNA may be synthesised separately. It is possible, of course, that the "28S" nucleolar RNA corresponds to the 30S RNA found in HeLa cells. A rapidly labelled extranucleolar RNA was described, sedimenting with a broad peak at 45S and which had a high A-U content.

Hastings, Parish, Kirby and Klucis (1965) have found high molecular weight precursor-type RNA in Esch.Coli. On heating the complex it degraded to the two ribosomal RNAs and smaller highly labelled material. It has been suggested that the high molecular weight precursor is merely a complex between the r-RNA and rapidly labelled RNA.

The situation is obviously complex and further experiments will be required before a solution is apparent.

Tamaoki (1966) has described the presence of ribonucleoprotein particles in the nuclei of cells which sediment between 60S and 100S and which contain up to 60% of the rapidly labelled 45S RNA. In HeLa cells these particles behave in an identical manner to ribosome sub-units during electrophoresis, (Tamaoki and Mueller, 1965). The formation of the particles containing 45S RNA precedes the formation of ribosomes, and it has been suggested that they are ribosome precursors.

When disrupted HeLa cells are analysed by sucrose gradient centrifugation, ribonucleoprotein particles of approximately 45S and 60S are observed in the cytoplasm. The particles have

identical sedimentation coefficients to the particles obtained from 74S ribosomes in low Mg. Girard et al (1965), Jocklick and Becker (1965a) and McConkey and Hopkins (1966) have shown that the 45S particle contains the newly synthesised cytoplasmic 18S RNA, but no 28S RNA. A similar 45S particle has been reported in rat liver cytoplasm (Monshaw, Revel and Hiatt, 1965). It is the first cytoplasmic particle to become labelled when HeLa cells are exposed to radioactive RNA precursors. At later times radioactivity also appears in the region of the 60S particle. Jocklick and Becker (1965a) have reported that this new RNA is exclusively 28S RNA. However, both Girard et al (1965) and McConkey and Hopkins (1966) showed the presence of both labelled 28S and 18S RNA in the cytoplasmic 60S particle.

At the moment these conflicting views are difficult to reconcile. The point is of considerable importance to the mechanisms of genesis of ribosomes and polyribosomes. Strong suggestive evidence has recently been derived that newly synthesised ribosomal subunits, perhaps containing m-RNA, enter the polysomes as subunits before equilibrating with the 74S ribosome pool (Girard et al 1965; Jocklick and Becker, 1965a; McConkey and Hopkins, 1966). A 60S particle containing both 28S and 18S RNA would not be consistent with such a mechanism since 60S particles derived from 74S ribosomes contain only 28S RNA (Girard et al, 1965).

Little is known about the labelling characteristics of the 5S ribosomal RNA from animal or bacterial cells. In Esch. Coli

it appears to be more rapidly synthesised than transfer RNA, (Rosset, Monier and Julien, 1964). In the aquatic fungus Blastocladiella emersonii, nuclear 5S is very rapidly labelled (Comb, and Katz, 1964). The 5S RNA from ascites cells is rapidly labelled and also metabolically stable (Lal and Burdon, 1966.)

3.(iii) Messenger RNA

Following the infection of Esch. Coli with bacteriophage the normal high rate of bacterial RNA synthesis is rapidly arrested, but the synthesis of a small fraction of RNA with a high rate of metabolic turnover remains. The base composition of this RNA was found to be complementary to that of the bacteriophage DNA (Volkin and Astrachan, 1956; Volkin, Astrachan and Countryman, 1958; Astrachan and Volkin, 1958; Nomura, Hall and Spiegelman, 1960). The nucleotide sequence of this RNA was also complementary to sequences in the invading bacteriophage DNA (Hall and Spiegelman, 1961; Spiegelman, Hall and Storck, 1961).

This was the first demonstration of the "messenger" RNA proposed by Jacob and Monod (1961) as an agent for the transfer of genetic information from DNA to the site of protein synthesis. The original observations in phage infected Esch. Coli have been extended to uninfected cells, and m-RNA is now well characterised in micro-organisms as a rapidly labelled RNA with a high rate of metabolic turnover, which becomes associated with ribosomes to direct the synthesis of specific polypeptides.

The situation is more complex in animal cells. The criteria

normally employed to identify mammalian m-RNA are inadequate (i.e. DNA-like base composition, rapid synthesis and the ability to stimulate the incorporation of amino acids into non specific peptides in a cell-free protein synthesising system). There is no reason to believe that all m-RNA must necessarily reflect the base composition of total DNA. Not all rapidly labelled nuclear RNA is transferred to cytoplasmic polyribosomes. More definitive methods of describing m-RNA are needed.

In animal cells it is difficult to detect pulse labelled RNA with a DNA-like base composition in the bulk RNA. Physical separation of minor components is often required. Phenol extraction of RNA provides the basis for such a fractionation. It has been shown that the extraction of tissue homogenates with cold phenol at neutral pH removes the bulk of the RNA, but leaves behind a fraction different in base composition and metabolic behaviour (Sibitani, Yamani, Kimura and Takahashi, 1960). The basis of this fractionation is believed to be due to the association of such RNA with the chromatin matrix. In calf thymus nuclei the RNA was rapidly labelled and had a DNA-like base composition (Sibitani, deKloet, Allfrey and Mirsky, 1962).

The refractory RNA can be extracted by phenol at elevated temperatures (Georgiev, and Mantieva, 1962; Georgiev and Lozman, 1964) or increased pH, (Brawerman, Gold and Eisenstadt, 1963; Hadjivassiliou and Brawerman, 1965). All species of RNA can be extracted with phenol in the presence of sodium dodecyl sulphate.

Brawerman et al (1963) isolated a fraction from rat liver

nuclei at pH 8.3 which actively stimulated the uptake of amino acids into an Esch. Coli cell free protein synthesising system. This template RNA was present as several peaks in sucrose gradients with sedimentation coefficients of 16S and higher (Hadjivassiliou and Brawerman, 1965). The most recent report from this group states that the nuclear template material sedimonts at 9S - 16S and is rapidly labelled (Hadjivassiliou and Brawerman, 1966). Di Girolamo et al (1964) have isolated nuclear RNA from rat liver and claim that template material sedimonts at values greater than 28S.

Working with ascites tumour cells Georgiev and Lerman, (1964) isolated refractory RNA from the nucleus by the thermal extraction method. At 40 - 55°C the RNA consisted of two peaks; 30S RNA had a low G.C. content and 45S had a high G.C. content. At 55-63°C polydisperse RNA with a low G.C. content was extracted. Muramatsu, Hodnett and Busch, (1966) have described a rapidly labelled RNA from the extra nucleolar region of rat liver nuclei. This RNA sedimented in a polydisperse fashion from 4S - 6S to greater than 45S. At each region in the gradient a high A U content was observed. Attardi et al (1966) have described the formation of RNA greater than 100S in the nuclei of immature duck erythrocytes.

It is not known what relationship this rapidly labelled high molecular weight RNA bears to the template RNA of Brawerman or Di Girolamo, or to cytoplasmic m-RNA. However the work of Harris (1963), Harris et al (1963), Paul and Struthers (1963) and Attardi et al (1966) makes it clear that a substantial proportion of rapidly labelled nuclear RNA is degraded in the

nucleus and may never be transferred to the cytoplasm.

The cytoplasmic m-RNA of animal cells appears to have a much longer half life than in bacteria. Estimates of the half life of m-RNA in animal cells are 30 min. in mouse mammary adenocarcinoma, (Trakatellis, Montjar and Axelrod, 1965), 2-3 hours in rat diaphragm muscle (Manchester, 1963), 3-4 hours in HeLa cells (Penman et al, 1963), 4-6 hours in mouse fibroblast L cells (Reich, Franklin, Shatkin and Tatum, 1962). In rat liver no diminutions in cytoplasmic protein synthesis was observed up to 17 hours after RNA synthesis was blocked with actinomycin D, (Revel and Hiatt, 1964a) However, using different methods Wettstein, Staehelin and Noll (1963) and Villa-Trevino, Farber, Staehelin, Wettstein and Noll (1965) have estimated a half life of 60 min. for rat liver m-RNA. Reticulocytes continue to synthesise haemoglobin for some days after RNA synthesis has ceased (London, Shemin and Rittenberg, 1950; Pinheiro, Leblond, Droz, 1962; Burny and Chantrenne, 1963).

After short exposure of HeLa cell cultures to radioactive RNA precursors a major portion of the cytoplasmic label is found in the polysomes (Penman et al, 1963; [illegible], [illegible] and [illegible], 1964). When the polysomal RNA is analysed the label is found in a broad peak from 6S to 25S in the sucrose density gradient. The nucleotide composition of this RNA is not unlike that of HeLa DNA. At later times labelled r-RNA predominates. When the cytoplasmic polysomes are fractionated, the largest structures contain the largest m-RNA, while the small polysomes contain a mixture of small and large polysomes (Latham and Darnell, 1965a)

Similar experiments have been performed in rat liver by Stacheliin et al (1964). Once again the size of the m-RNA was related to the size of the polysome from which it was derived. The m-RNA from a trimer (a polysome containing three 74S ribosomes attached to one m-RNA strand) sedimented with a peak at 7.2S, while that from a polysome containing 14 ribosomes sedimented with a peak at 13.6S. The experiments also indicated that m-RNA is synthesised more slowly in liver than in HeLa cells. Studies with actinomycin D suggested that only a few minutes supply of m-RNA were available to the cytoplasm.

Di Girolamo et al (1964) measured the ability of rat liver cytoplasmic RNA to stimulate amino acid uptake into a cell free protein synthesising system. The greater part of the activity was found in the region of 18S RNA but the specific activity of a 6 - 11S fraction was much greater.

Hadjivassiliou and Brewerman (1966) isolated an RNA fraction from rat liver with phenol at pH 8.3. The bulk of this template activity sedimented in the 9 - 16S region in sucrose gradients. When hot phenol was used the template RNA complexed with 18S RNA and sedimented in the 18S region. Stacheliin et al (1964) have also reported complex formation between m-RNA and r-RNA.

It seems clear from these experiments that cytoplasmic m-RNA in animal cells has a fairly long half-life and is quite small in size. The relationship to rapidly labelled nuclear RNA remains obscure. The low molecular weight template RNA extractable at pH 8.3 from liver nuclei may be related to cytoplasmic m-RNA.

Recently evidence has been presented that in HeLa cells and

rat liver cytoplasmic m-RNA first appears in particles which sediment in the 45S region (Latham and Darnell, 1965b; McConkey and Hopkins, 1966; Henshaw, Revel and Hiatt, 1965). The authors tentatively concluded that this represented m-RNA attached to the small ribosomal subunit, and the first stage in the formation of polysomes. This is supported by the experiments of Jocklick and Becker (1965b) in vaccinia-infected HeLa cells. The viral m-RNA is synthesised in the cytoplasm and remains associated with particles in the 45S region for a few minutes before labelled m-RNA appeared in the polysomes. Henshaw et al (1965) demonstrated that in 5×10^{-4} M $MgCl_2$ poly U was bound preferentially to the 45S particles from rat liver.

On the other hand Spirin (1966 in the press) pointed out that attachment of a 15S - 18S RNA to a 45S ribosomal subunit should lead to a considerable increase in the density of the particle, and it seems improbable that its sedimentation coefficient would not alter appreciably as a result.

In fish and sea urchin embryos rapidly labelled RNA with the characteristics of messenger is found in cytoplasmic particles which sediment between 23S and 68S (Spirin, Belitsina, and Ajtkhozhim, 1964; Spirin and Nemer, 1965; Spirin, 1966.) The particles are less dense than ribosomes and are not considered to be ribosomal subunits. They have been termed informosomes by Spirin and he attributed to them the function of storing m-RNA.

Obviously further work is required to determine the nature of such m-RNA containing particles and to elucidate their relationship with ribosome subunits and polysomes.

3.(iv) Soluble RNA

Relatively little is known about the detailed kinetics of s-RNA synthesis in animal cells. Like other classes of RNA it is believed to be synthesised in the nucleus and the work of Sirlin, Jacob and Tandler (1963) implicates the nucleolus as the site of synthesis.

Sucrose gradient analysis of cytoplasmic RNA following brief exposure of cells to labelled adenosine, cytidine or inorganic phosphate usually results in the most rapid accumulation of radioactivity into 4S RNA. In the presence of actinomycin D such incorporation into s-RNA is inhibited to a much smaller extent than into other classes of RNA. In rat liver and L cells it has been shown that most of the resistant label could be accounted for in the terminal $\ldots_{p}C_{p}C_{p}A$ nucleotide sequence (Morits, 1963; Harel, Harel, Boer, Imbenotte and Carponi, 1964; Moule, and Landin, 1965; Franklin, 1963).

Turnover of the terminal adenosine of s-RNA *in vivo* has been demonstrated in rapidly dividing yeast (Rosset and Monier, 1963), *Esch.Coli* (Cannon, 1964), rat liver (Scholtissek, 1962), and in reticulocytes (Burney and Chantrenne, 1963; Holt, Jool and Herbert, 1966). In reticulocytes the rate of terminal CMP turnover was found to be four to eight times slower than the rate of terminal AMP turnover.

3.(v) Homopolymers

Reports on the base composition of pulse labelled RNA fractions frequently show a high content of adenine (Hoyer, McCarthey, and Bolton, 1963; Harris *et al*, 1963; Henshaw, Revel and Hiatt, 1965).

Hadjivassiliou and Brawerman (1965) described an RNA from rat liver, with a high adenine content, but which possessed poor template ability and sedimented in a rather homogeneous manner around 10S. Recently the same workers (1966 in press) have succeeded in isolating material from rat liver microsomes which apparently consists almost entirely of polyA and which sediments at about 10S. The function of this polynucleotide is not known. Extracts of thymus nuclei have also been shown to contain polyA, (Edmonds, and Abrams, 1962 and 1963).

Nothing is known about the mode of synthesis of the rat liver polyA. The thymus polynucleotide is apparently synthesised under the action of a specific polyA dependant polyA polymerase (see 5.(ii) of this section).

4. Templates for RNA Synthesis

The experiments discussed in section 3.(i) make it clear that the major portion of RNA synthesis in animal cells is localized in the nucleus, and in particular on the chromatin fraction. Chromatin comprises the bulk of cellular DNA, an approximately equal amount of basic histone, variable amounts of non histone protein and RNA, and a small proportion of phospholipid and phosphoprotein, (Dingman and Sporn, 1964; Frenster, 1965).

The dependance of RNA synthesis on chromatin, and the presence of the genetic material in that fraction, clearly suggest the possibility that sections of the DNA may act as templates for the synthesis of cellular RNA. That this is indeed the case is shown by the results to be discussed below.

4.(i) RNA synthesis and actinomycin

Actinomycin is a bacteriostatic, tumour-inhibiting chromopeptide antibiotic which inhibits the synthesis of nucleic in intact bacterial and animal cells, and by enzyme preparation. The most widely used actinomycin has been actinomycin D., (Figure 2). Low concentrations of actinomycin selectively suppress cellular (Reich, Franklin, Shatkin and Tatum, 1961 and 1962) and enzymatic RNA synthesis (Reich, Goldberg and Rabinowitz, 1962; Goldberg, Rabinowitz and Reich, 1962; Hurwitz, Furth, Malamy and Alexander, 1962), DNA synthesis being affected only at much higher concentrations (Kirk, 1960; Hurwitz et al, 1962; Keir, Omura and Shepherd, 1963).

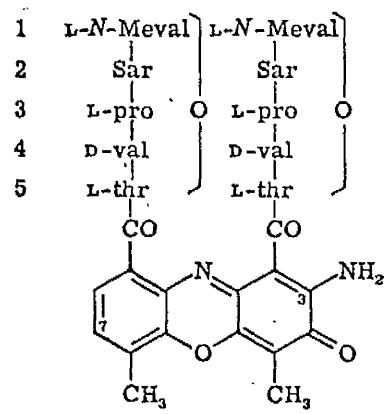
The inhibition of RNA synthesis requires the binding of

F I G U R E 2

The structure of Actinomycin D (C)

(From Reich, E., in "Progress in Nucleic Acid Research and Molecular Biology", volume 3, 1965, edited by Davidson, J.N. and Cohn, W.E., Academic Press, N.Y. and London, p.186)

Figure 2.



Actinomycin C_1

FIG. 1. Structure of actinomycin C_1 (D) (*3*, *4*). thr = L-threonine; val = D-valine; pro = L-proline; Sar = Sarcosine; Meval = L-*N*-Methylvaline.

actinomycin to DNA (Reich, Goldberg and Rabinowitz, 1962). When DNA is added to solutions of the antibiotic, its peak extinction in the visible region is reduced and shifted to longer wavelengths. This effect was used by Kersten to find out which constituents of DNA would bind with the inhibitor, (Kersten, 1961). He found that spectral changes could be reproduced using various purine derivatives in the following order of effectiveness;

deoxyguanosine guanosine adenine = AMP = ATP = adenosine = deoxyadenosine : pyrimidine derivatives were inactive. Kersten concluded that actinomycin reacts with DNA guenine.

Despite the subsequent proof of the validity of this conclusion, it would appear that the model reaction with purine derivatives differs in some respects from, and is less specific than, the actinomycin-DNA interaction, (Reich, 1964). For example, actinomycin complexes with neither DNA-adenine, (Goldberg, Rabinowitz and Reich, 1962; Kahan, Kahan and Hurwitz, 1963), nor with RNA, (Hamilton, Fuller and Reich, 1963; Haselkorn, 1964). DNA must provide more than a simple reactive nucleoside. Since s-RNA, which is highly double helical, but like all RNA in the A configuration, cannot bind the inhibitor, it seems probable that helical structure in the B configuration is required.

The importance of the helical configuration in the reaction of DNA with actinomycin has been confirmed by binding studies using different templates. Thus the maximum binding capacity of single-stranded or heated DNA is less than native DNA with a similar base composition (Goldberg et al, 1962; Kahan et al,

1963; Reich, 1964). Dissociation of the actinomycin-DNA complex begins at the onset of strand separation when the complex is heated (Reich, 1964). Apyrimidinic DNA, which contains no regular secondary structure, reacts much less efficiently than native DNA (Goldberg et al, 1962).

The presence of DNA guanine in the helical structure is obligatory for both binding and inhibition. The maximum amount of actinomycin bound by DNA preparations parallels their guanine content, although it is not directly proportional to it, (Goldberg et al, 1962; Kahan et al, 1963). Helical DNA which contains no guanine fail to bind the antibiotic.

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

Many of the studies on actinomycin have utilised a system comprising purified bacterial RNA polymerase in vitro. In such systems the susceptibility of a DNA-directed RNA synthetic reaction, is completely dependant on the capacity of the template to bind the antibiotic (Goldberg et al, 1962; Kahan et al, 1963). Actinomycin does not inhibit the enzyme directly, nor does it compete with nucleotide precursors or cofactors. Thus, the inhibition cannot be overcome by increasing amounts of enzyme or nucleotides; it can, however, be overcome by increasing amounts

DNA (Goldberg and Rabinowitz, 1962; Hurwitz et al, 1962).

The bacterial enzymes may also use RNA preparations as templates for RNA synthesis. In this case, however, the reaction is completely resistant to the action of actinomycin (Goldberg, Reich and Rabinowitz, 1963; Fox, Robinson, Haselkorn and Weiss, 1964; Krakow and Ochoa, 1963). In addition, cells infected with RNA viruses develop specific RNA dependant RNA synthesising systems which are also resistant to the inhibitor (see 5.(i) of this section).

These experiments make it clear that actinomycin is a powerful and specific inhibitor of DNA-dependant RNA synthesis and is an extremely useful tool in determining the dependance of RNA synthesis on DNA in vivo.

There is general agreement that actinomycin inhibits the in vivo synthesis in animal, plant and bacterial cells not only of nuclear, but of cytoplasmic RNA (Reich, Franklin, Shatkin and Tatum, 1962; Mach and Tatum, 1963; Merits, 1963; Franklin, 1963; Girard et al, 1964). When the RNA of actinomycin treated, pulse labelled cells is analysed by sucrose gradient centrifugation up to 99% inhibition of the synthesis of high molecular weight can be achieved under conditions where a much smaller inhibition of incorporation into 4S-RNA is observed. For example, in rats treated with 0.8 mg/Kgm of actinomycin D, 90 - 95% inhibition of liver r-RNA synthesis is achieved, but only 50 - 70% inhibition of liver s-RNA synthesis (Moule and Landin, 1965). Merits (1963) demonstrated that in the livers of rats labelled with P^{32} phosphate after administration of 4 mg/Kgm of actinomycin D, up to 70% of the label could be recovered in the terminal OMP of s-RNA, following

alkaline hydrolysis. This has been attributed to the turnover of the terminal nucleotides of s-RNA in liver (Harel et al, 1964), HeLa cells, (Tamaoki and Mueller, 1962) and L cells, (Franklin, 1963).

It should be noted, however, that in these experiments, a significant portion of radioactivity could be recovered in the AMP, GMP and UMP, even at fairly high levels of actinomycin. Following the administration of C^{14}-orotic acid to rats treated with 1.5 mg/Kgm of the inhibitor, the ratios of the specific activities of the cytidylic, uridylic and pseudouridylic acids from liver s-RNA were almost identical to those in control animals (Revel and Hiatt, 1964b). Since it is known that pseudouridylic acid is exclusively localized in non-terminal positions in s-RNA, the possibility of residual synthesis of complete chains cannot be excluded. In this connection it is of interest that it has recently been suggested that pseudouridylic acid may be incorporated into preformed RNA in Esch. coli spheroplasts (Weiss and Legault-Demare, 1965).

At higher concentrations of the inhibitor the "incorporation" of label into internal sites of the s-RNA molecule is inhibited by over 90%, (Moule and Landin, 1965; Revel and Hiatt, 1964b). Although s-RNA is relatively resistant to the action of actinomycin, nonetheless it would appear to be synthesised in a DNA-dependant reaction.

Not all other classes of RNA exhibit the same susceptibility to actinomycin. Perry (1963) found that the incorporation of H^3 nucleosides into nucleolar and cytoplasmic RNA was suppressed

by low concentrations of the drug, where as the incorporation into RNA of the extranucleolar chromatin continued unabated. A relatively resistant nuclear RNA associated with chromatin has been described in ascites cells (Popovic, 1965) and Paul and Struthers (1963) have described the synthesised of a nuclear RNA in LS cells pulsed with high levels of actinomycin which was apparently degraded in the nucleus.

Reports frequently show the residual synthesis of poly-disperse RNA with a DNA-like base composition (Georgiev et al, 1963; Harel et al, 1964; Kempf and Mandel, 1966). It is not clear if the 8 - 10S resistant RNA of rat liver described by Harel et al, is related to the polyA of Hadjivassiliou and Brawerman (1966). Nonetheless, in most cases virtually all RNA synthesis can be abolished by raising the concentration of actinomycin.

The reason for the varying susceptibility of different RNA fractions is not clear. Both the base composition and length of the individual DNA templates is likely to be critical.

The experiments with actinomycin D provide compelling evidence that the bulk of cellular RNA is synthesised in a DNA-dependant reaction.

4.(ii) Hybridisation

Further information on the source of RNA can be obtained using the technique of specific hybridisation. When heat denatured or single stranded DNA and RNA are heated together and cooled slowly, then, provided the sequence of bases in the RNA molecule is complementary to that in regions of the DNA

they will react to form a hybrid containing one strand of RNA and one of DNA. The RNA in such hybrids is resistant to the action of ribonuclease (Yankofsky and Spiegelman, 1962).

The technique has been used to demonstrate sequence homology between DNA and r-RNA in bacterial (Yankofsky and Spiegelman, 1962b; 1963 Ibid; Attardi, Huang and Kabat 1965a), plant (Chipchase and Birnstiel, 1963) and animal cells (McConkey, and Hopkins, 1964; Perry, Srinivasan and Kelly, 1964; Attardi, Huang and Kabat, 1965b). The results suggest that the two r-RNA molecules are each derived from distinct sequences of the DNA. Attardi et al (1965 a and b) estimated that Esch. coli DNA contains the equivalent of 1 - 6 stretches complementary to 16S RNA; the figures for HeLa cells are 200 - 400 and 400 - 600 respectively. Some uncertainty is introduced because of overlapping sequences in the two r-RNA species. Complementarity between the 5S RNA and DNA of Esch. coli has been reported (Zehavi-Willner, and Comb, 1966).

S-RNA has also been shown to form specific hybrids with its homologous DNA (Goodman and Rich, 1962; Giacomoni and Spiegelman, 1962). Approximately 40 stretches equivalent to s-RNA were found in Esch. coli DNA, supporting the known degeneracy of the genetic code.

In 1961, Hall and Spiegelman found that the rapidly labelled RNA formed in T2 phage infected Esch. coli hybridised only to the T2 DNA. These observations have been extended to demonstrate sequence complementary between the rapidly labelled RNA and the homologous DNA of uninfected bacterial cells (Hayashi and Spiegelman, 1964; McCarthy and Bolton, 1964).

Perry et al (1964) have shown that rapidly labelled RNA from L cells in culture can hybridise to L cell DNA. The ribosomal RNA competed with the 40S and 50S nucleolar RNA for sites on the DNA, supporting the hypothesis that these molecules are ribosomal precursors.

Paul and Gilmour (1966 a and b) used the hybridisation technique to demonstrate that, as a consequence of cytodifferentiation, different regions of the rabbit bone marrow and thymus genomes were available for transcription. From their figures for the percentage of the DNA being transcribed it can be deduced that RNA which was neither soluble nor ribosomal formed specific hybrids.

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

4.(iii) Asymmetric Synthesis of RNA.

Having established that RNA appears to be synthesised on a DNA template, the question then arises -- is one strand, or both strands of the DNA copied?

An indication of the most probable answer to this question was given by the experiments of Bautz (1963) who found that in T4 phage infected Esch. coli the phage specific RNA was always single stranded and would not anneal to itself. In both uninfected and T2 phage infected Esch. coli rapidly labelled RNA could hybridise to only one half of the homologous DNA (McCarthy and Bolton, 1964; Hall, Green, Nygaard and Boezi, 1963). Examination of the effect of fluorouracil on the phenotypic reversion of rII mutants of T4

phage, following infection of Esch. coli, showed about half of the T- A pairs estimated to be present in the mutant phage DNA were found to revert (Champe and Benzer, 1962). This would be expected if about half of the A residues, i.e. equivalent to one strand, could pair with fluorouracil in the formation of phage specific RNA. Fox and Meselson (1963) concluded from the effects of photo inactivation of ∝ phage DNA containing s-bromouracil in one strand, that only one strand of the DNA performed a vital function in vivo.

These experiments suggest that only one strand of DNA is transcribed in vivo. 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 Robinson, 1963).

More concrete evidence has been obtained by hybridisation of phage specific RNA from phage ∝ infected B. Megatherium and SP 8 infected B. subtilis with the homologous DNA. The constituent strands of the DNA from these phages can be separated by physical means, since one strand in each is rich in pyrimidines and the other rich in purines. The pyrimidine-rich strands are more dense than the purine-rich strands, and can be separated from them by density gradient centrifugation. Specific hybrids are formed only with the heavy strand in each case (Tocchini-Valentini, Stodolsky, Aurisicchio, Sarnat, Graziosi, Weiss, and Geiduschek, 1963; Marmur and Greenspan, 1963). Similarly m-RNA from phage ϕ ∝ 174 infected Esch. coli does not hybridise with the single stranded DNA of the mature phage, but only with the DNA of

the double stranded replicative form (Hayashi, Hayashi and Spiegelman, 1963). This indicates that the phage specific RNA is transcribed from the negative strand of the double helical replicative DNA.

More than 90% of the newly synthesised RNA of Krebs II ascites tumour cells is sensitive to traces of ribonuclease and will not hybridise to itself, (Shankin and Burdon, 1966). On the basis of this and the characteristics of hybridisation of the RNA to ascites DNA, it was concluded that RNA synthesis in these cells was asymmetric.

Highly purified preparations of bacterial RNA polymerases can transcribe template DNA in an asymmetrical manner. Using a crude supernatant fraction of B. megatherium and phage α DNA as template. Goldschek, Tocchini-Valentini and Samat (1964) found that the asymmetry of synthesis was dependant on the native configuration of the DNA, but not on the continuity of the phage chromosome. When fragmented, native T4 DNA serves as primer for a partially purified RNA polymerase from Esch. coli, over 90% of the synthetic RNA had nucleotide sequences identical to those found in T4 specific RNA synthesised in vivo (Green, 1964). Since only one strand of T4 DNA is transcribed in vivo, it was concluded that the same strand was utilised in vitro. The strand selection mechanism was destroyed by heat denaturation, but also by sonication. Asymmetric synthesis has also been obtained with a variety of heterologous enzyme-template systems (Colvill et al, 1965).

On the other hand the asymmetric synthesis occurring with a

highly purified, nuclease free, Esch. coli polymerase and double stranded ϕ x174 DNA was dependant on the circularity of the phage DNA. Breakage of the circle led to loss of the strand selection mechanisms (Hayashi, Hayashi and Spiegelman, 1964). This is in direct contrast to the experiments described above where there is no doubt that the major portions of the templates were degraded to some degree. The mechanisms of asymmetric synthesis remain obscure.

4. (iv) Exceptions to the Rule : RNA Viruses

Bacterial and animal cells infected with RNA containing viruses develop RNA dependant synthesising systems for the production of new viral DNA. On entry into the host cell, the viral RNA most likely serves as a protein program for the synthesis of a virus specific RNA dependant RNA polymerase, termed RNA replicase by Spiegelman (Spiegelman and Hayashi, 1963). The replicase must then synthesise new strands of virus RNA.

Theoretically, this could be achieved by two different kinds of mechanisms; 1) the replication could mimic RNA production in uninfected cells, a "copying" mechanism using Watson-Crick type base-pairing to produce replicas of viral RNA. 2) the virus systems could use a different kind of device.

The most popular model stem from the studies of virus ϕ x174 which contains single stranded DNA. On infection the single stranded DNA is converted to a double stranded form (Sinsheimer, Starman, Naglar and Guthrie, 1962). This serves as a template for the synthesis of virus specific m-RNA. It has been shown that the

RNA is complementary to the new RNA, or "minus"strand, (Hayashi, Hayashi and Spiegelman, 1963).

Ochoa et al proposed that the first step in the replication of viral RNA would be the conversion of the incoming RNA into a double stranded structure which could serve as a "replicating form" (RF) for the generation of single stranded copies of virus RNA (Ochoa, Weissmann, Borst, Burdon, and Billeter, 1964).

Montagnier and Sanders were the first to describe the formation of double stranded, virus specific RNA in ascites cells infected with EMC virus (Montagnier and Sanders, 1963). Reports of the same observation in other infected cells soon accumulated in the literature . (Kaesmer and Hoffmar-Berling, 1964; Monagama, Ikeda, 1964; Eriksson, Fenwick and Franklin, 1964; Amman, Delius and Hofschneider, 1964; Shipp and Haselkorn, 1964; Weissmann, Borst, Burdon, Billeter and Ochoa, 1964; Billeter, Weissmannand Warner).

In these cells a new RNA replicase appears (August, Cooper, Shapiro and Zinder, 1963; Weissmann, Simon, Borst and Ochoa, 1963; Eason, Cline, and Smellie, 1963; Baltimore and Franklin, 1963; Wilson and Bader, 1965; Haruna and Spiegelman, 1965).

The virus induced replicase from MS 2 infected Esch. coli has been partially purified (Weissmann, Simon and Ochoa, 1963; Weissmann, Simon, Borst and Ochoa, 1963). The reaction requires all four nucleoside triphosphates and Mg^{++} and is insensitive to actinomycin. No dependance on added RNA was observed, but the enzyme preparation contained quantities of RNA which Longridge

et al showed to be double stranded and similar to the RNA formed in vivo (Langridge, Billeter, Borst, Burdon and Weissmann 1964). In vitro the enzyme synthesised plus strands of viral RNA. The product is largely ribonuclease sensitive, but if protein denaturing agents or elevated temperature are employed, approximately 60% of the newly synthesised RNA becomes converted to a ribonuclease resistant form (Weissmann Borst, Burdon, Billeter and Ochoa, 1964b; Borst and Weissmann 1965). Similar results have been obtained in animal cells. When Krebs 2 ascites cells are infected with EMC virus, a new replicase appears in the large particle fraction of the cytoplasm (Horton, Lin, Martin and Work, 1966). The enzyme synthesised both single stranded and double stranded RNA in vitro, apparently from an endogenous template (Dalgarno, Martin, Lin and Work, 1966).

When P^{32} labelled MS2 phage are allowed to infect Esch. coli a double stranded RNA is formed which contains radioactive plus strands (Weissmann et al, 1964a). The radioactivity in the double stranded RNA reaches a peak soon after synthesis and then declines.

These results are so far consistent with a mechanism whereby incoming viral RNA strands are converted to a double stranded intermediate by the synthesis of a complementary (or minus) strand. Plus strands are then displaced by the synthesis of new plus strands into the RF RNA by the replicase. Extracts of Esch. Coli infected with SU II, a mutant of phage f2, are capable of forming a double stranded product from a single-stranded template, (August, Shapiro

and Hoyang, 1965; Shapiro and August, 1965).

However, it is not known if more than one enzyme is involved in the mechanism. By analysis of RNA phage mutants, Lodish and Zindor have shown that the two steps of RNA replication can be genetically dissociated, (Lodish and Zinder, 1966).

However, the experiments of Spiegelman and his colleagues with the phages Qβ and MS2 suggest an alternative solution. The replicases specific for these phages have been purified completely free of contaminating nuclease activity (Haruna and Spiegelman, 1965a; Overby, Barlow, Doi, Jacob and Spiegelman, 1966;) The following features distinguish the purified replicases from other preparations; 1) complete dependance on added RNA, 2) competence for prolonged synthesis of RNA, 3) ability to synthesise many times the input RNA, 4) saturation of the enzymes at low RNA concentration and 5) virtually exclusive requirement for homologous template, (Weissmann, Simon, Borst and Ochoa, 1963; Haruna and Spiegelman, 1965b).

The latter property is particularly striking and constitutes the first demonstration of a polymerase type of enzyme which exhibits complete and selective preferance for its homologous polynucleotide. Haruna and Spiegelman (1965b) have proposed that the recognition site is a secondary structure formed by two complementary sequences, one at the beginning and one at the end of the molecule.

There is no doubt that the replicases synthesize mature viral RNA <u>in vitro</u>. When the Qβ enzyme is primed with small amounts of Qβ RNA, autocatalytic synthesis of RNA is observed which becomes

linear when 1 µg of RNA per 40 µg of enzyme have accumulated (Haruna and Spiegelman, 1965a,). The RNA produced in the reaction is infectious and exhibits the same template properties in the replicase reaction as input RNA. When MS2 RNA is synthesised in vitro by serial transfer until less than one strand of input RNA per tube remains, the product is just as infectious as the RNA isolated from mature virus (Spiegelman, Haruna, Holland, Beaudreau and Mills, 1965).

No heat-sensitive ribonuclease-resistant material could be detected during the reaction. Neither the base composition of the product, nor its capacity to hybridise with plus strands of viral RNA, afforded compelling evidence for a double stranded intermediate (Haruna and Spiegelman, 1966). To the contrary, the results were explainable in terms of synthesis of product RNA from a template possessing a beginning sequence rich in adenine and another sequence complementary to it further on in the chain. In contrast, when purified Qβ replicase is presented with fragmented template RNA the reaction is slow and incomplete, and the product biologically inactive (Haruna and Spiegelman, 1965b). In these circumstances the product was found to be ribonuclease resistant.

These observations clearly throw some doubt on the interpretation of the experiments described earlier. Spiegelman maintains that although double stranded RNA is found in infected cells, there is no direct proof of its role in RNA replication. The experiments using fragmented Qβ-RNA obviously suggest that "RF" RNA may be produced as a by-product of an abnormal reaction brought on by the condition of the cells.

This question is of some importance to the question of the general mechanism of polymerase-type reactions. If Spiegelman is correct, a mechanism quite different to that proposed for other systems may have to be invoked. In order to synthesise "plus" strands from "plus" templates, a device with the ability to transcribe A as A and U as U etc. is necessary. Such a mechanism is, of course not without the known capabilities of enzyme protein catalysts, but would not employ normal Watson-Crick type base-pairings. The possibility that a "ghost" complementary chain, consisting of unpolymerised nucleotides hydrogen-bonded in the normal way to the template, might act as an intermediate in the reaction cannot be ruled out.

In conclusion it must be emphasised that the various areas of conflict raised by the two different points of view remain unresolved. The solution must await further experiments.

5. The *in vitro* synthesis of RNA

5.(i) Polynucleotide phosphorylase

The first enzyme isolated which catalysed a nett synthesis of nucleic acid was the polynucleotide phosphorylase from *Azotobacter vinelandii* (Grunberg-Manago, Ortiz and Ochoa, 1955). Enzymes with similar properties have since been demonstrated among a wide variety of aerobic and anaerobic bacteria in yeasts and in plant cells. Ribonucleoside diphosphates are required as substrates and are incorporated into polyribonucleotides from reaction mixtures containing a single ribonucleoside diphosphate or a mixture of ribonucleoside diphosphates and Mg^{++}. Neither nucleoside mono- nor nucleoside tri-phosphates will serve as substrates. Crude enzyme preparations do not require added RNA primers, but more purified preparations do.

The enzyme was believed for some years to be responsible for the *in vivo* synthesis of RNA. However in the RNA-primed reaction the base composition of the product reflects the base composition of the ribonucleoside diphosphates of the reaction mixture, rather than that of the primer (See Grunberg-Manago, 1963, for a review). The reaction proceeds by the addition of monomer units to the 3′ end of the primer in a chain extension mechanism (Singer, Heppel and Hilmoe, 1957 and 1960). The reaction requires a free 3′OH in the primer and stoichometric amounts of inorganic phosphate are released.

Accordingly the enzyme is no longer held to be responsible for RNA synthesis *in vivo*. Despite this, the enzyme has been a useful tool. In the absence of added primer polymerisation

proceeds after a lag. Using single ribonucleoside diphosphates in the reaction mixtures it has been possible to synthesise a variety of homopolymers.

The precise *in vivo* function of the enzyme remains unclear. It has been suggested that its primary function may be the elimination of the genetic information in m-RNA by stepwise degradation of the molecule (Cohen, 1961). However, the rate of phosphorolysis of most RNA preparations is low (Grunberg-Manago, 1959; Ochoa, 1957).

For many years there has been no satisfactory demonstration of polynucleotide phosphorylase activity in animal cells, possibly because the activities of adenylate kinase and related enzymes are so great as to destroy nucleoside diphosphates as they are formed. However, recently it has been possible to demonstrate the presence of activity in rat liver nuclei, which contain virtually no adenylate kinase (Siebert, Villabos, Suk Ro, Steele, Lindenmayer, Adams and Busch, 1966). Harris (1963) has described the breakdown of RNA in the nucleus of HeLa cells by an enzyme with the characteristics of a polynucleotide phosphorylase.

5. (ii) RNA polymerase

In view of the DNA-dependance of RNA synthesis *in vivo* it is not surprising that enzyme preparations can be isolated from a wide variety of sources which catalyse the synthesis of RNA in a DNA dependant reaction. Such enzymes are called RNA polymerases. Crude cell extracts which require the presence of the four ribonucleoside triphosphates and DNA have been described in bacterial cells (Hurwitz, Bresler and Diringer, 1960; Stevens, 1960;

Ochoa, Burma, Krüger and Weill, 1961; Weiss and Nakamoto, 1961; Colvill, Kanner, Tocchini-Valentini, Sarnat and Geiduschek, 1965) plant cells, (Rho and Bonner, 1961; Mans and Novelli, 1964; Semal, Spencer, Kim and Wildman, 1964), and from animal sources (Weiss, 1960; Goldberg, 1961; Burdon and Smellie, 1961; Strauss and Goldwasser, 1961; Baltimore and Franklin, 1962; Furth and Loh, 1963; Suk Ro, Moramatsu and Busch, 1964; Tsukada and Lieberman, 1964; Widnell and Tata, 1964; Mason and Smellie, 1965).

In many of these preparations the enzyme is particulate and strongly bound to DNA. In higher cells, the activity is associated with the nucleus and is found in both nucleolar (Suk Ro et al, 1963; Suk Ro and Busch, 1964) and chromatin fractions (Huang and Bonner, 1962). DNA-dependant uptake of ribonucleoside triphosphates into RNA has been reported in mitochondria (Kalf, 1964; Luck and Reich, 1964) and chloroplasts (Kirk, 1964) and these cellular organelles have been shown to contain DNA (Luck and Reich, 1964; Rabinowitz, Sinclair, Desalle, Haselkorn and Swift, 1965; Edelman, Cowan, Epstein and Schiff, 1964; Ray and Hanawalt, 1964).

Most of the studies on the characteristics and mechanism of RNA polymerase have utilised highly purified bacterial preparations. Recently soluble RNA polymerase preparations have been isolated from rat testes (Ballard, and Williams-Ashman, 1966) chicken embryo, (Furth and Loh, 1963) and Lymphosarcoma (Furth and Loh, 1965). Ramuz, Doly, Mandel, and Chambon (1965) describe the presence of soluble RNA polymerases from the nuclei of the rat liver, brain,

kidney, spleen, testes and prostate, and a soluble enzyme is found in pea seedlings (Stout and Kane, 1966). Preliminary results suggest that these enzymes have similar properties to the bacterial polymerases to be discussed below.

RNA polymerase has been isolated in a highly refined form from Micrococcus lysodeikticus (Nakamoto, Fox and Weiss, 1964), Esch. coli (Furth, Hurwitz and Anders, 1962; Chamberlin and Berg, 1962; Stevens and Henry, 1964) and Azotobacter vinelandii (Krakow and Ochoa, 1963). The enzymes require all four nucleoside triphosphates, a divalent metal ion (Mn^{++}, Co^{++}, Mg^{++}) and a DNA primer (Fox and Weiss, 1964; Stevens and Henry, 1964; Krakow and Ochoa, 1963). Denatured DNA is a somewhat less effective template than native DNA, the presence of polyamines enhances the activity of the native DNA directed reaction, and ribonucleotides are incorporated into RNA polymers containing all four ribonucleosides.

In addition to this "normal" reaction, RNA polymerase preparations are capable of catalysing the formation of homopolymers when only one ribonucleoside triphosphate is presented to the enzyme. The reaction is dependant on the presence of DNA, but denatured DNA appears to serve as a better primer than native DNA. Mn^{++} is required rather than Mg^{++}. (Fox and Weiss, 1964; Stevens, 1964; Chamberlin and Berg, 1962). The presence of the other ribonucleoside triphosphates inhibits homopolymer formation. It is not certain why the enzyme catalyses this reaction, or if it is significant *in vivo*. It has been suggested that synthesis might occur by a "slippage" mechanism over short

sequences of homopolymers in the DNA template. The response to denatured DNA, and the observation that polyamines fail to stimulate the reaction (Krakow, 1963; Fox and Weiss, 1964; Stevens 1964) suggests a mechanism different from that of heteropolymer formation.

Synthetic and natural polyribonucleotides can also serve as templates. Poly A, U and C have been shown to separately prime the incorporation of the complementary nucleotides (Krakow and Ochoa, 1963; Fox, Robinson, Haselkorn and Weiss, 1964). This type of reaction requires Mn^{++} rather than Mg^{++}, and polyamines are distinctly inhibitory. Competitive inhibition is observed between DNA and RNA for the enzyme, and this, along with data on the priming ability of various nucleic acids, at different stages of enzyme purification, suggests that only one enzyme is involved. Again, the *in vivo* significance of this activity is uncertain.

In the DNA primed reaction the base composition of the product RNA is dictated by the DNA template. The nearest neighbour frequencies of RNA product are in close agreement with those of template DNA (Weiss and Nakamoto, 1961 a and b; Furth, Hurwitz and Anders, 1962; Hurwitz, Furth, Anders and Evans, 1962; Hurwitz, Furth, Malamy and Alexander, 1962; Krakow and Ochoa, 1963). When synthetic polydeoxyribonucleotides such as dAT are used, only the ribonucleotides complementary to those in the templates are incorporated into RNA (Furth *et al*, 1962, Chamberlain and Berg, 1962; Krakow and Ochoa, 1963; Stevens and Henny, 1964). Finally, the RNA synthesised by the enzyme *in vitro* can form specific

hybrids with its template DNA (Geiduschek, Nakamoto and Weiss, 1961; Robinson, Hsu, Fox and Weiss, 1964; Colvill et al, 1965). These results are consistent with a mechanism in which the base sequence of DNA is rigorously copied in the synthesis of RNA.

In addition, when single stranded ϕ X 174 DNA is presented to the polymerase, the first product of the reaction is a DNA-RNA hybrid, providing further confirmation of a copying mechanism (Bassel, Hayashi and Spiegelman, 1964; Sinsheimer and Lawrence, 1964; Chamberlain and Berg, 1964). Similar formation of DNA-RNA hybrids have been described when heat denatured DNA is primer (Warner, Samuels, Abbotband Krakow, 1963). The DNA-RNA hybrid formed with ϕ X174 DNA can serve as a template for further RNA synthesis. The reaction, however, appears to be abnormal. Evidence has been presented that RNA in the hybrid is displaced during the formation of new RNA in a semi-conservative type of mechanism (Chamberlain and Berg, 1964).

The assymetric synthesis of RNA both in vivo and in vitro (4(iii) of this section) indicates that only one strand of the DNA template is copied. The mechanism of strand selection remains obscure. Asymmetric synthesis of RNA, however, does suggest assymetry in the enzyme and a selective mechanism of attachment of the enzyme to the DNA template.

Electron micrographs of Esch. coli RNA polymerase reveal a structure consisting of 6 sub-units arranged in a hexagonal configuration round an empty centre (Fuchs, Zillig, Hofschneider, and Preuss, 1964; Colvill, von Bruggkee and Fernandez-Moran, 1966). The enzyme sediments through sucrose gradients with a

sedimentation coefficient of 24S. The hexagonal structure was also observed in crude extracts of the cells. Aggregates of the enzyme and breakdown products can be observed, which also have enzymatic activity. The results suggest that an 18S particle consists of 3 of the subunits. It is not known if the subunits differ in any way from each other. It was possible to observe the enzyme attached to native DNA in the electron microscope.

Crawford, Crawford and Richardson and Slayter (1965) also observed enzyme-DNA complexes in the electron microscope. Under conditions where the DNA was saturated with enzyme, only 8 - 9 polymerase molecules could be observed per molecules of polyoma DNA (M.wt = 3×10^6) and approximately 11 - 14 per molecule of papilloma DNA (m.wt = 5.3×10^6). The ratio of enzyme to template was approximately the same in each case. The amount of enzyme bound was considerably smaller than would be expected if polymerase could bind at any point on the DNA, and obviously suggests restricted sites of attachment. Bremer, Konrad and Bruner (1966b) found that only 180 growing chains of RNA could be accommodate on phage T7 DNA <u>in vitro</u>. In the synchronous transcription of circular RFϕX174 DNA by an <u>Esch. coli</u> RNA polymerase <u>in vitro</u>, five discrete sizes of RNA were synthesised, up to and including an RNA which represented transcription of the complete ϕX174 genome (Hayashi, 1965). This result provides strong confirmatory evidence for the existence of specific sites for the initiation of RNA synthesis in the

ϕx174 molecule. The nature of such sites remains obscure, but may be specific sequences of nucleotides in the DNA. The sites are likely to be similar throughout the bacterial world, since assymetric synthesis of RNA has been observed in a number of heterologous enzyme-template systems (Colvill et al, 1965).

When the DNA template is heat denatured, the number of sites available for the initiation of RNA synthesis as measured by the number of growing RNA chains per DNA molecule, increases greatly, (Maitra and Hurwitz, 1965; Bremer, Konrad and Bruner, 1966b). Under these circumstances much shorter RNA chains are formed, loss of assymetric transcription is observed, and it is considered that the selectivity of the attachment mechanism has been lost.

It is of interest that Bremer et al (1965 and 1966b) report the presence of two classes of polymerase in their preparations. One which leads to abortive synthesis of RNA and which forms only very short chains, they term an "early quitter", while the enzyme responsible for the synthesis of long RNA chains is a "late quitter". It is not known if "early quitters" are related to the fragments of polymerase described by Fuchs et al (1964) and Colvill et al, (1966).

If the model described for actinomycin action is correct (4.(i) of this section), then it may be considered that RNA polymerase binds to the minor groove of the double helix of DNA. The polymerase binds extremely tightly to the template. This has been borne out by experiments where the ability of RNA to inhibit the reaction was measured. Both r-RNA and s-RNA inhibit the DNA-

dependant incorporation of nucleotides into RNA (Tissiéres, Bourgois and Gros, 1963). The inhibition is only effective, however, if the RNA is allowed to react with the polymerase before the addition of DNA. When RNA is added after the reaction has started no inhibition is observed (Fox, Gumport and Weiss, 1965; Bremer, Yegian and Konrad, 1966a). Krakow has demonstrated that the RNA formed in situ in the in vitro reaction can inhibit the enzyme (Krakow, 1966). By measuring the release of radioactive inorganic pyrophosphate from γ-labelled P^{32} ribonucleoside triphosphates, it was possible to follow the reaction in the presence of ribonuclease. Remarkable stimulation, which was dependant on DNA and sensitive to actinomycin, was obtained in the presence of the nuclease.

Using glycerol gradient centrifugations, it could be demonstrated that the inhibition by s-RNA involved binding of the RNA to the polymerase (Fox et al, 1965). The inhibition by RNA may involve competition with DNA for the active site for DNA attachment on the enzyme molecule. There is, however a separate site for product RNA, as the growing RNA chains and Template DNA can remain attached to the polymerase simultaneously (Hayashi, 1965; Bremer et al, 1966b).

From the kinetics of inhibition of RNA polymerase by acridines at varying concentrations of DNA and ribonucleoside triphosphates, Nicholson, B. and Peacock, A., (1966) concluded that the inhibitor could combine reversibly with the polymerase and occupy sites which both the free nucleotide bases and the DNA bases normally employ. It can be considered, therefore, that there are at least three

different active sites on the molecule, one for DNA bases, one for RNA bases and one for free nucleotide bases.

Polyribonucleotide synthesis starts at the 5′ end and grows in the direction of the 3′ end of the product RNA. The first nucleotide incorporated retains the triphosphate group esterified to the 5′ hydroxyl and can be identified as such throughout synthesis in vitro (Maitra and Hurwitz, 1965; Bremer, Konrad, Davies and Stent, 1965). Using phage T2, T5 and SP_3 DNA, the first nucleotide incorporated was predominately adenosine, followed by guanosine; very little cytidine or uridine was incorporated into the 5′ terminal position. Where a number of bacterial DNA preparations or thymus DNA were used as primers, guanosine was the predominant starting nucleotide. These findings implicate pyrimidines on the DNA in the initiation sites. No evidence for a homopolymer sequence was obtained.

The newly synthesised chains start as small sequences and grow during the reaction, remaining attached to the polymerase. During the transcription of double stranded ϕX174 DNA Hayashi (1965) observed the formation of RNA-DNA hybrids during the reaction which survived protein denaturing agents. The RNA in the hybrid region was approximately 50 nucleotides in length and was always located at the growing point of the chain. This confirms a number of reports over the years of the isolation of DNA-RNA hybrids in vivo (Spiegelman, Hall and Storck, 1961; Finamore and Volkin, 1961; Schulman and Bonner, 1962; Mead, 1964). Recently Hayashi and Hayashi, M. (1966) isolated a

DNA-RNA hybrid from ϕx174 infected Esch. coli and made the striking observations that the RNA from the hybrid was complementary only to the negative strand of the RF ϕx174 DNA.

These experiments provide strongly suggestive evidence that the formation of base pairs is part of the mechanism involved in polymerase action, and one consistent with a mechanism in which the DNA nucleotide bases swing out from the interior of the helix to facilitate base-pairing.

Finally the question of the release of the newly formed RNA remains. Much less is known about this. In some circumstances *in vitro* the RNA remains attached to the polymerase which can enter another cycle of transcription with the production of extra long RNA (Bremer *et al*, 1966b). It has been suggested recently that ribosomes may complex with the newly formed RNA and facilitate its removal from the template (Byrne, Leven, Bladen and Nirenberg, 1964; Stent, 1964). Whatever the significance of such a mechanism *in vivo*, it is not mandatory *in vitro*, since free RNA is released during the transcription of double stranded RF ϕx 174 DNA by highly purified Esch. coli RNA polymerase (Hayashi, 1965).

HOMOPOLYMER FORMATION

As well as the DNA dependant and virus specific RNA dependant polymerases, whose biological functions are well defined, there have been many reports in the literature of enzyme fractions which incorporate single ribonucleoside triphosphates into homopolymers.

A system incorporating AMP residues from ATP into RNA has been described in the soluble fraction of chick embryos (Chung, Mahler and Enrione, 1960). The enzyme requires Mg^{++} and RNA as primer. Terminal incorporation into the primer chain occurs. A similar system has been partially purified from the chorio allantoic membranes of eleven day old chick embryos. Chains of 8 - 11 residues are formed (Venkataraman and Mahler, 1963).

An enzyme from the pH 5 fraction of rat liver cytoplasm has been partially purified (Klemperer, 1963a). In the presence of Mg^{++}, AMP residues are incorporated terminally into a variety of polyribonucleotide primers, including r-RNA, s-RNA, poly U and poly C. Sequences of 3'-5 uridilate residues were formed. In the presence of Mn^{++} AMP is incorporated into r-RNA giving chains 20 residues long (Klemperer, 1963b). Other primers such as s-RNA, poly U and poly C also stimulated actively, but the best primers were found to be short chain oligonucleotides (Klemperer, 1964 and 1965). Poly A failed to serve as primer possibly because of its secondary structure.

Cytoplasmic fractions from ascites tumour cells which incorporate UTP into poly U have also been reported (Burdon and

Smellie, 1961 and 1962). An enzyme synthesising poly A chains up to 100 residues long has been purified from calf thymus nuclei. The purified preparation contained a naturally occurring poly A primer (Edmonds and Abrams, 1962 and 1963). An enzyme which was capable of incorporating CMP from CTP into internal and terminal positions of RNA has been described from calf thymus nuclei. The preparation contained an RNA which appeared to be heterogeneous and was active as primer (Edmonds, 1965). Chains up to 13 residues long could be synthesised. S-RNA preparations from a variety of sources were active primers but r-RNA was inactive. It is not known if this system is related to the synthesis of the terminal sequence of s-RNA.

A number of reports have described particulate fractions from a wide variety of cells which can support the uptake of nucleotides into RNA. An enzyme from Esch. coli ribosomes catalysed the RNA dependant incorporations of ribonucleotides into RNA. The enzyme has been purified and requires Mg^{++} and added RNA (August, Ortiz and Skalka, 1962; August, Ortiz and Hurwitz, 1962). Extensive polymer synthesis was observed only with ATP although the other nucleotides were incorporated to a small extent. The product was predominately poly A. (Strauss and Goldwasser, 1961). reported the ability of pigeon liver microsomes to incorporate UMP from UTP into RNA. The activity was stimulated by the presence of the other three triphosphates, but the nature of the product was not elucidated.

Cytoplasmic ribosome of microsomal preparations have been shown to support the RNA-dependant incorporation of nucleotides

into RNA, (Baltimore and Franklin, 1963; Mason and Smellie, 1965; Dalgarno, Martin, Liu and Work, 1966; Wykes and Smellie, 1966). In the case of ascites cells incorporation was predominately terminal addition. Preparations of nuclear ribosomes also support such incorporation (Burdon, 1963; Noora, 1966).

It is not considered that such RNA dependent RNA synthesis is connected with RNA-virus specific RNA replicases, since the characteristics of the two systems are quite distinct, and on deliberate infection of both animal and bacterial cell cultures, the replicases appear in a separate particulate fraction (Weissman, Simon, Barat and Ochoa, 1963; Baltimore and Franklin, 1963; Mason and Smellie, 1965; Horton, Liu, Martin and Work, 1966).

Also of note is an enzyme system which incorporates ribonucleotide triphosphates into polydeoxyribonucleotides. (Krakow, Kammen and Canellakis, 1961; Canellakis and Krakow, 1962). Any of the four ribonucleoside triphosphates are incorporated terminally into DNA. The enzyme is distinct from DNA polymerase and adds only one residue onto the ends of the DNA chain. The ribotidyl-DNA will then act as a primer in the rat liver system which incorporates UMP residues into poly U.

Whilst the function of these homopolymer synthesising systems is not well understood the formation of the pCpCpA sequence in s-RNA is of obvious biological importance. Cytoplasmic fractions of rat liver cytoplasm can incorporate AMP

from ATP into terminal positions of s-RNA chains (Heidelberger, Harbers, Leibman, Takagi and Potter, 1956). If the enzyme is first preincubated in the absence of substrate, the ability to incorporate AMP is reduced. This ability can be restored by the presence of CTP and it appears that the two nucleotides are incorporated in a ratio of one to two. No other ribonucleotides are incorporated and it appears that s-RNA is a specific acceptor for these enzymes (See Smellie, 1963, for a review).

Daniel and Littauer (1963) have purified an enzyme system from the 105,000 x g supernatant of rat liver, the main activity of which was the formation of the CCA terminal sequence of s-RNA. In addition, however, the uptake of UMP from UTP into s-RNA was noted. R-RNA, microsomal RNA, phage RNa and poly A or Poly C were inactive. The main product of this reaction was s-RNA..... XpCpU. s-RNA..... XpCpC failed to act as an acceptor.

AIMS OF THE PRESENT WORK

In a number of experimental systems, the RNA-dependant synthesis of RNA in cytoplasmic fractions of cells have been described. The present work is concerned with the elucidation of some of these enzyme activities in rat liver cytoplasm.

EXPERIMENTAL

1. Animal Strain

Mature, unmated female rats, 10-12 weeks old, of the Wistar strain were used. The animals were random bred in the Department animal house. Synthetic diet 41B, supplemented with minerals, was supplied by Oxoid.

2. Removal of the livers

The livers were first perfused with saline to remove blood. Animals were anaesthetised with ether, and the abdomens opened. The hepatic portal vein was closed, using artery forceps. A syringe needle, attached to a large reservoir of ice-cold 0.9% saline, was introduced into the vessel on the liver side of the block. The inferior vena cava was then severed and the saline allowed to perfuse through the liver until all the blood had been washed out. The organs were then quickly excised and placed in ice-cold vessels to be chilled, then weighed.

3. Preparation of Enzyme Fractions

3. (i) Cell fractionation using Tween 80

The method was derived from that described by Fisher and Harris, (1962) [4]. The chilled livers were diced with scissors and homogenised in a chilled Sirvica homogeniser (Sirvica, New York, U.S.A.) with 5 volumes of ice-cold 0.01M-tris-HCl buffer, pH 8.0 + 0.003 M - MgCl2 in 0.1 % (v/v) Tween 80. (L. Light & Co., Colnbrook, England). Seven passes sufficed to disrupt the cells and leave the nuclei intact.

The nuclei were obtained as a pellet on centrifugation at

600 g for 5.0 min. at $0^{o}C$, and supernatant material was removed with a pasteur pipette. The supernatant fluid was centrifuged at 10,000 x g for 15 min. at $0^{o}C$ to obtain the 10,000 x g sediment. The 10,000 x g supernatant liquid was centrifuged at 105,000 x g for 120 min. at $0^{o}C$ to obtain the microsome fraction. Particulate fractions were suspended in 0.01M-tris-HCl, pH 8.0.

3. (ii) Cell fractionation in sucrose solutions

A modification of the conditions of Munro et al (1965) were used. No Ca^{++} was included in the medium.

The chilled livers were diced with scissors and then homogenised gently in a Sirofca homogeniser with 2.5 volumes of 0.25M Sucrose, 0.001M-tris-HCl, pH. 8.0. It was observed that the nuclei in such homogenates were rather fragile. Five passes at half speed were sufficient to disrupt the cells leaving most of the nuclei intact.

The homogenate was centrifuged at 12,000 x g for 10 min. at $0^{o}C$ and the sediment discarded. The supernatant was centrifuged at 105,000 x g for 120 min. at $0^{o}C$ to obtain the microsome fraction. For enzyme assays the particulate fraction was suspended in 0.01M-tris-HCl, pH. 8.0.

3. (iii) Subfractionation of rat liver microsomes

The following method was employed to fractionate microsomes into Rough Surfaced Vesicles (RSV), Smooth Surfaced Vesicles (SSV) and "free" or "natural" ribosomes unattached to membranes. The RSV are believed to derive from fragments of the granular endoplasmic reticulum; i.e. endoplasmic reticulum

bearing adherent ribosomes on its outer surface (Moule, Y., Rouiller and Chauveau). The SSV probably include a variety of membranous forms devoid of ribosomes, such as the agranular endoplasmic reticulum, the plasma membrane and pinocytotic or secretory vesicles (Hallinan, 1965).

The method is derived from that of Hallinan and Munro (1965). The chilled, diced livers were homogenised in a Sirvica homogeniser in 4 volumes of 30% Sucrose, 0.01M̄-tris-HCl, pH 8.0, 0.003M̄ $MgCl_2$ and 0.0005 M̄ EDTA. About fifteen passes were necessary to disrupt the cells, leaving the nuclei intact. Particles larger than microsomes were removed by centrifugation at 18,500 x g for 20 min. at $0^{o}C$.

Rough surfaced vesicles (RSV) were obtained as a pellet on centrifuging the supernatant at 78,000 x g for 60 min. at $0^{o}C$. Two particulate fractions were observed at this stage. A heavier glutinous opaloscent pellet at the bottom of the centrifuge tube was termed the Rough Surfaced Vesicle Heavy (RSVH) fraction. A lighter fluffy pellet above this was called the Rough Surfaced Vesicle Light (RSVL) fraction. The RSVL fraction suspended very easily in buffer and could be separated from the RSVH. However it was almost impossible to avoid contamination of the RSVL with 78,000 x g supernatant.

SSV and "free" ribosomes were isolated from the 78,000 x g supernatant fraction by homogenization with 0.5 volumes of ice-cold 2:2:4-trimethyl-pentane (isooctane). Homogenisation was carried out in a Sirvica homogeniser until an emulsion had formed

(3-4 strokes). The system was centrifuged at 150,000 x g for 120 min. at 0°C. The SSV fraction centrifuged centripetally and formed a pellicle at the isooctane-aqueous interphase. "Free" ribosomes formed a pellet at the bottom of the centrifuge tube.

The particulate fractions were suspended in 0.01M-tris-HCl, pH. 8.0, for enzyme assays.

4. Enzyme Assays

4. (i) Nucleotidyl Transferase Assays

4. (i).1. Nucleotidyl Transferase activity was measured by estimating the incorporation of radioactive labelled ribonucleotides into acid insoluble products. All assays were performed in 5.0 x 0.5 m. or 5.0 x 0.25 in test tubes. Where possible a special mixture was made up to include all reagents common to a given assay. This was pipetted into ice-cold tubes followed by any variable reagents. The enzyme fraction was added last. Total reaction volume was 0.5 ml. After incubation the reaction was terminated either by rapidly freezing the tubes in a mixture of solid CO_2 in ethanol, and storing at -15°C until required, or by the addition of 5.0 ml of ice-cold 5% TCA and processing immediately. All assays were performed in duplicate.

4. (i).2. Preparation of samples for counting

The general procedure consisted of precipitating the RNA with acid followed by repeated washing of the precipitate to remove unincorporated nucleotides. The level of unincorporated nucleotides was measured by estimating the radioactivity in unincubated samples, (zero time counts).

Very high zero time counts were given by particulate fractions. Various methods of washing the RNA precipitate were tested. Unsatisfactory results were obtained using the methods of NaCl extraction of RNA (Davidson and Smellie, 1952)precipitation of the RNA followed by washing with a mixture of TCA and $Na_4P_2O_4$ precipitation of reaction mixtures on filter paper discs and washing and precipitation of RNA and trapping and washing on Millipore filters or Teflon coated glass fibre discs.

Finally a completely satisfactory method was evolved from a modification of the procedure described by Baltimore and Franklin (1963).

All operations were carried out in an ice bath. To the thawed reaction tubes was added 0.5 ml of ice-cold saturated $Na_4P_2O_7$ and 1.0 mg of carrier yeast RNA or bovine serum albumen. The tubes were shaken on a Vortex mixer and allowed to equilibrate for 10 secs. 5.0 ml of ice-cold 5% T.C.A. were then added and the tubes shaken once more. After equilibration for 10 min. the precipitate was collected by centrifugation at 600 x g for 10 min. at 0^oC and the supernatant fluid discarded.

The precipitates were broken up by shaking on a Vortex mixer and dissolved in 0.5 ml of ice-cold saturated $Na_4P_2O_7$. After 10 secs. 5.0 ml of ice-cold 5% T.C.A. were added and the solutions mixed thoroughly again. 10 min. were allowed for equilibration and the precipitates were collected by centrifugation for 10 min. at 600 x g at 0^oC. This step was repeated three times.

In experiments with ^3H or ^{14}C ribonucleotides the precipitate

of RNA and protein was drained and taken up in 0.1 ml. concentrated formic acid. The RNA was hydrolysed at 100°C for 15 min. The solutions were then diluted with 1.0 ml of deionised water, and aliquots removed for counting.

If the incorporation of P^{32} labelled nucleotides was being measured, the precipitates were washed with 5.0 ml of 1% potassium acetate in 95% ethanol, and the tubes drained. The precipitate was then dissolved in 1.0 ml concentrated formic acid, and aliquots removed for counting.

4. (i). 3. 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 counting of almost 50 per cent, and a background of 14-17 counts per min. An aliquot of the sample to be counted was dried onto a stainless steel planchette under an infra red lamp. The planchette was fitted into a planchette holder and placed in the machine. ^{32}P on chromatograms was detected using a Nuclear Chicago Actigraph fitted with a windowless gas flow counter.

For ^{3}H and ^{14}C labelled samples an aliquot (usually 0.5 ml) of the diluted hydrolysate in formic acid was dissolved in 8.0 ml of dioxan-based scintillation fluid (0.7% 2.5 diphenyloxasole [P.P.O.], 0.03% 1.4-Di-[2-(5-phenyloxazethyl)]- benzene [P.O.P.O.P.] and 10% naphthalene in scintillation grade dioxan). The samples were then counted in either a Nuclear Chicago Model 725 liquid scintillation spectrometer, or a Packard series 4,000 liquid scintillation spectrometer.

Efficiency of counting was determined by the channels ratio method and was 10-18% for ^{3}H and 75-80% for ^{14}C. Occasionally the radioactivity of RNA in column effluents was measured using Millipore filters (see section 10.) In this case the dried filters were counted in scintillation vials containing 10 ml of toluene based scintillation fluid, (0.5% P.P.O. plus 0.03% P.O.P.O.P. in ANALAR toluene).

4. (ii) Assay of alkaline phosphatase

E. coli alkaline phosphatase was supplied commercially as a satd. ammonium sulphate precipitate. This was diluted 1:100 for assay purposes. 0.1 ml diluted enzyme, 2 μ moles p-nitrophenyl-phosphate and 20 μ moles tris-HCl buffer, pH. 8.0, in a total volume of 0.5 ml were incubated for various times at 37°C. The reaction was terminated by cooling the tubes in crushed ice and adding 2.5 ml ice-cold 0.1M NaOH. The extinctions at 400 mμ was then measured. The amount of phosphate hydrolysed was calculated assuming that under these conditions Emax for p-nitrophenol is 12,000 (Ramsel, and Khorana, 1961). The activity of the undiluted enzyme was calculated to be 120 umoles/min/ml of phosphate hydrolysed.

4. (iii) Assay of snake venom diesterase

The snake venom diesterase was assayed as follows: 0.1 ml of an appropriate dilution of the enzyme was incubated at 37°C for the appropriate time with 2 μ moles p-nitrophenyl-pT, 1 μ mole of $MgCl_2$ and 20 μ moles of tris-HCl buffer pH 8.5 in a total volume of 0.5 ml. The reaction was stopped by the addition of 2.5 ml of ice-cold 0.1M NaOH and the extinction at 400 mμ measured. The

undiluted enzyme released 0.5 μmoles p-nitrophenyl/min./ml.

4. (iv) Assay of Nuclease activity of cell extracts

Nuclease activity was determined using the same reaction conditions as for nucleotide incorporation except that ribonucleoside triphosphates were omitted. When highly polymerised yeast RNA was used as substrate the reaction was stopped by the addition of 3.5 ml of ice-cold 1.0 M $HClO_4$ after the tubes had been cooled in crushed ice. The resulting precipitate was centrifuged down at 600 x g for 10 mins. at 0°C, and the extinction of the supernatant determined at 260 mμ. The amount of nucleotide hydrolysed was calculated assuming Emax 10,600. When p-nitrophenyl-pT was used as substrate the reaction was terminated by cooling the tubes in crushed ice followed by the addition of 2.5 ml of ice-cold 0.1M NaOH. The extinction at 400 mμ was determined and the amount of p-nitrophenyl released calculated assuming Emax 12,000.

5. The preparation of (^{32}P) UTP

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

5. (i) The preparation of (^{32}P) CTP

A solution of ^{32}P-labelled phosphoric acid (100 M curies carrier free ^{32}P and 1 m-mole phosphoric acid in aqueous solution) was concentrated to dryness *in vacuo* at 40°C to remove traces of HCl. 10 ml of anhydrous pyridine (prepared by storing over calcium hydride) and 1 ml. cyanoethyl alcohol were added and

the solution concentrated *in vacuo* to an oil at 40°C. A second portion of anhydrous pyridine was added and the solution again concentrated to an oil. Then 5 ml of anhydrous pyridine and 2.1 gm dicyclohexylcarbodiimide (DCC) were added and the reaction set aside overnight at room temperature in a well-stoppered flask.

Water (5.0 ml) was added to stop the reaction and the resulting mixture heated in a boiling water bath for 30 min. The mixture was concentrated to dryness *in vacuo* and 10 ml of water and 10 ml of saturated barium hydroxide was added to the residue. After 5.0 min. at room temperature, the pH was adjusted to 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 (CEP) which was collected after one hour at 0°C by centrifugation. The crystals were redissolved in water (5.0 ml) by adding a minimal volume of glacial acetic acid, neutralised with $Ba(OH)_2$, centrifuged to remove traces of insoluble material and recrystallised by adding 10 ml. of ethanol. The product was collected by centrifugation in a pre-weighed tube, washed with ethanol, acetone and finally ether. The yield at this stage was normally about 60%. The product was dissolved in water with the aid of acetic acid to a final volume of 20 ml., and passed through a Dowex-50-H^+ column (8 cm. x 2 cm.). The effluent was taken to dryness and dissolved in anhydrous pyridine to which isopropylidene uridine had been added in the ratio 1 m-mole isopropylidene uridine per 0.5 m-mole cyanoethylphosphate.

5. (ii) The preparation of (^{32}P) UMP

The above solution was concentrated to an oil *in vacuo* at 40°C. 10.0 ml. of anhydrous pyridine was added and the solution again concentrated to dryness. The process was repeated once more and the residue was dissolved in 5.0 ml of anhydrous pyridine and DCC was added in the proportion 2.0 m-moles DCC per m-mole isopropylidene uridine.

After 20 hr. at room temperature, the well stoppered flask was opened and 10 ml of water added. After one hour the mixture was concentrated to dryness *in vacuo*. The residue was hydrolysed for 90 min. in 10 per cent acetic acid (40 ml) at 100°C to remove the isopropylidene groups and cleave phosphoamide bonds. The acetic acid was then removed by evaporating the solution to dryness with the last traces being removed by a second evaporation after adding 10.0 ml of water. The residue was heated with 40 ml 9 N-ammonium hydroxide (to remove cyanoethyl groups) at 60°C for 90 min. and the ammonia removed by concentrating the mixture to dryness. 10 ml. of water was added to the residue and the insoluble dicyclohexylurea removed by filtration under reduced pressure. The precipitate was washed with a small volume of water and a sample of the combined filtrates was taken for paper chromatography of the reaction products. The precipitate was then washed very thoroughly with 100 - 200 ml. of water, the filtrates being combined. Descending chromatography was performed in one dimension on sheets of Whatman No. 1 chromatography paper. UMP, uridine and isopropylidene uridine were used as markers. The

chromatogram was developed for 16 hours in the ammonium iso-butyrate system, consisting of 100 ml isobutyric acid, 55.8 ml. water and 4.2 ml ammonia. The sheet was then dried and scanned for ultraviolet-absorbing spots using a Manovia chromatolite.

The combined filtrates were diluted to about 250 ml. and applied to a Dowex-1-Cl$^-$ column and washed with water until the optical density at 265 mu was less than 0.05. The (^{32}P) UMP was eluted with 0.05M-HCl (500 ml). The total extinctions of the UMP fraction was determined and the yield of UMP calculated. The eluate was concentrated *in vacuo* at 40°C.

5. (iii) *The preparation of* (^{32}P) UTP

The reaction mixture for UTP contained the following components for each 100 µmole of (^{32}P) UMP:-

1.2 ml tri-n-butylamine, 6 ml of pyridine, 0.2 ml. 85 per cent (w/v) phosphoric acid and 5.0 gm. 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 vol. of water were added to precipitate DCU from unreacted DCC. The flask was shaken and left for one hour at 0°C. 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 five 10 ml. portions of water, these water washes being added to the main aqueous phase.

The combined aqueous phases were concentrated at 40°C *in vacuo*. The material was applied to a Dowex-50-Na$^+$ column

(4 cm. by 10 cm.) to remove tri-n-butylamine. The uridine derivatives were eluted by washing with water until the extinction of the eluate at 260 mμ was less than 0.05. The total extinction of the eluate at 260 mμ was determined and the amount of uridine derivatives calculated. The effluent was diluted to one litre and adsorbed onto a Dowex-1-Cl^- column (2 cm. by 20 cm.). The column was washed with water to remove ultra-violet absorbing material, the total extinction being recorded.

Gradient elution of the Dowex-1-Cl^- column was then carried out with 1.3 litres of 0.01 $\bar{M}$ HCl in the mixing vessel and 2 litres of 0.3 $\bar{M}$ - LiCl in 0.01$\bar{M}$ - HCl in the reservoir. The eluate was collected in 2 or 3 ml fractions which were scanned automatically for radioactivity. The fractions containing (^{32}P) UTP were pooled and the volume reduced *in vacuo* at 25° C, the yield having been determined. Li Cl, HCl and inorganic phosphate were removed on activated charcoal which was prepared in the following manner. A column of charcoal was washed with ethanol-ammonia (70 per cent [v/v] aqueous ethanol containing 10 ml. concentrated ammonia per litre) until the absorbancy at 260 mμ fell to less than 0.01. The column was then washed with water, 0.01 $\bar{M}$ - $NaHCO_3$ and then with approximately 5 column volumes of 1$\bar{M}$ - HCl. The charcoal was suspended in water, fine particles were decanted off and washing was continued until neutral. The column was then repacked.

The mixture containing (^{32}P) UTP was absorbed on the

charcoal column which was washed with water until chloride ions were completely eluted. Washing was continued with small volumes of 0.01 $\bar{M}$ - $NaHCO_3$ to remove inorganic ortho- and pyro-phosphate until ultraviolet absorbing material began to be eluted. The column was allowed to drain and was washed with 2 column volumes of water. (^{32}P) UTP was eluted with about one litre of ethanol-ammonia and was concentrated in vacuo at 25°C. The material was redissolved in water and passed through a small Dowex-50-Na^+ column then checked for purity by chromatography in the ammonium isobutyrate solvent. The chromatogram was tested for the presence of phosphate thus: one gram of ammonium molybdate was dissolved in 5.0 ml. of water. 3.0 ml. of 72% (v/v) perchloric acid and 3.0 ml. concentrated HCl were added and made up to 100 ml with acetone. The chromatogram was dipped in this mixture, dried and exposed to ultraviolet light for 30 min. Inorganic phosphate gives a yellow spot on dipping; inorganic phosphate and ester phosphate give blue spots after exposure to ultraviolet light.

6. Preparation of Bentonite

Brownhill, Jones and Stacey (1959) have reported that bentonite can absorb and inhibit ribonuclease activity during extraction of RNA from cells. Bentonite was used in the preparation of RNA from cell extracts and was prepared as follows.

Bentonite was obtained from the British Drug Houses Limited. 2 g. of crude bentonite was suspended in 40 ml. water and centrifuged at 750 x g for 15 min. The sediment was discarded and the

supernatant material was centrifuged at 8,700 x g for 20 min. The sediment so obtained was resuspended in 0.1M-EDTA, pH 7.0, and set aside at room temperature for 48 hours. The material was centrifuged once more at 750 x g and 8,700 x g. The 8,700 x g sediment was suspended in 0.01M-sodium acetate, pH 6.0, centrifuged at 8,700 x g and the sediment taken up in the acetate buffer at a concentration of 2.5 per cent. (w/v)

7. Preparation of RNA

7.(i) Extraction of RNA from cellular subfractions and incubations mixtures

RNA was extracted from cell extracts or incubation mixtures by a modified version of the method described by Perman (1966). All extracts or incubation mixtures were chilled in crushed ice before fractionation.

The solution containing the RNA to be extracted was added to a chilled tube containing 10% (with respect to RNA) of washed bentonite suspension. The solution was made 1% with respect to sodium dodecyl sulphate (SDS) and allowed to stand for 5.0 min. One volume of a 90% aqueous redistilled solution of phenol that had been containing 0.1% 8-hydroxy-quinolene and buffered to pH 7.0 with 0.01 M-tris-HCl was then added, and the mixture shaken vigorously at room temperature for 5.0 min. One volume (with respect to original RNA solution) of chloroform containing 1% isoamyl alcohol was then added, and the mixture shaken vigorously for a further 5.0 min.

The solution was then resolved into an aqueous phase and

a phenol-chloroform phase by centrifugation at 1,500 x g for 5.0 min. A flocculent precipitate formed at the interphase.

The phenol-chloroform layer was discarded, leaving the interphase material, and the phenol-chloroform extraction repeated. The extraction of the aqueous phase and the precipitate was repeated another three times, using chloroform-isoamyl alcohol mixture alone.

At this point no flocculent precipitate remained. A thin interfacial layer was observed which probably comprised denatured protein (Penman, 1966).

The aqueous phase was added to two volumes of redistilled ethanol, the solution made 1% with respect to potassium acetate and allowed to stand at -20°C for one hour. The RNA precipitate was collected by centrifugation at 15,000 x g for 10 min. and reprecipitated from 66% ethanol, 1% potassium acetate.

For further manipulation the RNA was finally dissolved in dilute buffer of the appropriate pH.

7. (ii) Preparation of "short" polyuridylic acid

Poly U (K^+ salt) was obtained commercially, had a sedimentation value greater than 4S and was completely excluded from particles of Sephadex G-100 equilibrated to 0.01M tris-HCl buffer, pH 7.0 (see section 10 under "Experimental").

In order to distinguish polyu from RNA which was also excluded from the gel under these conditions, the molecular size of the polyu was reduced by alkaline degradation.

7. (ii) 1. Alkaline hydrolysis of poly U

40 mg of polyu were dissolved in 4.0 ml of 0.1M-KOH and

incubated at 37°C for 9.0 min. The hydrolysate was then adjusted to pH 7.0 with 1.24 $\bar{M}$ $HClO_4$, and after standing in ice for 20 min., insoluble potassium perchlorate was removed by centrifugation at 600 x g for 20 min. at 0°C. The supernatant was subjected to gel filtration on a 35 x 2.7 cm column of G-100 Sephadex equilibrated to 0.01$\bar{M}$ tris-HCl pH 7.0. Elution was by the same buffer and 2.0 ml fractions of the eluate collected and monitored for extinction at 260 mμ. The hydrolysed poly U was recovered over a broad region of the effluent. Fractions from the appropriate region of the effluent were pooled, the polynucleotide material precipitated for 1 hour at -20°C. from 66% redistilled ethanol, 1% potassium acetate, and collected by centrifugation at 15,000 x g for 10 min. at 0°C. The precipitate was dissolved in about 2.0 ml of 0.01$\bar{M}$ tris- HCl pH 8.0, and the total number of o.D units at 260 mμ recovered estimated (200).

7.(ii) 2. Dephosphorylation of shortened poly U

Alkaline degradation of polyribonucleotides leaves the 2′- 3′ end of the molecule phosphorylated (Davidson, 1965). In order to dephosphorylate the molecules to give free 2′- 3′- OH ends the poly U was subjected to the action of alkaline phosphatase.

The 200 oD units of poly U were incubated at 37°C with 2.0 ml. of a 1 in 10 dilution of E. Coli alkaline phosphatase (equal to 20 μmoles of p-nitro phenol released/min.) under the incubation conditions described in 4.2 of this section. After 4.0 min. the solution was cooled in crushed ice and the enzyme inactivated as follows.

7. (ii) 3. Inactivation of alkaline phosphatase

The solution was refluxed with an equal volume of re-distilled ethanol for one hour. The ethanol was then allowed to evaporate. An equal volume of chloroform was then added and the mixture shaken vigourously for 2.0 hours, after which the two layers were separated by centrifugation at 1,000 x g for 10 min. The upper aqueous layer was removed, the chloroform washed 2x with small aliquots of water and the washings combined with the aqueous phase.

RNA was collected after standing for one hour at -20°C in 66% ethanol, 1% potassium acetate, by centrifugation at 15,000 x g for 10 min. at 0°C. The poly U was dissolved in 0.01M tris-HCl pH 7.0. The preparation exhibited no detectable phosphatase activity when incubated with p-nitrophenylphosphate under the conditions of 4.2 of this section.

7.(ii) 4. Final chromatography on G-100 Sephadex

The poly U was once again subjected to gel filtration on the same G-100 Sephadex column under identical conditions. The fractions from the desired region of the effluent were pooled and the poly u precipitated for 1 hour at -20°C from 66% ethanol, 1% potassium acetate, and collected by centrifugation at 15,000 x g for 10 min. at 0°C.

The material was dissolved in 0.01M tris-HCl pH 8.0 and the extinction at 260 mu adjusted to 100 o.D/ml. 150 o.D units of "short" poly U were finally recovered. The solution was stored at -20°C.

8. Snake Venom Diesterase digestion of ^{3}H labelled RNA

A modification of the procedure described by Naora (1966) was adopted. 10 o.D units (at 260 mμ) of ^{3}H labelled RNA, extracted from incubation mixtures as in 7.(i) of this section, was incubated at 37°C in a total volume of 5.0 ml with the following; 500 μmoles of tris-HCl buffer, pH 8.5, 25 μmoles of $MgCl_2$ and 1.0 ml of diluted snake venom phosphodiesterase (equivalent to 30 μmoles of p nitrophenol released/min. under these conditions).

At appropriate times 0.5 ml aliquots were removed, and rapidly chilled in crushed ice. 2 mg of bovine serum albumen (free of acid soluble o.D. at 260 mμ) was added, followed by sufficient ice-cold perchloric acid to obtain a final concentration of 0.5 $\bar{N}$, and a final volume of 2.25 ml.

The resulting precipitate was collected by centrifugation at 600 x g for 10 min. at 0°C. A sample of the supernatant was analysed for extinction at 260 mu. The precipitate was washed, dissolved in formic acid and water, and the radioactivity counted as described in 4.(i).2. and 4.(i).3. of this section.

The percentage of total nucleotides and radioactivity released in an acid soluble form was estimated from the o.D and the radioactivity measurements.

9. Ultracentrifugational studies.

9.(i) In sucrose density gradients

9.(i).1. Analysis of RNA

Sedimentation analysis of RNA solutions were performed by centrifugation in linear sucrose density gradients. A mixing

device (Beck and Line, 1954) was used routinely to deliver 4.6 ml. of sucrose, linearly graded from 5 to 20 per cent, in 0.5 x 2 in. cellulose nitrate tubes. The sucrose was buffered by 0.01$\bar{M}$ - potassium acetate, pH 5.2, 0.1$\bar{M}$ - NaCl and 10^{-4} $\bar{M}$-$MgCl_2$.

The tubes were then chilled in crushed ice. 0.05 - 0.1 ml. aliquots containing about 5.0 o.D units (at 260 mμ) of RNA dissolved in the same buffer were carefully layered on top of the gradients. The tubes were then centrifuged at 0^{o}C in the swinging bucket rotor (SW.39) of the Spinco Model L, the SW.40 rotor of the M.S.E. 50, or the SW.40 rotor of the Griffin-Christ Model Omega II ultracentrifuges. The length of time and speed of the runs are indicated in the legends to the appropriate figures.

The bottoms of the tubes were punctured by a syringe needle and 40% sucrose pumped through it. The contents of the tubes were forced upwards through a Spectronic 505 linear flow spectrophotometer, and the optical extinction at 260 mμ recorded automatically.

If the RNA had been labelled with ^{3}H ribonucleotides the effluents from the Spectronic 505 were collected in 5 drop fractions. Nucleic acids were precipitated by the additions of 5.0 ml of ice cold 5 per cent TCA and 1 mg of bovine serum albumen. The precipitates were then washed and prepared for scintillation counting as in 4.(i).2. and 4.(i).3. of this section. Pyrophosphate was used only in the first wash.

9.(i).2. Analysis of "free" ribosomes

The "free" ribosomes were also analysed by sucrose gradient

analysis. 5-15 or 5 - 20 per cent sucrose gradients containing 0.01$\bar{M}$-tris-HCl, pH 8.0, and 0.0025$\bar{M}$-$MgCl_2$ were prepared using a mixing device as described above.

0.1-0.2 ml. aliquots of solutions containing about 5.0 o.D. units (at 260 mμ) of the "free" ribosomes suspended in the same buffer, or straight from incubation mixtures, were layered on the gradients and centrifuged as above.

The spectrophotometric and radioactivity analyses of gradients was performed as already described. Occasionally, to detect the presence of ferritin, the optical extinction at 320 mμ was monitored (Munro, Jackson and Korner, 1964).

9.(ii). Analytical ultracentrifuge studies

Solutions containing nucleic acids or cell fractions in the appropriate buffers were centrifuged in a model E Spinco analytical ultracentrifuge equipped with both Schlieren and ultraviolet optical systems.

Using ultraviolet optics concentrations of 1 O.D. (at 260 mμ) per ml. in 0.15$\bar{M}$ NaCl, and using Schlieren optics 5 - 10 mg/ml in 0.01$\bar{M}$ tris pH 8.0, 0.15 $\bar{M}$ NaCl were employed. Other constituents, such as Mg^{++}, are indicated opposite the appropriate figures.

The sedimentation values of components were calculated from the formula,

$$S = \frac{1}{w^2} \cdot \frac{d \log_e x}{dt}$$

where w is the angular velocity in radius/sec., x is the radius in cms. of the boundary from the centre of rotation, and t is the time in secs. One Svedberg unit (S) is equal to 10^{-13} sec.

10. Gel filtration on Sephadex

The uncharged dextran gel Sephadex G-100 was used as a molecular sieve in the separation of RNA molecules according to their size and shape.

A weighed amount of dry dextran powder was suspended with stirring in an excess of buffer, normally 0.01M -tris -HCl pH 7.0, and the resulting gel allowed to equilibrate at room temperature for 24 hours. After equilibration the fine particles were removed by resuspending the gel and allowing it to resettle. When a sharp boundary had been obtained the supernatant was removed by suction. This was repeated a number of times until the supernatant was clear.

Two columns were used, both supplied by Pharmacia Fine Chemicals, Uppsala, Sweden, and designed specifically for use with Sephadex gels; a 2.54 x 40 cm column and a 2.54 x 100 cm. column fitted with a water jacket. The gel is supported by a nylon mesh at the bottom of the column and the "dead space" below this is very small. The surface of the gel can be stabilised by a nylon mesh fitted tightly over the end of a hollow tube which fits tightly inside the column.

The gels were poured at room temperature. The column was mounted vertically, filled to a height of about 5 cm with the buffer to be used and some allowed to pass through the "dead space" at the bottom of the column. A suspension of the gel in the same buffer was then poured into the column to a height of about 20 cm. The flow rate of the effluent was adjusted to about 0.8 - 0.9 ml/min.

As the gel settled the packing was continued until the desired column height was achieved. If the column was to be used in the cold the gel was poured to a height 2-3 cm. above that ultimately required.

The column was then connected to a large reservoir of the appropriate buffer and the gel washed for two days at a flow rate of about 0.5 ml/min. In the case of the large column the water jacket was connected to a refrigeration unit supplied by Grant Instruments (Cambridge) Limited, and 20% glycol at 1-2°C circulated round the column.

Before use the void volume of the column was determined by measuring the elution volume of material completely excluded from the gel particles. Blue Dextran 2000, (supplied by Pharmacia Fine Chemicals), dissolved in a little water, was used for this purpose.

Samples were applied as follows. The level of buffer in the column was allowed to fall just below the surface of the gel. The sample was applied gently using a pasteur pipette and the level of fluid once again allowed to fall just below the surface of the gel. The sample was washed into the gel with two small aliquots of buffer in a similar fashion, and then the top of the column filled with buffer and the reservoir connected.

The flow rate was adjusted to 0.4 - 0.5 ml. per min. and 2.0 ml. fractions of the effluent collected on an Almex Central fraction collector. The o.D. at 260 mμ of each fraction was determined using an Unicam SP 500 fitted with a micro-cell attachment.

For measurement of radioactivity, nucleic acids were precipitated by the addition of 5.0 ml. of ice cold 5% TCA and 1 mg of bovine serum albumen carrier. The precipitate was washed and prepared for scintillation counting as in 9.(i).1. of this section.

Occasionally an alternative method was used. Nucleic acids were precipitated by the addition of 0.2 ml 50% TCA and 100 ug of bovine serum albumen carrier. The precipitate was trapped on 2 mm Millipore filters (0.45 μ pores) and washed with three 5.0 ml aliquots of ice cold 5% TCA. The filters were dried and counted in Toluene based scintillation fluid as in 4.(i).3. of this section.

11. Analysis of ribonucleotides

11.(i) Chromatographic Analysis

11.(i).1. Separation of ribonucleoside mono, di and triphosphates

The acid soluble fractions of incubation mixtures labelled with ^{3}H-ribonucleotide triphosphates were analysed as follows. Incubation mixtures set up as in section 4.(i).1. were precipitated with 0.5 ml of 0.3$\bar{M}$-perchloric acid in the cold. The precipitates were removed by centrifugation at 600 x g for 10 min. at 0^{o}C, and the supernatants quickly adjusted to pH 6.0 with 5.0 $\bar{M}$-KOH. After 1 hour in crushed ice insoluble potassium perchlorate was removed by centrifugation.

0.05 ml aliquots of each supernatant were applied to sheets of Whatman No. 1 chromatography paper and markers of the appropriate ribonucleoside mono, di and triphosphates were superimposed on the

samples. Descending chromatograms were developed for 36 hours in the Ethanol-ammonium acetate solvent (60 ml of 1.0M-ammonium acetate containing 0.01M versene and 140 ml of 90 per cent ethanol). The paper sheets were dried and the ultraviolet-absorbing spots located with the aid of a "Chromatolite" low pressure mercury lamp, whose radiation was screened to give an emission maximum at 253.5 mu.

The spots were cut out and eluted into vials with distilled water and the radioactivity estimated in a scintillation spectrometer in the dioxan based scintillation fluid as in 4.(i).3. of this section.

11.(i).2. Separation of nucleotides from nucleosides

Acid washed, insoluble material from incubation mixtures labelled with ^{14}C ^{3}H ribonucleoside triphosphates as in 4.(i).1. and 4.(i).2. of this section were hydrolysed for 16 hours at 37°C in 0.3M-KOH. The hydrolysates were neutralised with 12.4M-perchloric acid and insoluble potassium perchlorate removed as above.

0.05 aliquots of the supernatants were applied to sheets of Whatman's No. 1 chromatography paper with the appropriate markers. Ribonucleotides were separated from ribonucleosides on descending chromatograms developed overnight in isopropanol:water:0.88 ammonia (35:15:1). Ultraviolet-absorbing spots were eluted and counted as above.

11.(ii) Electrophoretic separation of ribonucleoside monophosphates

The acid washed insoluble material from reaction mixtures

labelled with (^{32}P) UTP was hydrolysed in KOH and neutralised (to about pH 3.5) as above. Aliquots were applied as a narrow band on to 8 cm x 70 cm strips of 3 MM Whatman's chromatography paper. The strips were moistened with 0.02 M-citrate buffer, pH 3.4, (0.02M-citric acid adjusted to pH 3.4 with 0.02M ammonium citrate). 4 Kv were applied across the paper for 1¼ hours, using a Shandon high voltage electrophoresis apparatus.

The ultraviolet spots were located as above, cut out and counted directly in a Nuclear Chicago gas flow counter. Alternatively the strips were scanned in the Nuclear Chicago Actigraph, and the radioactivity in each peak estimated by cutting it out and weighing it.

12. Estimation procedures

12.(i) Protein

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951). This involves the use of a modified Biuret reagent, and the method is in fact a modification of that first introduced by Wu (1922). The reagents used were as follows:-

A. 2 per cent $Na_2 Co_3$ in 0.1M-NaOH.

B. 0.5 per cent $CuSO_4$.

C. 2 per cent sodium potassium tartrate (Rochelle salt).

D. 50 ml of A. mixed with 0.5 ml. of B. and 0.5 ml. of C.

E. Folin Ciocalteau reagent, (Folin, and Ciocalteau, 1927).

The Folin Ciocalteau reagent, obtained from British Drug Houses Limited, was titrated with standard NaOH to a phenolphthalein end point, and then diluted to 1.0N with respect to acid.

The standard used was a bovine serum albumen preparation routinely used in the department. All assays were in duplicate.

To a sample of 5 to 400 µg. of protein in 2.0 ml was added 5 ml. of reagent B. The solution was mixed well and allowed to stand for ten minutes at room temperature. 0.5 ml of reagent B was added and the solution mixed immediately. After 30 min. the colour intensity was read in a Unicam S.P. 600 spectrophotometer. For solutions containing low amounts of protein it was desirable to take readings at 750 mµ, the absorption maximum. With stronger solutions the readings were taken at 500 mµ to allow a greater working range.

12.(11) Estimation of RNA

RNA was measured by the orcinol method (W. Mejbaum, 1939) as modified by Slater (1956).

This method measures the colour produced on the interaction of pentose liberated on the hydrolysis of RNA (particularly from the purines) 5:5 dihydroxytoluene (orcinol) and ferric ions. Protein does not interfere with the assay, although RNA can contribute to the colour (Dische, (1955) and Shettles and Davidson, 1955).

Ferric chloride reagent was prepared by dissolving 0.1 g. of $FeCl_3$ in 100 ml. molar concentrated HCl. Orcinol reagent was prepared by dissolving 1 g. of orcinol in 20 ml. redistilled absolute alcohol (reagent grade orcinol was used but was discarded when it became tinted deep pink).

To duplicate 3.0 ml. samples, containing 20 - 300 µg RNA, was added 3.0 ml of $FeCl_3$ reagent and 0.3 ml of orcinol reagent.

Standard solutions of ribose were incorporated in each estimation. The mixtures were heated in a boiling water bath for 45 min.

The tubes were cooled under the tap and the o.D. at 670 mμ determined in a Unicam SP 600. 4.3 mg of yeast highly polymerised RNA gave the same o.D as 1 mg ribose (the final o.D depends on the purine content of the RNA).

12.(iii) Estimation of DNA

The diphenylamine method of Burton (1956) was employed.

Diphenylamine reagent was prepared by dissolving 1.5 g. of steam distilled diphenylamine dissolved in 100 ml of re-distilled glacial acetic acid and 1.5 ml of Analar concentrated sulphuric acid. On the day of use 0.1 ml of 16 mg/ml redistilled acetic acid in water was added to 20 ml of the diphenylamine solution.

To duplicate 1 ml samples 2.0 ml of diphenylamine reagent was added. The tubes were placed in a water bath or other dark place overnight and the o.D. at 600 mμ read in a Unicam SP 600 spectrophotometer. Standard DNA solutions were incorporated in each assay.

1 mg of yeast highly polymerised RNA gave the same o.D at 600 mμ as 5.0 μg of DNA.

13. Materials

Ribonucleoside mono, di and triphosphates and deoxyribonucleotides were purchased from Pabst Laboratories, the Sigma Chemical Company and British Drug Houses Limited. Phosphocreatine

was obtained from Calbiochem, highly polymerised yeast RNA and carrier yeast RNA from British Drug Houses Limited. Bovine serum albumen for co-precipitation purposes was purchased from the Armour Pharmaceutical Company, and in a crystalline form for standard assays from Calbiochem. Phosphocreatine kinase, deoxyribonuclease, ribonuclease and snake venom diesterase were purchased from the Sigma Chemical Company and E. Coli alkaline phosphatase from the Nutritional Biochemical Corporation.

Actinomycin D was a gift from Merck, Sharp and Dohme, Inc. Landschutz ascites DNA was a gift from Dr. J.B. Shepherd.

^{3}H ribo- and deoxyribonucleoside triphosphates were obtained from Schwartz Bioresearch Inc. Carrier free ^{32}P orthophosphate and ^{32}P cyanoethylphosphate were purchased from the Radiochemical Centre, Amersham, England.

Iso-octane (2:2:4)(trimethylpentane) was obtained from British Drug Houses Limited, and tween 80 (polyoxyethylene sorbitan monooleate) from Light and Company Limited.

AnalaR dioxan, purchased from the British Drug Houses Limited, was purified by passing up to seven litres through a 130 x 8 cm. column of activated alumina. The activated alumina was obtained from the Alumina Company of America. P.O.P.O. and scintillation grade naphthalene were purchased from Nuclear Enterprises (G.B.) Limited, P.P.O. from Koch-Light Laboratories Limited and AnalaR toluene from British Drug Houses Limited.

RESULTS

1. Preliminary investigations

Polyribonucleotide synthesis was measured by following the incorporation of radioactive ribonucleotides into acid-insoluble material. Initially experiments were performed to determine the most suitable method for fractionating the cells. Reaction mixtures contained (α-^{32}P) UTP, highly polymerised yeast RNA as primer and Mg^{++} ions to fulfil metal ion requirements. An energy generating system comprising phosphocreatine and phosphocreatine kinase was included, and the reaction was buffered by tris-HCl.

Total microsomes and the 105,000 x g supernatant were prepared by either the Tween 80 method or the Sucrose method as described under Experimental. The levels of incorporation measured on two separate occasions are given in Table 1. Cell fractionation in Tween 80 yielded the most active preparations and this method was used thereafter unless otherwise stated.

The ability of the 10,000 x g sediment, the 105,000 x g sediment and the 105,000 x g supernatant to catalyse the uptake of UTP was then tested. As can be seen in Table 2, activity was found in each fraction. While there was marked differences in the characteristics of incorporation into the 105,000 x g supernatant and sediment (the microsomes), the activity in the 10,000 x g sediment closely resembled that of the microsomes. The specific activity of the 10,000 x g sediment, was, however, much lower. This fraction is probably rather heterogenious and will contain the bulk of the mitochondria. The probability of contamination

with the microsome fraction is very high. The 10,000 x g sediment was not investigated further.

Incorporation of UTP into the microsomes was dependant on the presence of ATP, GTP and CTP, while added RNA only stimulated the reaction slightly. This experiment has been repeated many times with the same pattern of results. The dependance of the reaction on the presence of all four ribonucleoside triphosphates obviously suggests the formation of heteropolymers. On the other hand, the pattern of UTP uptake by the 105,000 x g supernatant was quite different. The reaction showed a marked dependance on added RNA. This is more noticeable in other experiments shown in Table 3. The dependance of the reaction on the presence of all four ribonucleoside triphosphates varied considerably, in contrast to the microsomes.

The characteristics of the two fractions were investigated in more detail.

2. Further characterisation of the 105,000 x g supernatant

In the following experiments the 150,000 x g supernatant obtained during the preparation of free ribosomes was used. Frequently small amounts of EDTA were included in the reaction mixtures to remove traces of heavy metal ions sometimes found in the buffer systems.

The dependance of the reaction on added enzyme protein is shown in Figure 3. Unincubated control tubes were set up for each concentration of protein used. A linear response up to 1.0 mg. of protein per tube was obtained and thereafter all protein concentrations used were within this range.

TABLE 1

Effect of different methods of cell fractionation on the ability of rat liver microsomes and 105,000 x g supernatant fraction to incorporate α-^{32}P-labelled UTP into RNA

The reaction mixture contained 50 μmoles tris-HCl buffer, pH 8.0, 5 μmoles $MgCl_2$, 0.1 μmole each of α-^{32}P-UTP, (1.0×10^6 counts/min/μmole), ATP, GTP and CTP, 50 μg RNA, 1.8 μmoles phosphocreatine, 45 μg phosphocreatine kinase, and up to one mg of enzyme protein in a total volume of 0.5 ml.

Incubation was at 37°C for 10 min. The incorporation of ^{32}P-UMP into acid insoluble material was as described under Experimental.

TABLE 1

Experiment	Method of fractionation	Cell fraction	mumoles UMP incorporated per mg. pr.
1	Sucrose	supernatant fraction	0.102
		microsome fraction	0.084
	Tween 80	supernatant fraction	0.172
		microsome fraction	0.160
2	Sucrose	supernatant fraction	0.033
		microsome fraction	0.155
	Tween 80	supernatant fraction	0.108
		microsome fraction	0.330

TABLE 2

The incorporation of (α-^{32}P) UTP into the 10,000 x g sediment, the 105,000 x g sediment and the 105,000 x g supernatant fraction of rat liver cytoplasm

The reaction mixture contained 50 μmoles tris-HCl buffer, pH 8.0, 5 μmoles of $MgCl_2$, 0.1 μmole of α-^{32}P labelled UTP, (1.0×10^6 cpm/μmole), 1.8 umoles phosphocreatine, 45 μg phosphocreatine kinase and up to 1 mg of enzyme protein in a total volume of 0.5 ml.

Where indicated 50 μg of highly polymerised yeast RNA and 0.1 μmole each of ATP, GTP and CTP were included.

Incubation was for 10 minutes at 37°C. The incorporation of ^{32}P-UTP into acid insoluble material was measured as before.

TABLE 2

cell fraction	mμmoles UMP incorporated per mg. of enzyme protein			
	no additions	plus RNA	plus ATP, GTP, CTP.	plus RNA, ATP, GTP, CTP.
10,000 x g sediment	0.005	0.004	0.02	0.041
105,000 x g supernatant	0	0.049	0.006	0.176
105,000 x g sediment	0.023	0.025	0.225	0.253

TABLE 3

The incorporation of UMP from UTP into the cell sap under various conditions

Reaction mixtures as in Table 2, except that in experiment 4, no phosphocreatine, or phosphocreatine kinase was present, and H^3-UTP was employed (20 μc/μmole). With the exception of experiment 3 the fractions were obtained following cell fractionation in sucrose.

Incubations were at 37°C for 20 minutes with the exception of experiment 4 where the tubes were incubated for 30 minutes.

Incorporation of UMP into acid insoluble material was measured by the method appropriate to the isotope used.

T A B L E 3

Experiment	mμmoles UTP incorporated into acid insoluble material per mg. protein			
	no additions	+ ATP, GTP CTP	+ RNA	+ RNA; ATP, GTP CTP
1	0.007	0.005	0.092	0.102
2	0.001	0	0.031	0.033
3	0.002	0	0.083	0.172
4	0.026	0.014	0.158	0.133

FIGURE 3

The effect of enzyme protein concentration on the incorporation of (α-^{32}P) UTP by the 150,000 x g supernatant of rat liver cells

The reaction mixture contained 50 µmoles of tris-HCl buffer pH 8.0, 3 µmoles $MgCl_2$, 10 µmoles EDTA, 0.1 µmole (αP^{32}) UTP, 50 µg highly polymerised yeast RNA, 2 µmoles phosphocreatine, 20 µg phosphocreatine kinase and the indicated amount of enzyme protein in 0.5 ml.

Incubations were performed at 37°C for 10 minutes.

FIGURE 4

The fine course of the incorporation of (α^{32}P) UTP by the 150.000 x g supernatant of rat liver cells

The reaction mixtures were as in Figure 3 except H^3 UTP (20 µc/µmole) was used and each tube contained 0.7 mg. of enzyme protein. 0.1 µmole each of ATP, GTP and CTP were added where indicated.

Incubation was at 37°C for the appropriate time.

- - - - ● - - - - ^{3}H-UTP only

———— Δ ———— ^{3}H-UTP + ATP, GTP and CTP

Figure 3.

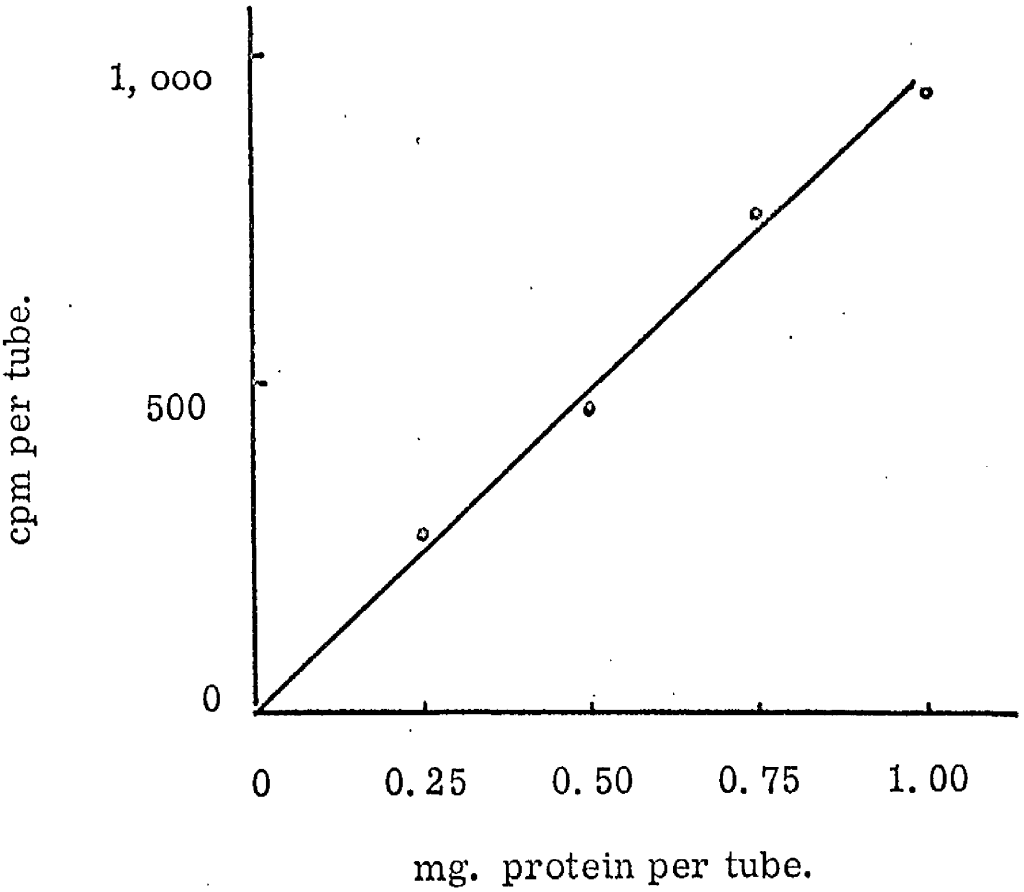


Figure 4.

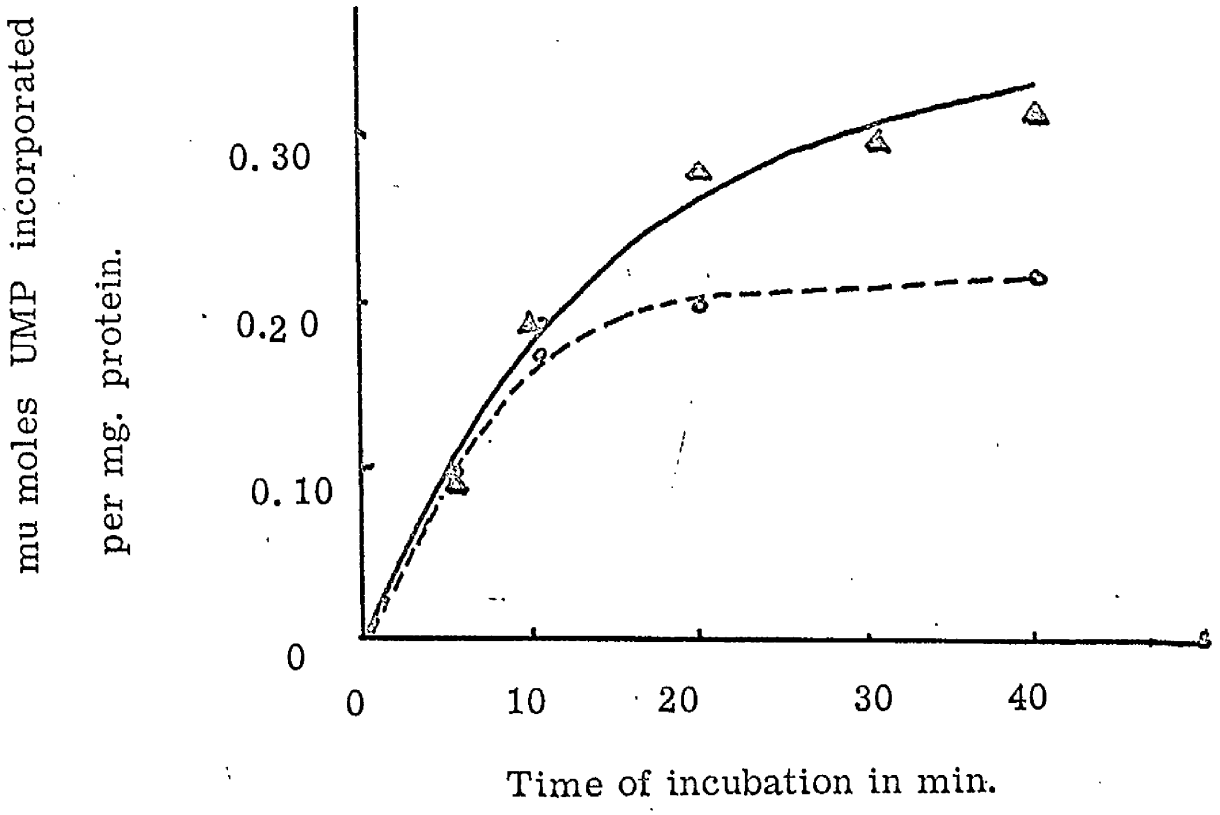


Figure 4 shows the time course of the uptake of UTP alone and in the presence of ATP, GTP and CTP. The reaction rate fell away after 20 min. and appeared to be more extensive in the presence of all four ribonucleoside triphosphates, although in this experiment the initial reaction velocities were identical.

The dependance of the reaction on added Mg^{++} is shown in Figure 5. The reaction reaches a peak at 2.0 µmoles of added Mg^{++} per tube and then declines to a plateau region extending from 3.0 µmoles of Mg^{++} onward. It will be observed that the reaction curve does not intersect the ordinate at zero. This is probably due to the presence of 0.28 µmole of $MgCl_2$ per tube derived from the homogenising medium. A similar Mg^{++} response curve was observed with a 78,000 x g supernatant fraction obtained during the preparation of the RSV fraction. The reason for this type of response is not known. In all the following experiments 3.0 - 4.0 m µmoles of added $MgCl_2$ per tube were used.

Table 4 shows the effect of including an ATP generating system in the reaction mixture. 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, 1961). The results show that the uptake of UTP is stimulated by the presence of phosphocreatine and phosphocreatine kinase. Therefore, either UDP, produced from UTP by phosphatase activity during the reaction, can be utilised as substrate at the high levels of phosphocreatinase kinase employed, or, more likely, traces of ADP in the presence of

ribonucleoside diphosphate kinases in the 150,000 x g supernatant, act catalytically in rephosphorylating UDP. The ATP generating system stimulated the reaction to a greater extent in the absence of ATP, GTP and CTP. It is possible that the stimulatory action of ATP, GTP and CTP in the absence of phosphocreatine and phosphocreatine kinase may be partly due to the protection of UTP by transphosphorylation.

The dependance of the supernatant enzyme on added RNA is demonstrated in Figure 6. The reaction was carried out at two different Mg^{++} concentrations because it is known that RNA is capable of binding divalent cations (Bannerjee and Perkins, 1962). The reason for the biphasic nature of the curve is not known, although it may reflect the presence of different enzymes.

The location of ^{32}P-UMP residues incorporated into acid insoluble material was then studied. Alkaline hydrolyses releases the α-^{32}P of an incorporated ribonucleoside triphosphate as the 2′, 3′ phosphate derivative of the neighbouring nucleoside residue. In this way it is possible to determine the extent to which UMP residues are incorporated adjacent to the other ribonucleosides in the polynucleotide product. When UMP is incorporated into a true heteropolymer chain the radioactivity should be recovered more or less randomly in all four ribonucleoside triphosphates, whereas if homopolymer chains are formed radioactivity should be recovered predominantly in UMP.

Reaction tubes were set up containing the normal incubation mixtures and the extent of (α-^{32}P) UTP incorporation in the presence and absence of ATP, GTP and CTP measured in the normal

TABLE 4

The effect of phosphocreatine and phosphocreatine kinase on the uptake of (α^{32}P) UTP by the 150,000xg supernatant fraction of rat liver cells

Reaction mixtures contained 50 μmoles tris-HCl buffer, pH 8.0, 4 μmoles $MgCl_2$, 0.1 μmole (α-^{32}P) UTP (0.8×10^6 cpm per μmole), 50 μg highly polymerised yeast RNA and 0.69 mg of enzyme protein in a total volume of 0.5 ml.

0.1 μmole each of ATP, GTP and CTP, and 2 μmoles of phosphocreatine and 20 μg of phosphocreatine kinase were added where indicated.

Incubation was at 37°C for 20 minutes.

T A B L E 4

Additions	mμmoles UMP incorporated per mg. protein
NIL	0.195
PC/PCK	0.360
ATP, GTP, CTP	0.267
ATP, GTP, CTP, PC/PCK.	0.368

FIGURE 5

The effect of increasing concentrations of $MgCl_2$ on the incorporation of ^{3}H-UTP by the 150,000 x g supernatant of rat liver cells

Reaction mixtures contained 50 μmoles tris-HCl pH 8.0, 0.1 μmole H^3UTP (20 μc/μmole), 0.1 μmole each of ATP, GTP and CTP, 50 μg of highly polymerised yeast RNA and 0.424 mg. of enzyme protein in 0.5 ml.

Where indicated various amounts of $MgCl_2$ were included in the reaction mixture.

Incubation at 37°C for 10 minutes.

FIGURE 6

The effect of increasing concentrations of RNA on the uptake of (α-^{32}P) UTP by the 150,000 x g supernatant of rat liver cells

Reaction mixtures contained 50 μmoles tris-HCl buffer, pH 8.0, either 3.5 or 4.0 μmoles $MgCl_2$, 0.1 μmole UTP, (0.5×10^6 cpm per μmole); 0.1 μmole each of ATP, GTP and CTP, 2 μmoles phosphocreatine, 20 μg phosphocreatine kinase and 0.86 mg of enzyme protein in a total volume of 0.5 ml.

———o——— 3.5 μmoles $MgCl_2$ per tube

- - - -Δ- - - - 4.0 μmoles $MgCl_2$ per tube

Figure 5

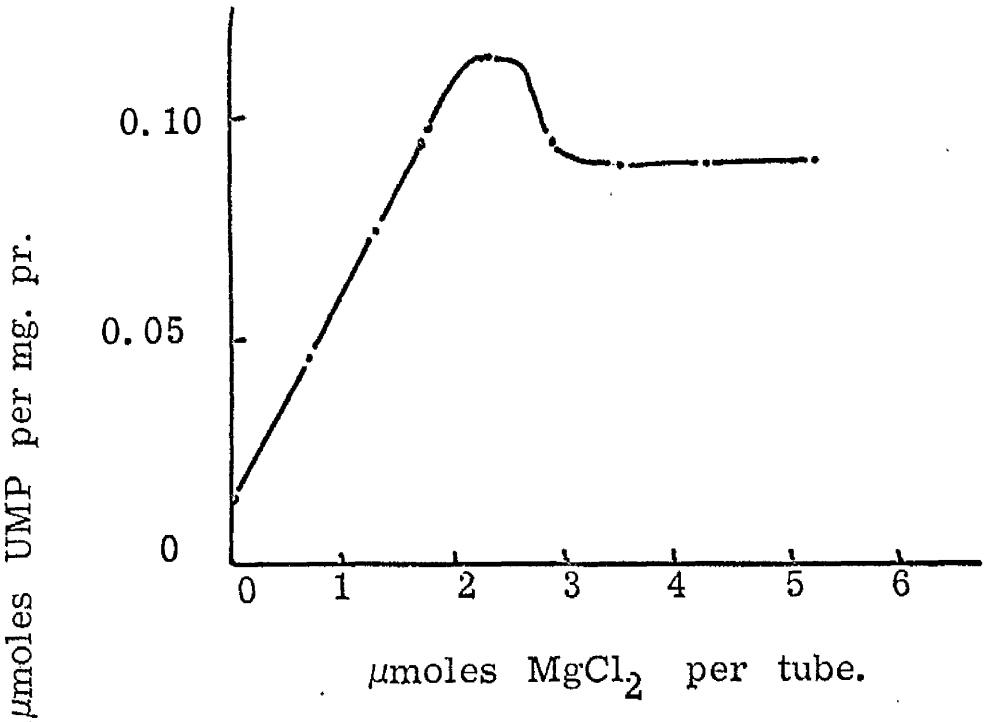
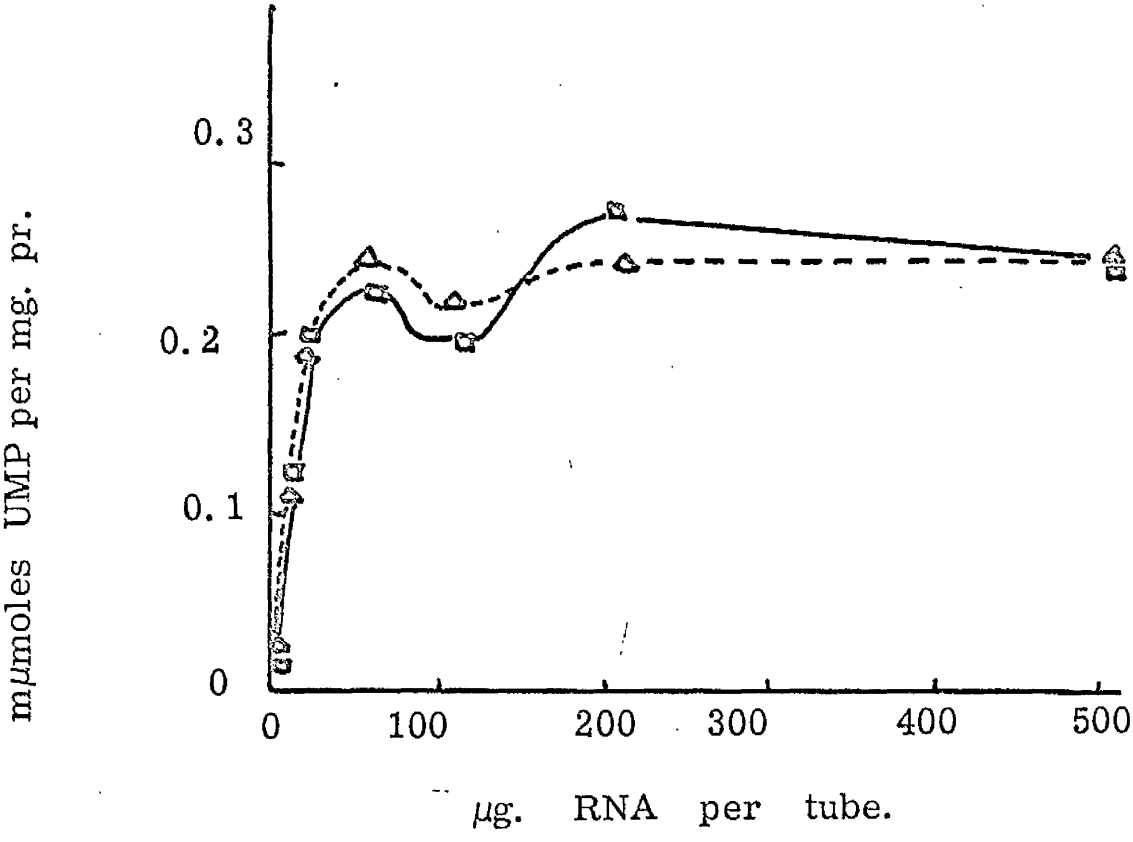


Figure 6



way (Figure 7). Simultaneously, reaction tubes, containing (α-^{32}P)UTP of ten times the specific radioactivity, were incubated under identical conditions. The acid insoluble products from these tubes were taken through the usual washing procedure, and the RNA hydrolysed in 1.5 N KOH for four hours at 37°C. The hydrolysate was neutralised and the ribonucleoside monophosphates separated electrophoretically as described in section 11(ii) under Experimental. The percentage of the total radioactivity recovered in UMP, CMP, AMP and GMP is given in Table 5. The figures are corrected for the error due to the presence of unincorporated UTP, (zero time counts).

As can be seen approximately 80 per cent of the radioactivity was recovered in UMP, suggesting the synthesis of predominantly polyuridylate sequences. The presence of ATP, GTP and CTP did not decrease this figure but rather increased it, further evidence for homopolymer, rather than heteropolymer formation. The results do not, however, preclude a small amount of heteropolymer formation, since 20 per cent of the radioactivity was recovered in CMP, AMP and GMP.

In order to clarify this point, the average length of the newly synthesised chains was estimated as follows. The ability of the supernatant enzyme to incorporate ^{3}H-UTP into an acid insoluble product was measured in the usual way (Figure 8). At the same time, identical reaction mixtures were set up containing ^{3}H-UTP with specific radioactivity ten times greater. The acid insoluble material from these tubes was taken through the usual washing procedure and the RNA hydrolysed overnight at 37°C in 1.5 N KOH.

FIGURE 7

The extent of incorporation of (α^{32}P) UTP by the 150.000 x g supernatant of rat liver cells

Reaction mixtures contained 50 μmoles tris-HCl pH 8.0, 4.0 μmoles $MgCl_2$, 10 μmoles EDTA, 0.1 μmole (α^{32}P) UTP (2×10^6 cpm per μmole), 200 μg RNA, 2 μmoles phosphocreatine, 20 μg phosphocreatine kinase, 0.4 mg of enzyme protein in 0.5 ml total volume.

0.1 μmole each of ATP, GTP and CTP was included where indicated.

Incubations were at 37°C for the appropriate time.

The distribution of ^{32}P in AMP, GMP, UMP and CMP were estimated as in the text at times indicated by the arrows and are given in Table 5.

——Δ—— ^{3}H-UTP + ATP, GTP and CTP

——▲—— ^{3}H-UTP only

Figure 7

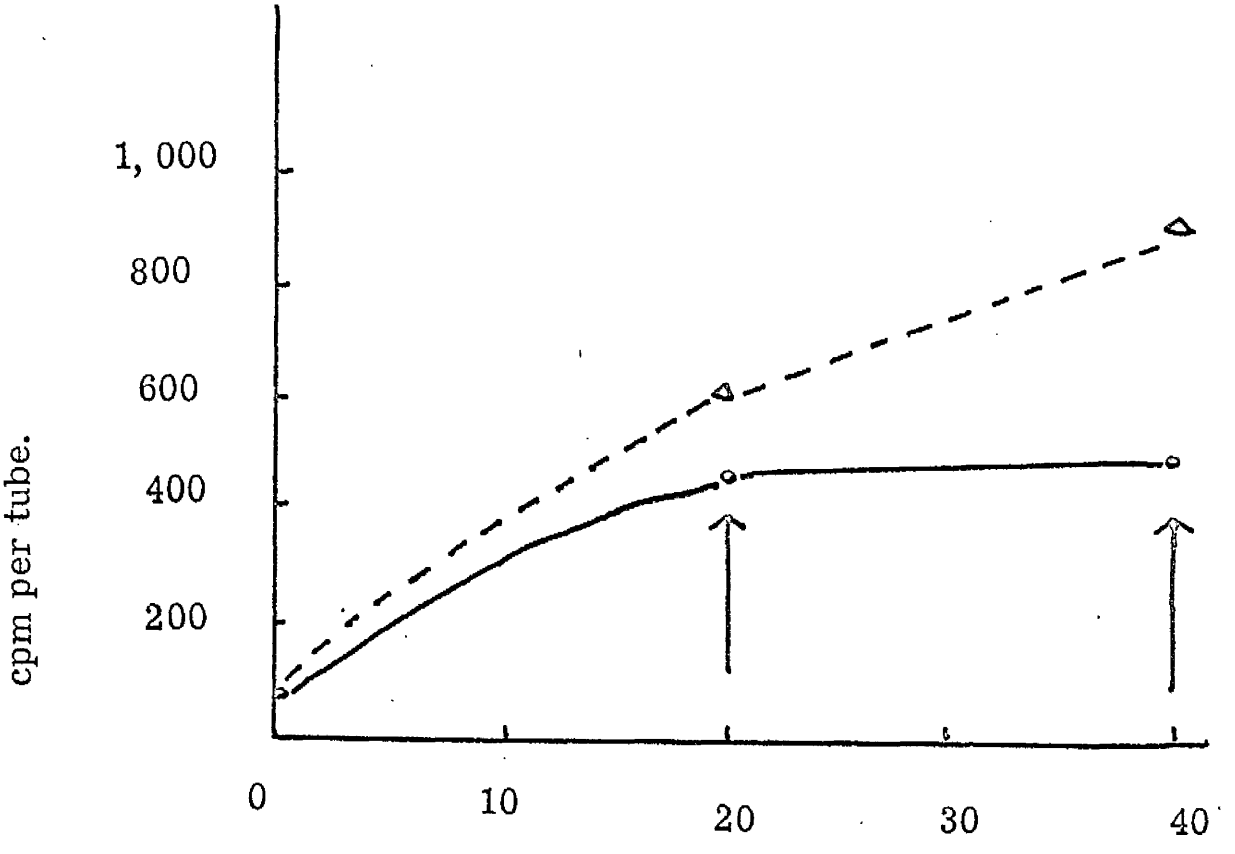

1, 000
800
600
400
200
cpm per tube.
0
10
20
30
40
Time of incubation in mins.

TABLE 5

Distribution of the ^{32}P in the ribonucleotides obtained on alkaline hydrolysis of the product of the uptake of ($\alpha^{32}P$) UTP by the 150,000 x g supernatant of rat liver cells

Reaction mixtures contained 500 μmoles tris-HCl buffer pH 8.0, 40 μmoles $MgCl_2$, 0.1 μmole EDTA, 1 μmole ($\alpha^{32}P$) - labelled UTP. (20 x 10^6 cpm per μmole), 2 mg RNA, 20 μmoles phosphocreatine, 200 μg phosphocreatine kinase and 4 mg of enzyme protein in 5.0 ml total volume.

1 μmole each of ATP, GTP and CTP were included where indicated.

The tubes were incubated at 37°C and the reaction stopped by rapid cooling at the times indicated in Figure 7.

T A B L E 5

Time of incubation	Additions	percentage of counts recovered in			
		UMP	CMP	AMP	GMP
20	NIL	79.9	5.0	6.1	9.0
20	ATP, GTP, CTP	84.7	4.8	0.4	10.1
40	ATP, GTP CTP	81.1	4.3	5.1	9.5

FIGURE 8

Time course of the uptake of ^{3}H-UTP into the 150,000 x g supernatant of rat liver cells

Reaction tubes contained 50 μmoles tris-HCl buffer, pH 8.0, 3.0 μmoles $MgCl_2$, 0.1 μmole ^{3}H-UTP (20 mc/μmole), 200 μg highly polymerised yeast RNA, 2 μmoles phosphocreatine, 20 μg phosphocreatine kinase, 10 mμmoles EDTA, and 0.46 mg of enzyme protein in a total volume of 0.5 ml.

0.1 μmole each of ATP, GTP and CTP were included where indicated.

Incubations were at 37°C for the appropriate time.

The average lengths of the newly synthesised chains were estimated during the reaction at the time indicated by the arrows, and are presented in Table 6.

———Δ——— ^{3}H-UTP only

- - - -○- - - - ^{3}H-UTP + ATP, GTP and CTP

Figure 8.

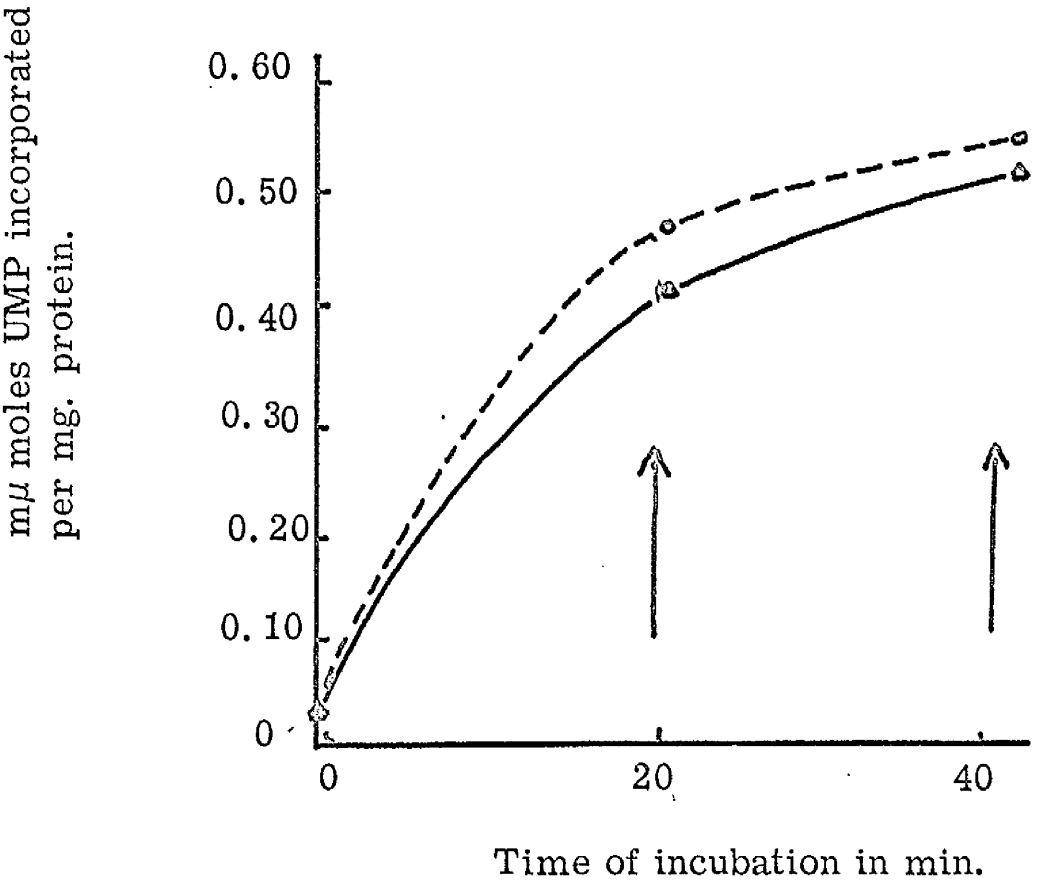

mμ moles UMP incorporated per mg. protein.
0. 60
0. 50
0. 40
0. 30
0. 20
0. 10
0
0
20
40
Time of incubation in min.

TABLE 6a

Average length of newly synthesised chains following incorporation of ^{3}H-UTP by the 150.000 x g supernatant of rat liver

Reaction mixtures were identical to those in Figure 6, except that the specific radioactivity of the ^{3}H-UTP was 200 µc/µmole.

The average chain lengths were estimated at the times indicated by the arrows in Figure 6 by the method described in the text.

TABLE 6b

Average length of newly synthesised chains following incorporation of ^{3}H-UTP by the 150,000 x g supernatant of rat liver

As for Table 6a.

0.85 mg of enzyme protein per tube was used.

T A B L E 6 a

Time of incubation	Addition	Ratio nucleotide nucleoside	average chain length
20	NIL	0.95	2
20	ATP, GTP CTP	1.2	2-3
40	NIL	1.23	2-3

T A B L E 6 b

Time of incubation	Addition	Ratio nucleotide nucleoside	average chain length
20	NIL	0.83	2
20	ATP, GTP, CTP	0.95	2
40	NIL	0.93	2
40	ATP, GTP, CTP	1.76	3

F I G U R E 9

Incorporation of H^3 UTP, ^3H ATP and ^3H CTP into the 150,000 x g supernatant of rat liver cells

Reaction mixtures contained 50 μmoles tris-HCl buffer pH 8.0, 4.0 μmoles $MgCl_2$, 10 mμmoles EDTA, 0.1 μmole of the appropriate ribonucleoside triphosphate, 200 μg of highly polymerised yeast RNA, 2 μmoles phosphocreatine, 20 μg phospho creatine kinase, 0.9 mg of enzyme protein.

Where indicated 0.1 μmole each of the other three unlabelled ribonucleoside triphosphates were included.

Incubations at 37°C.

———o——— ^3H-UTP only

- - - -Δ- - - - ^3H-UTP + ATP, GTP + CTP

———•——— ^3H-ATP only

- - - -▲- - - - ^3H-ATP + UTP, GTP, CTP

———x——— ^3H-GTP only

- - - -□- - - - ^3H-CTP + UTP, ATP, GTP

Figure 9.

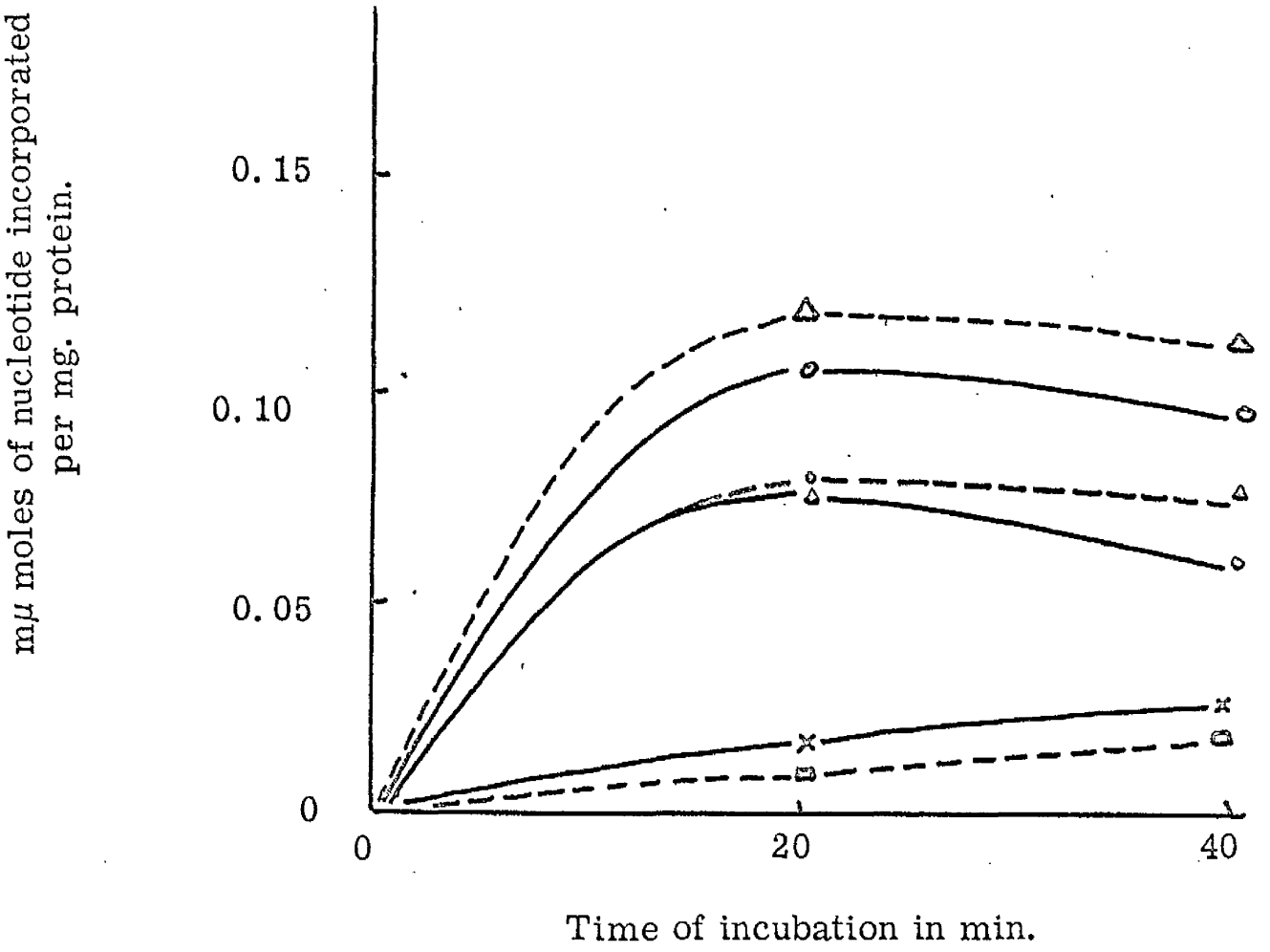

mμ moles of nucleotide incorporated per mg. protein.
0.15
0.10
0.05
0
0
20
40
Time of incubation in min.

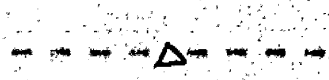
FIGURE 10

The Effect of increasing concentration on the uptake of ^{3}H UTP and ^{3}H ATP by the 150,000 x g supernatant of rat liver cells

Reaction mixtures contained 50 μmoles tris-HCl, pH 8.0, 4.0 μmoles $MgCl_2$, 10 mμmoles EDTA, 200 μg of highly polymerized yeast RNA, 2 μmoles phosphocreatine, 20 μg phosphocreatine kinase, and 0.734 mg of enzyme protein.

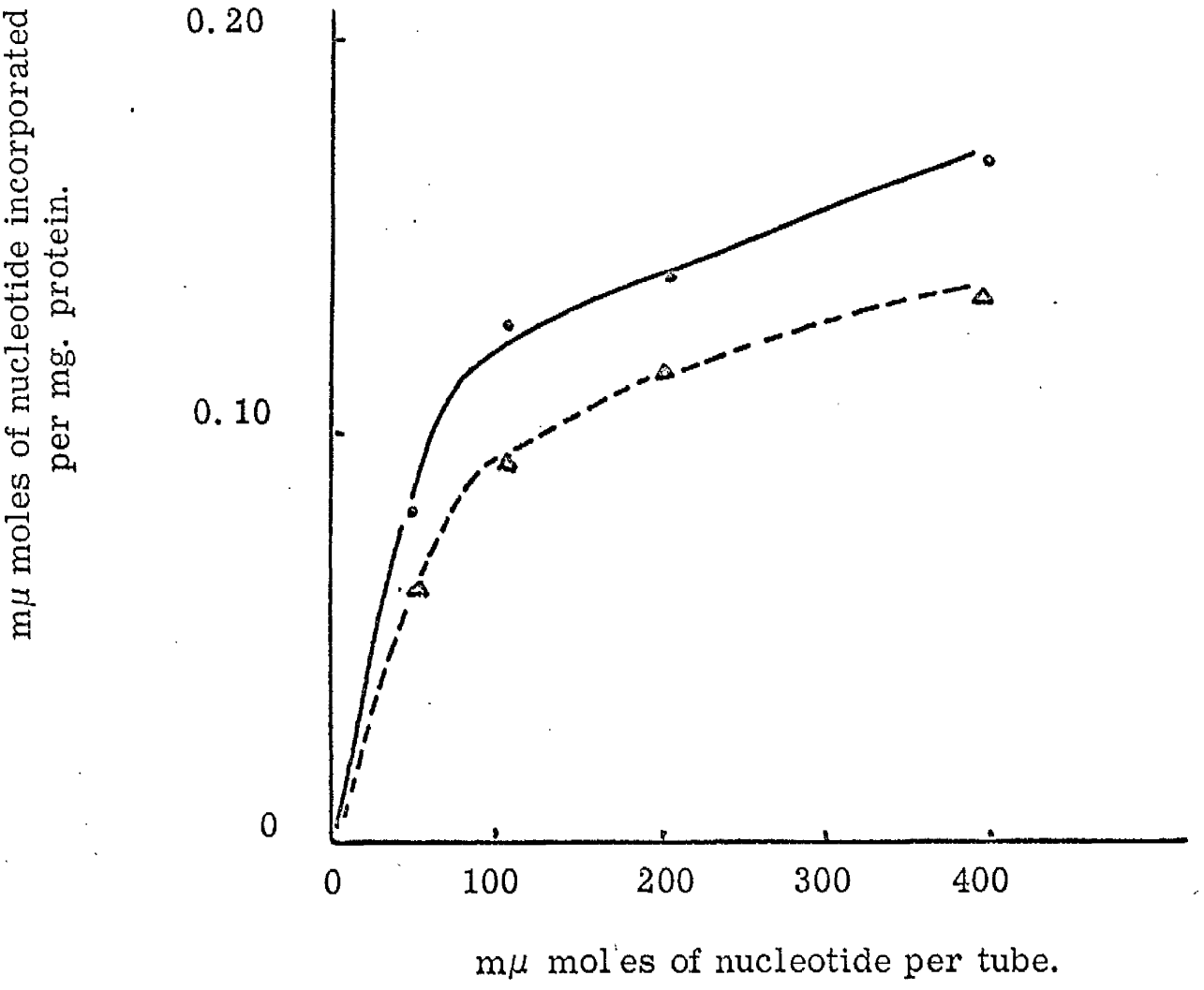
^{3}H-UTP or ^{3}H-ATP (20 μc/μmole) were included as indicated. Incubations were at 37°C for 10 minutes.

^{3}H-UTP



^{3}H-ATP

Figure 10.


0.20
0.10
0
0
100
200
300
400
mμ moles of nucleotide incorporated per mg. protein.
mμ moles of nucleotide per tube.

The extract was neutralised and the resulting ribonucleosides and ribonucleoside monophosphates separated by developing descending chromatograms on Whatman No. 1 paper overnight in the isopropanol/water/NH_4OH solvent (85/15/1). The resulting spots were eluted and the radioactivity counted as described in Section 4(i).3 under Experimental.

Following this procedure 3′ terminal ribonucleotides are released as free nucleosides, while those incorporated into internal positions are released as 2′ 3′ ribonucleoside monophosphates. The ratio of radioactivity between the free nucleosides and the nucleoside monophosphates therefore provides a convenient method for estimating the average length of the newly synthesised chains.

The results are presented in Table 6a. The figures are corrected for the radioactivity due to unincorporated UTP. The results of an identical independant experiment are given in Table 6b. It can be seen that the average length of the newly synthesised chains is very short, only 2-3. Addition of ATP, GTP and CTP had little effect, perhaps stimulating the addition of one more nucleotide. The results of the ^{32}P distribution experiment are exactly what one would expect if 2 - 3 uridylate residues were being incorporated terminally into a population of RNA molecules ending in all four ribonucleosides.

The ability of the 150,000 x g fraction to incorporate the other ribonucleoside triphosphates is presented in Figure 9. ATP is incorporated to an appreciable extent but GTP uptake is rather poor. No incorporation of CTP could be detected. Substrate

FIGURE 11

G-75 Sephadex chromatography of alkali hydrolysed highly polymerised RNA

50 Mg of highly polymerised yeast RNA was hydrolysed for 8.0 min at 37°C in 0.1M KOH. The extract was neutralised with PCA, insoluble salt removed and the hydrolysate applied to a 35 cm x 2.5 cm of Sephadex which had been equilibrated at room temperature to 0.01M-tris-HCl. ph 8.0. Elution was with the same buffer.

Material between the lines was pooled and treated as in text.

Blue Dextran dye, used as marker, and which is completely excluded from the gel, is marked by cross-hatching.

Figure 11.

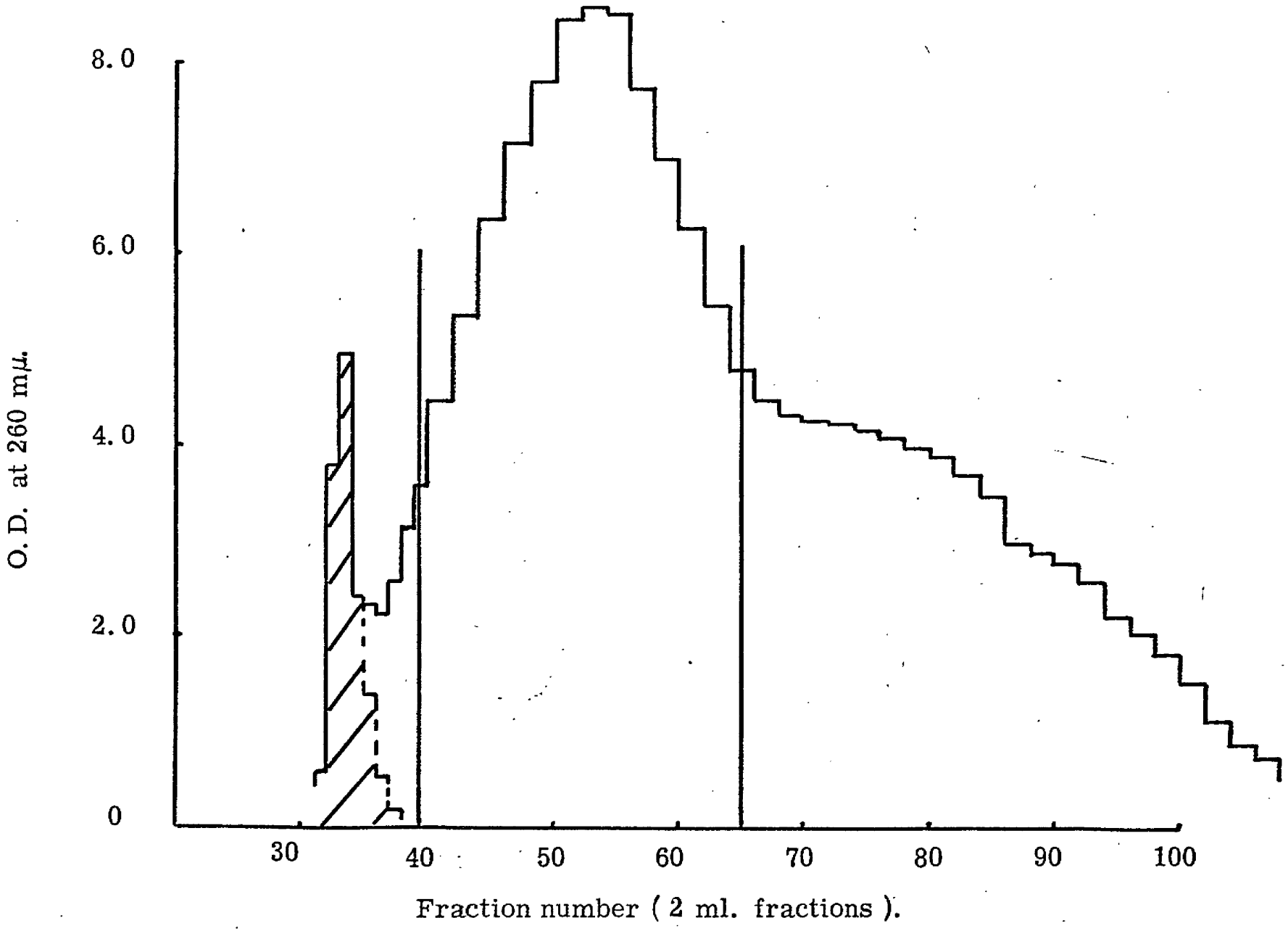

8.0
6.0
4.0
2.0
0
O. D. at 260 mμ.
30
40
50
60
70
80
90
100
Fraction number (2 ml. fractions).

FIGURE 12

Elution profile of dephosphorylated RNA

from G-75 Sephadex

25 O.D units of dephosphorylated RNA were applied in 2.5 ml to a 35 cm/2.4 cm column of G-75 Sephadex. Elution was by 0.01M tris-HCl buffer pH 8.0.

Dextran Blue marker (completely excluded from the gel) is marked in cross-hatching.

Figure 12.

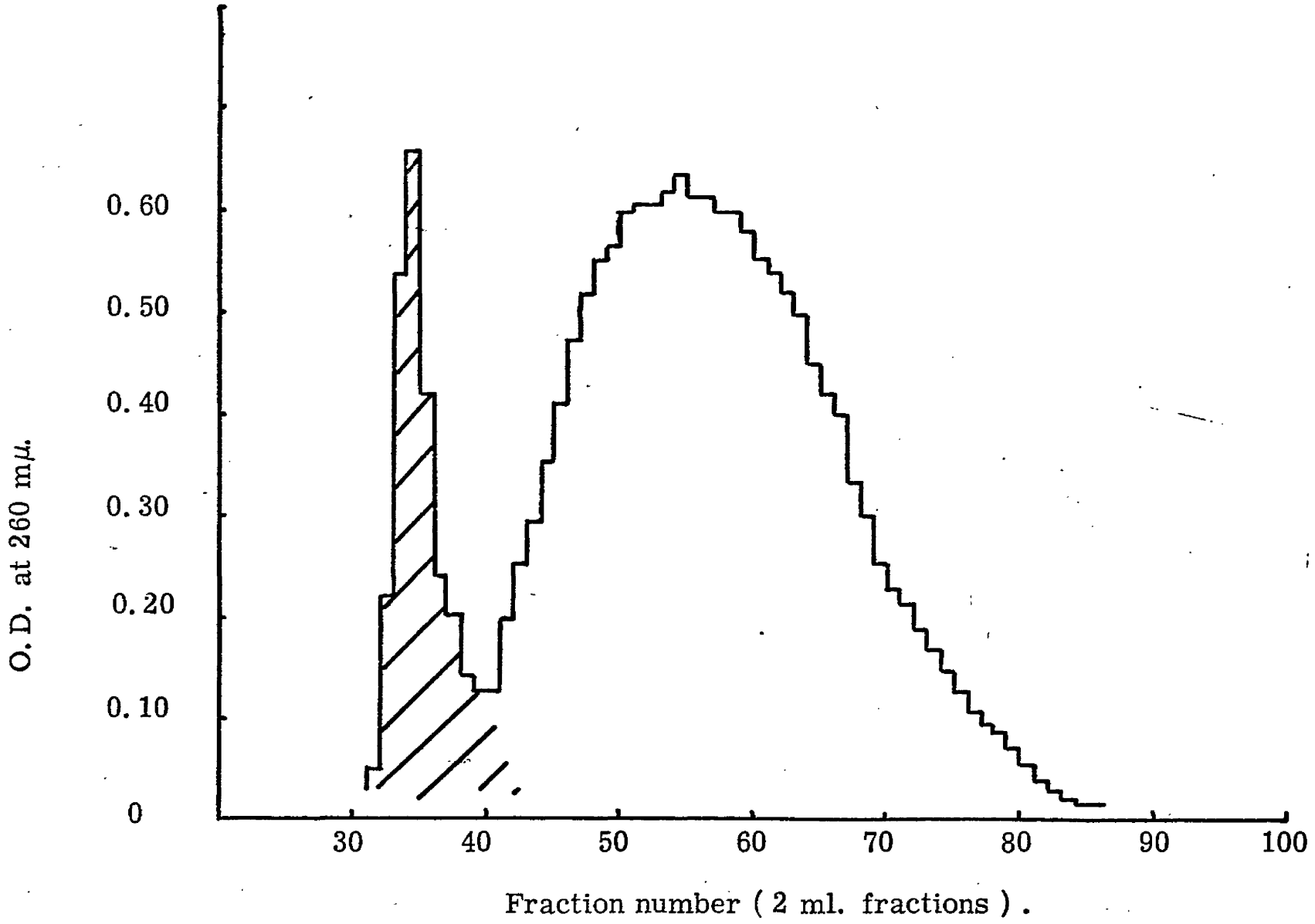

0. 60
0. 50
0. 40
0. 30
0. 20
0. 10
0
O. D. at 260 mμ.
30
40
50
60
70
80
90
100
Fraction number (2 ml. fractions) .

FIGURE 13

Effect of increasing amounts of RNA primer, containing either a 3' hydroxyl or predominantly 3' phosphate ends, on the uptake of ^{3}H UTP by the 150,000 x g supernatant of rat liver cells

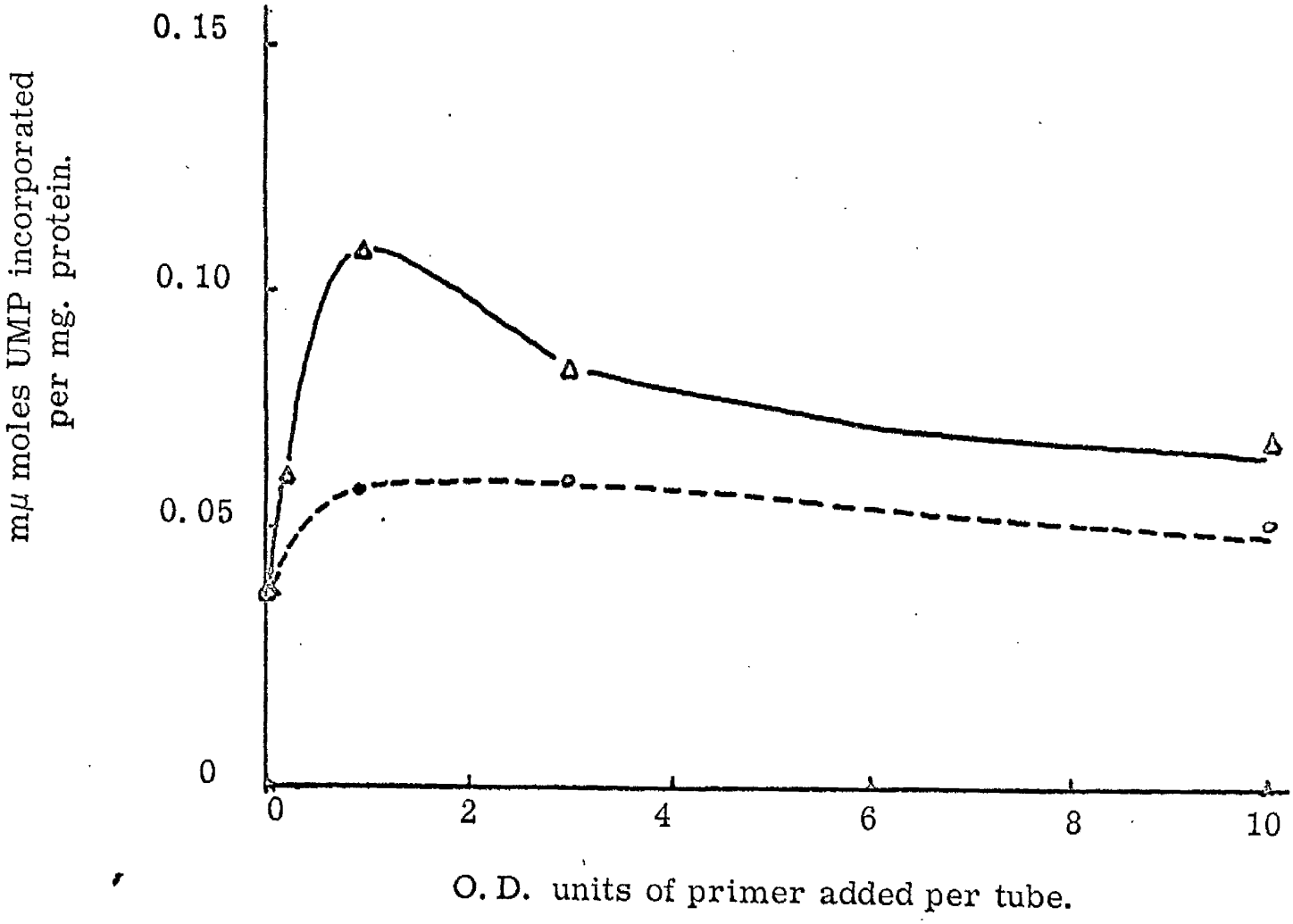
Reaction mixtures contained 50 μmoles tris-HCl buffer pH 8.0, 4.0 μmoles $MgCl_2$, 10 mμmoles EDTA, 0.1 μmole of ^{3}H UTP (20 μc/μmole), 0.1 μmole each of ATP, GTP and CTP, 2 μmoles phosphocreatine, 20 μg phosphocreatine kinase, 0.702 enzyme protein per 0.5 ml total volume.

The incubations were at 37°C for 5.0 minutes.

———△——— short dephosphorylated RNA

- - - -O- - - - short untreated RNA

Figure 13.


0.15
0.10
0.05
0
mμ moles UMP incorporated per mg. protein.
0
2
4
6
8
10
O. D. units of primer added per tube.

concentration curves for ATP and UTP are given in Figure 10, and it can be seen that there is little difference in the response of the enzyme to varying concentrations of the two substrates. With ATP as substrate it was found that the average chain length was only about one.

Taking these facts together it is possible to conclude that no heteropolymer formation occurs, and that the results are best explained by the incorporation of a few nucleotide residues into terminal positions of RNA.

The priming ability of RNA chains ending in 3′ hydroxyl, or 2′, 3′ phosphate nucleotides was then tested. Phosphorylated primers were prepared by controlled alkaline hydrolysis of highly polymerised yeast RNA. This treatment produces new 3′ phosphorylated ends. A portion of the RNA could then be treated with alkaline phosphatase to remove terminal phosphate groups, (section 7(ii)2. under Experimental). Alkaline phosphatase was inactivated before use of dephosphorylated RNA as primer.

50 mg of highly polymerised yeast RNA was hydrolysed in 0.1 M KOH at 37°C for 8.0 min. The KOH was neutralised with perchloric acid and insoluble salt removed by centrifugation in the cold. The hydrolysate was filtered through a 35 cm x 2.5 cm column of G-75 Sephadex (prepared as in section 10 under Experimental). Elution was with 0.01 M tris-HCl pH 7.0, the O.D. at 260 mμ of the effluent material was monitored and the results are shown in Figure 11. The contents of tubes 40 - 65 were pooled and the RNA precipitated from 66 per cent ethanol and 1.0% potassium acetate. The RNA was divided in two portions and dissolved in

0.01 M tris-HCl pH 8.0. One portion was treated with an excess of alkaline phosphatase, for 4.0 min at 37°C, and then the enzyme was inactivated as previously described. That this treatment did not seriously degrade the molecule further is shown in Figure 12. The original yeast RNA sedimented in the ultracentrifuge with a sedimentation value of 3.3S. After alkaline hydrolysis and Sephadex chromatography the sedimentation coefficient of the shortened product was 1.8S. Although it is not possible to make exact calculations, this probably represents a four to five fold reduction in molecular size. If the original highly polymerised yeast RNA contained only 3′ hydroxyl ends, alkaline hydrolysis would therefore produce a product in which 75 - 80% of the 3′ ends would be phosphorylated.

The effect of adding increasing amounts of the shortened primers on the uptake of UTP by the 150,000 x g supernatant is shown in Figure 13. It can be seen that at low primer concentrations the dephosphorylated RNA is a much more effective primer than the untreated RNA. It was concluded that the enzyme requires free 3′ hydroxyl ends and the results also suggest that nucleotides are incorporated into the 3′ ends of the primer itself, although this was not demonstrated directly. It will be observed that the untreated primer can support approximately 25% of the activity of that of dephosphorylated RNA. This agrees closely with the estimated 20-25% of 3′ hydroxyl ends which this primer contains.

3. Characterisation of the microsomal activity

The effect of increasing amounts of microsomes on the uptake of (α-^{32}P)UTP is shown in Figure 14. A linear response was

observed up to 1 mg of protein per tube. Table 2 shows the general substrate requirements for the microsomal enzyme. The requirement for the presence of all four triphosphates was very noticeable, although that for RNA less so. It can be seen from Table 7 that phosphocreatine and phosphocreatine kinase stimulate the reaction even in the presence of ATP, GTP and CTP. The pH dependance of the microsomal enzyme is shown in Figure 15. A fairly well defined optimum was observed in the range pH 7.5-8.0. Thereafter the reaction was carried out at pH 8.0. From figure 16 it can be seen that the reaction is dependant on added Mg^{++} ions, while Mn^{++} had no stimulatory effect whatsoever. The activity observed in the absence of added Mg^{++} is probably due to the presence of 0.51 μmoles of $MgCl_2$ per tube added with the enzyme and derived from the homogenising medium. In subsequent experiments 4.0 μmoles of $MgCl_2$ were added per tube. The time course of the reaction is shown in Figure 17. Incorporation increases rapidly till about 10 min and then drops sharply. The rapid loss of newly formed RNA after 10 min suggests nuclease activity. However, the presence of 10 μg of Bentonite per tube made no significant difference to the reaction kinetics.

Table 8 shows that while the addition of 5 μmoles of inorganic phosphate per tube inhibited the reaction by only about 50%, 2.5 umoles of inorganic pyrophosphate abolished incorporation completely. This experiment suggests that pyrophosphate may be released during the reaction, and UMP incorporated into polynucleotides. It further suggests that UTP, rather than UDP or UMP is the true substrate for the enzyme.

FIGURE 14

Effect of adding increasing amounts of the enzyme protein on the uptake of ^{3}H-UTP by rat liver microsomes

Reaction mixtures contained 50 μmoles of tris-HCl buffer, 4 μmoles $MgCl_2$, 0.1 μmole EDTA, 0.1 μmole ^{3}H-UTP (20 μc per μmole), 0.1 μmole each of ATP, GTP and CTP, 50 μg of highly polymerised yeast RNA, 2 μmoles phosphocreatine and 10 μg phosphocreatine kinase in 0.5 ml total volume.

Various amounts of microsome were included as indicated.

Incubations were at 37°C for 10 minutes.

FIGURE 15

pH dependance of the uptake of ^{3}H-UTP by rat liver microsomes

Reaction mixtures contained 4 μmoles $MgCl_2$, 10 mμmoles EDTA, 0.1 μmole ^{3}H-UTP (20 μc/μmole), 0.1 μmole each of ATP GTP and CTP, 100 μg of RNA, 2 μmoles phosphocreatine, 20 μg phosphocreatine kinase and 0.769 mg of microsomal protein in a total volume of 0.5 ml.

Where indicated 50 μmoles of tris-HCl buffer of the appropriate pH was included.

Incubations at 37°C for 6.5 minutes.

Figure 14.

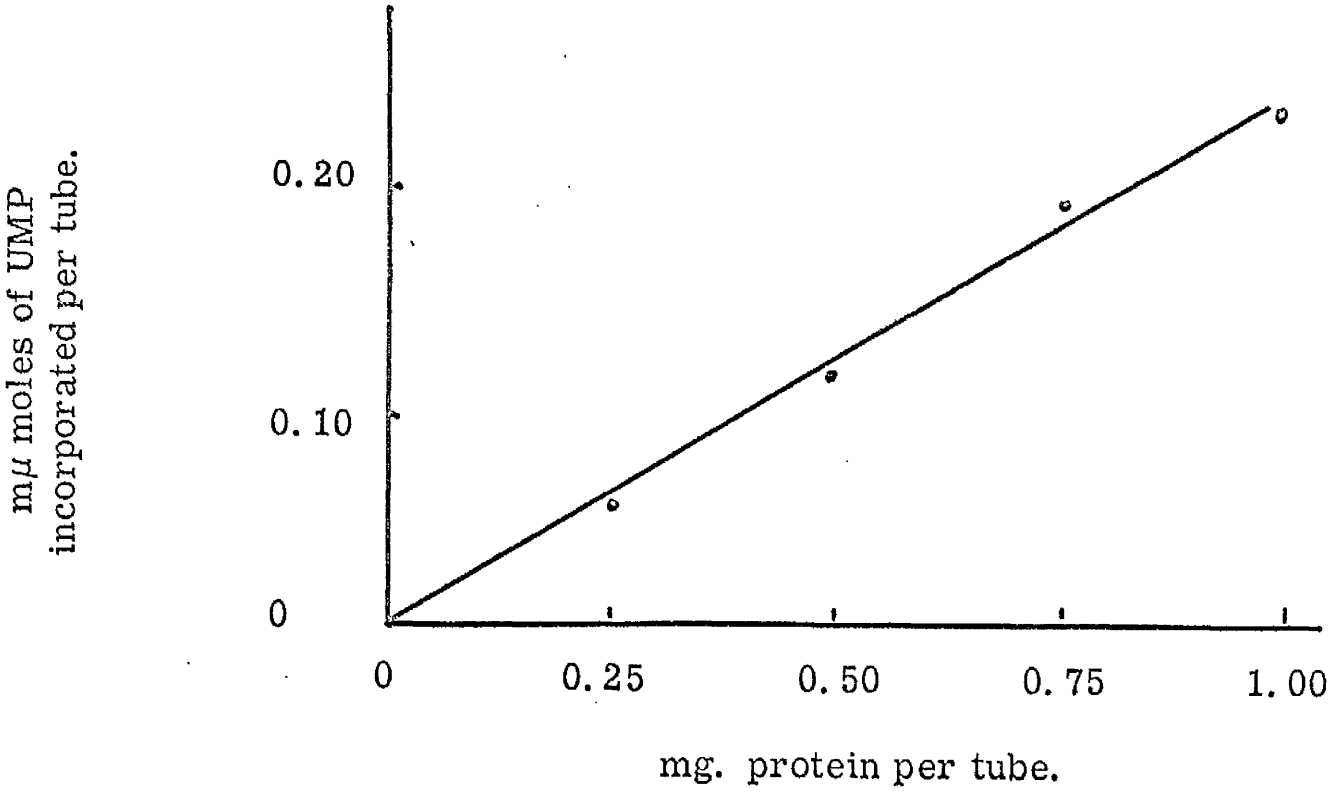


Figure 15.

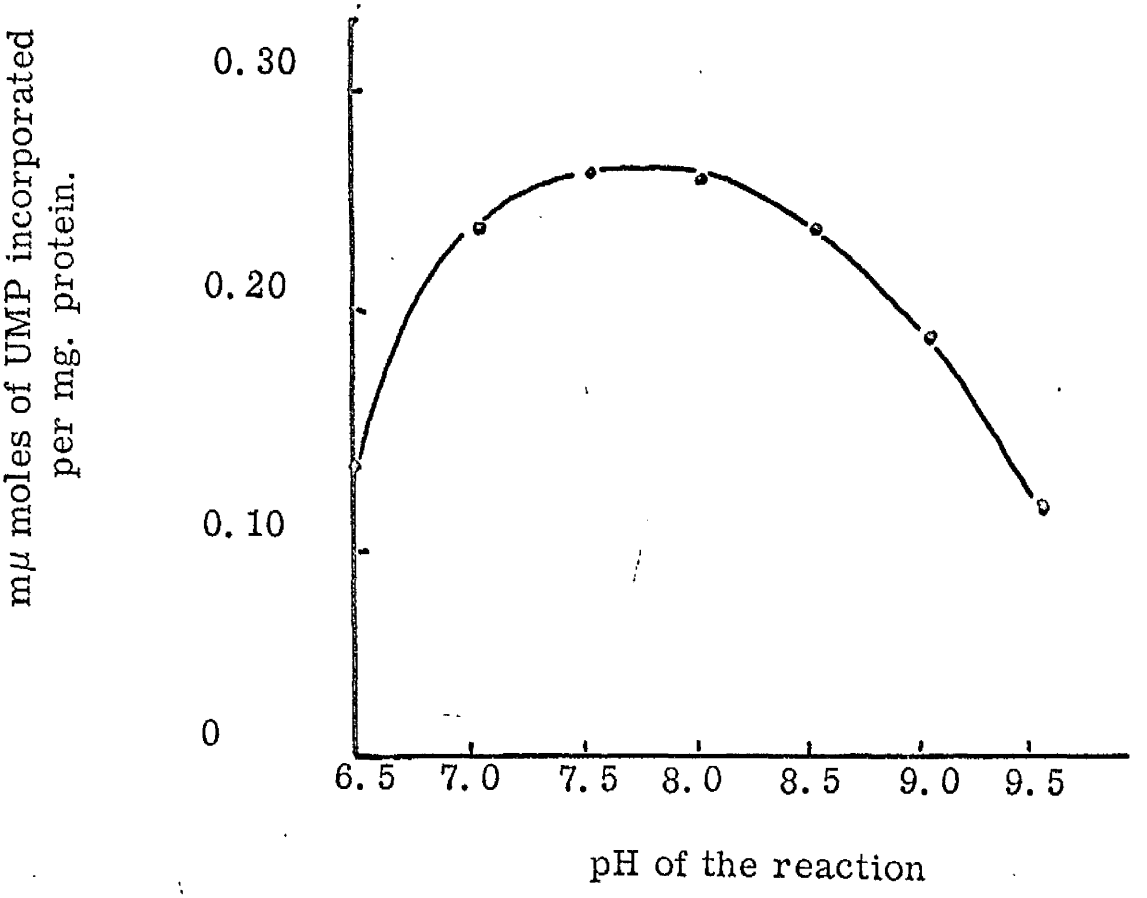


TABLE 7

Effect of phosphocreatine and phosphocreatine kinase on the uptake of UTP by rat liver microsomes

Reaction mixtures contained 50 μmoles tris-HCl buffer, pH 8.0, 4.0 μmoles $MgCl_2$, 0.1 μmole (α-^{32}P) UTP, (0.8×10^6 cpm/μmole), 50 μg of RNA and 0.745 mg of microsomal protein in a total volume of 0.5 ml.

Where indicated 0.1 μmole each of ATP, GTP and CTP and 2 μmoles phosphocreatine and 50 μg of phosphocreatine kinase were included.

Incubations were for 15 minutes at 37°C.

TABLE 8

The effect of inorganic phosphate and inorganic pyrophosphate on the uptake of UTP by rat liver microsomes

Reaction mixtures contained 50 μmoles tris-HCl buffer, pH 8.0, 4 μmoles $MgCl_2$, 0.1 μmole of ^{3}H-UTP (20 μc/μmole) 0.1 μmole each of ATP, GTP and CTP, 2 μmoles phosphocreatine, 50 μg phosphocreatine kinase and 0.632 mg microsomal protein in a total volume of 0.5 ml.

5.0 μmoles of disodium hydrogen phosphate on 2.5 μmoles of tetrasodium pyrophosphate, buffered to approximately pH 8.0 were included where indicated. Incubations for 6 min. at 37°C.

T A B L E 7

Additions	Incorporation as a percentage of the 15 minutes' activity
NIL	100
ATP, GTP & CTP	112
PE/PCK; ATP, CTP & GTP	116

T A B L E 8

Additions	mumoles UMP incorporated per mg. protein
NIL	0.183
5.0 mmoles Pi	0.930
2.5 umoles PPi	0

FIGURE 16

The effect of increasing concentrations of both Mg^{++} and Mn^{++} on the uptake of ^3H-UTP by rat liver microsomes

Reaction mixtures contained 50 μmoles tris-HCl buffer, pH 8.0, 0.1 μmole ^3H-UTP (20 μc/μmole), 0.1 μmole each of ATP, GTP and CTP, 50 μg of highly polymerised yeast RNA, 2 μmoles phosphocreatine, 20 μg of phosphocreatine kinase and 0.43 mg of protein in a total volume of 0.5 ml.

Various amounts of either $MgCl_2$ or $MnCl_2$ were included as indicated.

Incubations were at 37°C for 10 minutes.

———o——— $MgCl_2$

- - - -Δ- - - - $MnCl_2$

Figure 16.

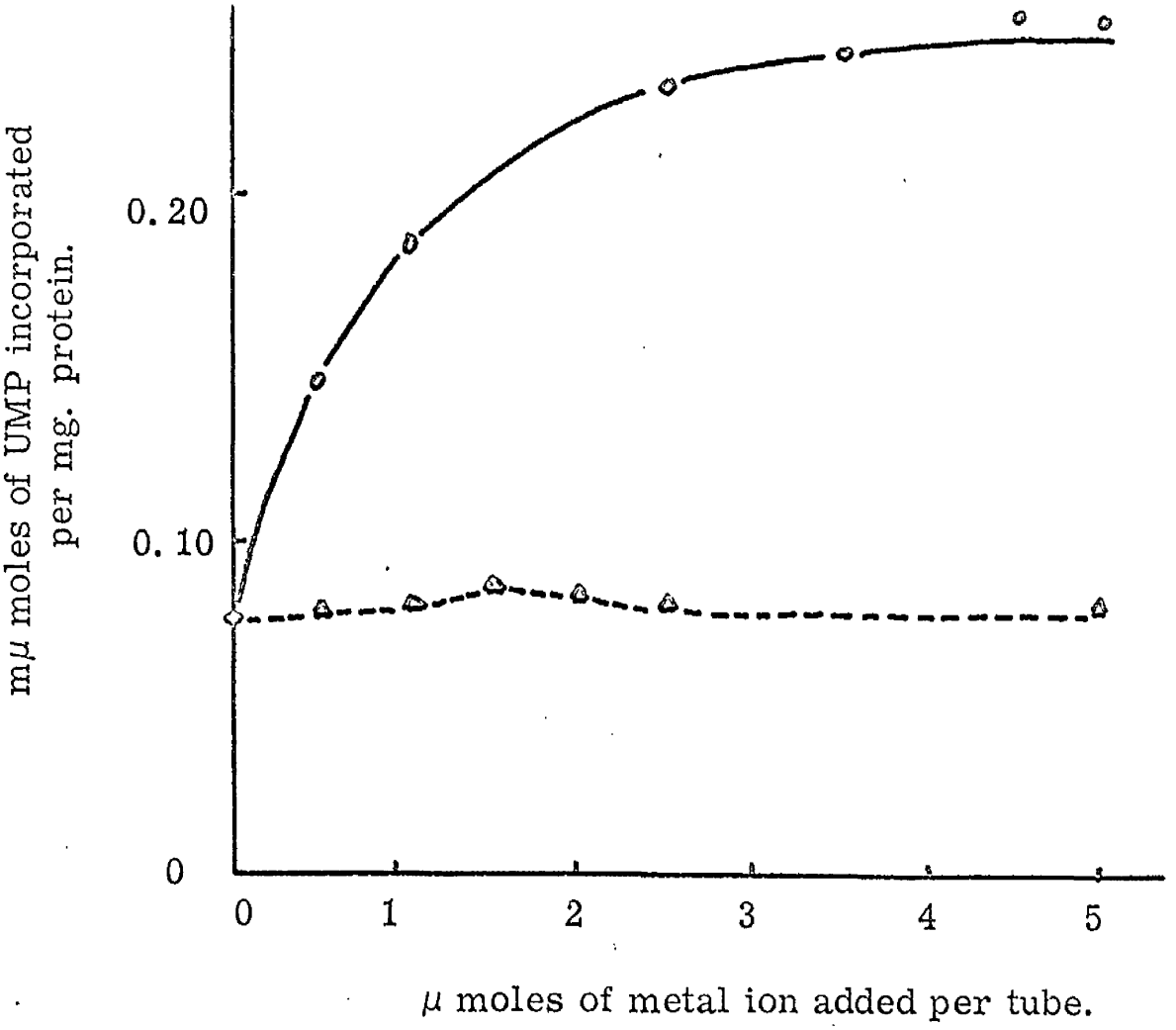

mμ moles of UMP incorporated per mg. protein.
0.20
0.10
0
0
1
2
3
4
5
μ moles of metal ion added per tube.

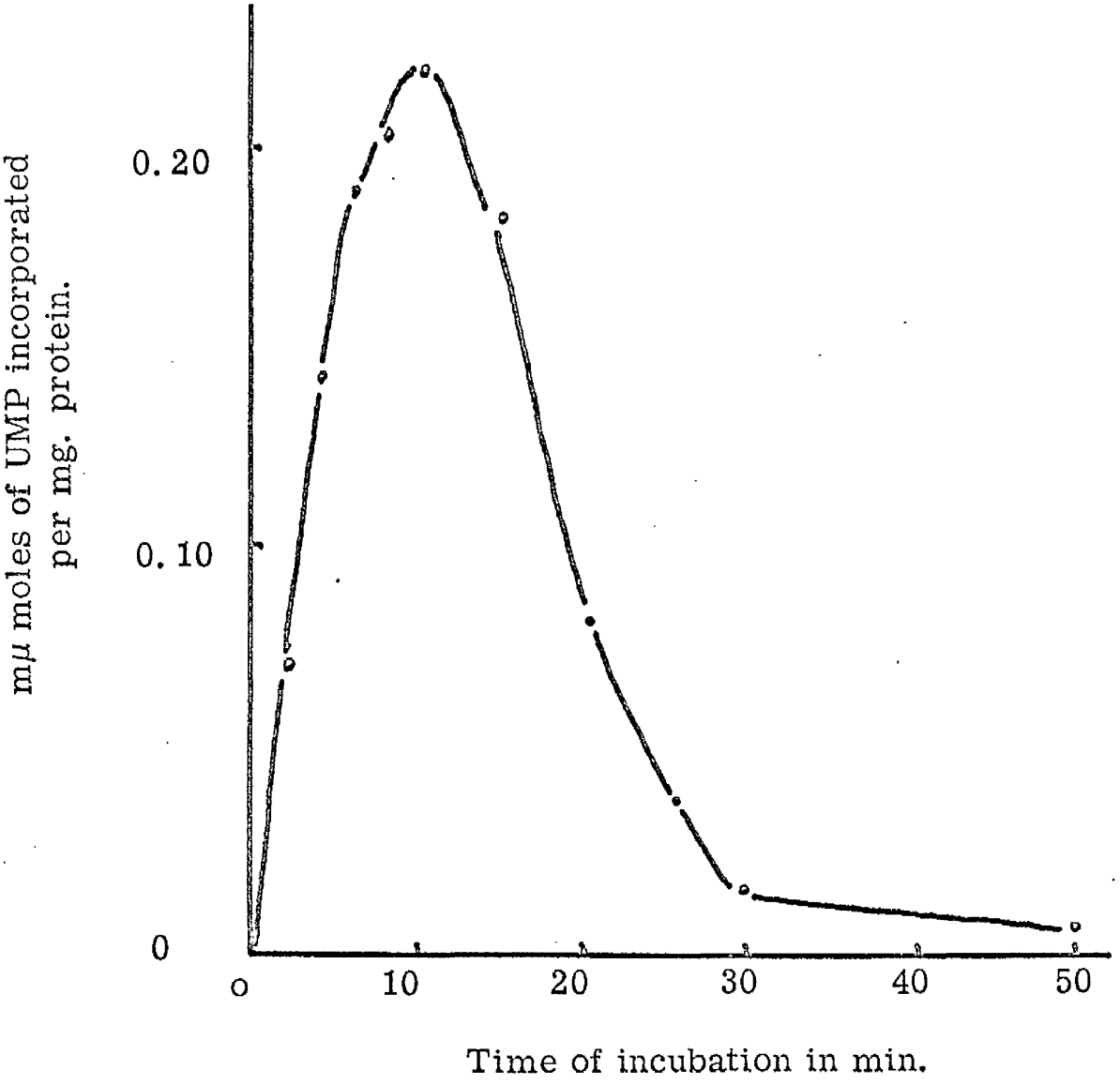
FIGURE 17

Time course of the incorporation of ^{3}H-UTP into acid insoluble products by rat liver microsomes

Reaction mixtures contained 50 μmoles tris-HCl buffer pH 8.0, 4 μmoles $MgCl_2$, 0.1 μmole ^{3}H-UTP (30 μc/μmole), 0.1 μmole each of ATP, GTP and CTP, 50 μg highly polymerised yeast RNA, 2 μmoles phosphocreatine, 50 μg phosphocreatine kinase and 0.87 mg of microsomal protein in a total volume of 0.5 ml.

Incubations were at 37°C for the appropriate time.

Figure 17.


mμ moles of UMP incorporated per mg. protein.
0.20
0.10
0
o
10
20
30
40
50
Time of incubation in min.

Experiments were carried out to study the effects of RNA, DNA, ribonuclease, deoxyribonuclease and actinomycin D on the uptake of UTP by the microsomes. From Table 9 it can be seen that while the addition of 50 µg of yeast RNA stimulated the reaction, 50 µg of DNA from Krebs II ascites cells, (a gift from Dr. J.B. Shephord), inhibited incorporation by about 20 per cent. Deoxyribonuclease had little effect, being mildly stimulatory if anything, while 10 µg of ribonuclease abolished incorporation completely. Actinomycin D (section 4 (i) under Introduction) over a wide range of concentrations had no effect on the reaction. These results provide very convincing evidence that the uptake of UTP is by an RNA, rather than a DNA dependant reaction.

The dependance of UTP uptake on the presence of ATP, GTP and CTP obviously suggests the formation of a heteropolymer product. In order to test this possibility the distribution of radioactivity in UMP, GMP, AMP and CMP was measured, following alkaline hydrolysis of the product of (α-^{32}P) UTP uptake by the microsomes. The experimental approach was identical to that already described for the 150,000 x g supernatant (see Table 5 and accompanying text). The results of several different experiments are presented in Table 10. As can be seen, the major portion of the radioactivity was recovered in UMP in each case, suggesting extensive incorporation of UMP adjacent to UMP in the product. However, a significant percentage of the ^{32}P was recovered in the other ribonucleotides, especially when ATP, GTP and CTP were included in the reaction mixture. There is a tendancy for UMP to be incorporated adjacent to GMP. From the results, then, it would not be possible to

TABLE 9

The Effect of RNA, DNA, ribonuclease, deoxyribonuclease and actinomycin D on the uptake of UTP by rat liver microsomes

Reaction mixtures contained 50 μmoles tris-HCl buffer pH 8.0, 4.0 μmoles $MgCl_2$, 20 μmoles EDTA, 0.1 μmole ^{3}H-UTP, (30 μc/μmole), 0.1 μmole each of ATP, GTP and CTP, 2 μmoles phosphocreatine, 50 μg phosphocreatine kinase and 1.10 mg of microsomal enzyme per tube.

Where indicated 50 μg of highly polymerised yeast RNA, 50 μg of DNA from Krebs II ascites tumour cells, 10 μg ribonuclease, 10 μg deoxyribonuclease and various amounts of actinomycin D were included.

Incubations were at 37°C for 5 minutes.

T A B L E 9

Additions	mμmoles UMP incorporated per mg. pr.
NIL	0.368
RNA	0.407
DNA	0.286
RNA ase	0
DNA ase	0.376
Act. D 1 μg	0.361
Act. D. 10 μg	0.368
Act. D. 50 μg	0.342
Act. D. 100 μg	0.382

TABLE 10

Distribution of radioactivity in UMP, GMP, AMP and CMP obtained following alkaline hydrolysis of the product of (α-^{32}P) UTP uptake by rat liver microsomes

Reaction mixtures contained 50 µmoles tris-HCl buffer, pH 8.0, 4 µmoles $MgCl_2$, 0.1 µmole (α-^{32}P) UTP, (20-50 x 10^6 cpm per µmole), 2 µmoles phosphocreatine, 50 µg phosphocreatine kinase and up to 1 mg of enzyme protein per total volume of 0.5 ml.

Where indicated 0.1 µmole each of ATP, GTP and CTP and 50 µg of highly polymerised yeast RNA were included.

Incubations were at 37°C for 10 minutes.

The correction for the radioactivity due to unincorporated UTP was made as before (See Table 5).

T A B L E 1 0

Addition	Nucleotide	% radioactivity recovered in the various nucleotides on different occs.				
		I	II	III	IV	V
NIL	UMP		87.6	89.6	77.2	71.6
	CMP		8.7	3.9	22.8	28.4
	AMP		0	1.0	0	0
	GMP		3.7	5.5	0	0
RNA	UMP		81.1	100	100	100
	CMP		9.3	0	0	0
	AMP		4.5	0	0	0
	GMP		5.1	0	0	0
AMP, GMP, CMP	UMP	56.1	58.0	84.8	61.4	62.4
	CMP	25.0	23.6	10.4	27.3	17.7
	AMP	7.4	7.2	0.2	3.8	12.7
	GMP	12.5	11.2	4.6	7.5	7.2
RNA, AMP, GMP, CMP	UMP	57.6	64.3	83.4	66.6	59.3
	CMP	16.1	15.6	8.2	17.7	24.1
	AMP	16.8	10.4	3.3	4.4	11.1
	GMP	9.5	9.7	5.1	11.3	5.5

exclude some synthesis of heteropolymer chains, especially in the presence of all four ribonucleoside triphosphates.

It was decided, therefore, to investigate the characteristics of each ribonucleoside triphosphate in greater detail. Substrate concentration curves for ATP and UTP are shown in Figure 18. The curves are similar, a rather broad, sloping plateau being observed between 100 and 400 m umoles of nucleotide per tube. The characteristics of incorporation of the four ribonucleoside triphosphates are presented in Table 11. UTP, CTP and GTP showed similar patterns of incorporation, stimulation being obtained on the addition of RNA and the other three ribonucleoside triphosphates in each case. On the other hand, the uptake of ATP was noticeably inhibited in the presence of GTP, UTP and CTP, both in the presence and absence of added RNA. In addition the incorporation of GTP was much lower than that of the other ribonucleotides, so that any heteropolymer formation would account for only a small proportion of the total activity.

The incorporation of each ribonucleotide was measured in the presence and absence of the other, both singly and together, and the results are presented in Table 12. The pattern which emerges from this experiment is obscure. The main point, however, is that in no case is the maximum incorporation achieved in the presence of all four ribonucleoside triphosphates, indicating again that the major portion of the activity had the characteristics of homo-polymer formation.

There was the possibility that the stimulation obtained in the presence of the other triphosphates reflected protection of the

FIGURE 18

The effect of increasing amounts of UTP or ATP on the incorporation of UTP or ATP into acid-insoluble products by rat liver microsomes

Reaction mixtures contained 50 μmoles tris-HCl buffer, pH 8.0, 4 μmoles $MgCl_2$, ^3H-ribonucleoside triphosphate (either UTP or ATP; 20 μc/μmole), 0.1 μmole of each of the other three ribonucleoside triphosphates, 50 μg of RNA, 2 μmoles phosphocreatine, 20 μg phosphocreatine kinase, and 0.73 mg of microsomal protein in a total volume of 0.5 ml.

Increasing amounts of the ^3H ribonucleoside triphosphate were added as indicated.

Incubations were at 37°C for 10 minutes.

———△——— ^3H-UTP

- - - - o - - - - ^3H-ATP

Figure 18.

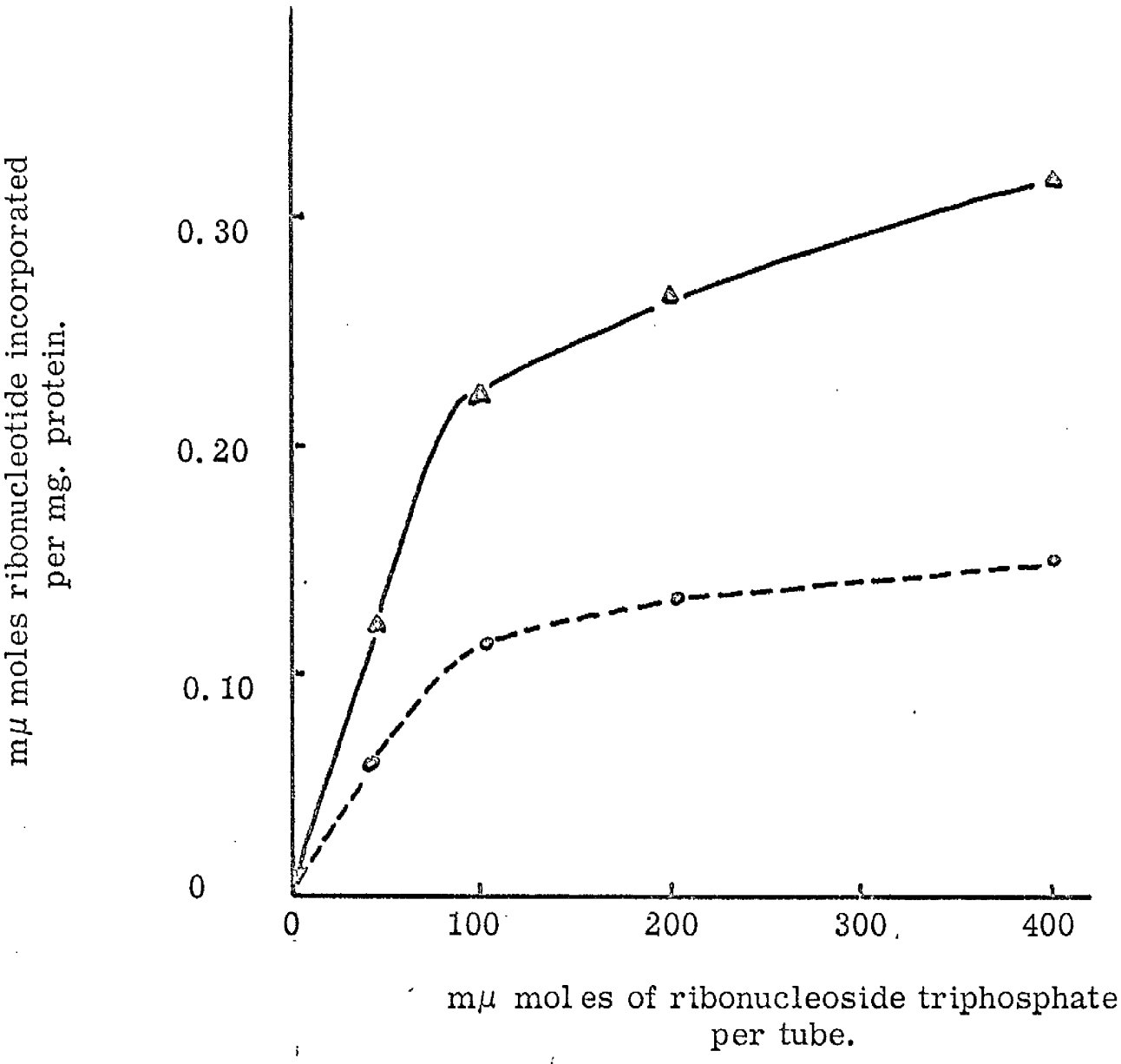

mμ moles ribonucleotide incorporated per mg. protein.
0.30
0.20
0.10
0
0
100
200
300
400
mμ moles of ribonucleoside triphosphate per tube.

TABLE 11

The characteristics of incorporation of ATP, GTP, CTP and UTP by rat liver microsomes

Reaction mixtures contained 50 μmoles tris-HCl buffer pH 8.0, 3 μmoles $MgCl_2$, 100 mμmoles EDTA, 0.1 μmole of ^3H-ribonucleoside triphosphate (20 μc/μmole), 2 μmoles phosphocreatine, 50 μg phosphocreatine kinase and 0.475 mg of protein in a total volume of 0.5 ml.

50 μg of highly polymerised and 0.1 μmole each of the other three ribonucleoside triphosphates were added where indicated.

Incubations were at 37°C for 10 minutes.

T A B L E 1 1

^{3}H-nucleotide	Additions	mumoles nucleotide incorporated per mg. pr.
UTP	Nil	0.059
	ATP, GTP, CTP	0.576
	RNA	0.199
	RNA, ATP, GTP, CTP.	0.803
CTP	Nil	0.201
	ATP, GTP, CTP	0.723
	RNA	0.409
	RNA, ATP, GTP, CTP.	0.953
ATP	Nil	0.211
	ATP, GTP, CTP	0.147
	RNA	0.361
	RNA, ATP, GTP, CTP.	0.188
GTP	Nil	0.000
	ATP, GTP, CTP	0.022
	RNA, ATP, GTP, CTP	0.057
	RNA	0.028

TABLE 12

The incorporation of each ribonucleoside triphosphate by the rat liver microsomes in the presence and absence of each of the others, singly and together

Reaction mixtures contained 50 μmoles tris-HCl buffer pH 8.0, 4 μmoles $MgCl_2$, 10 mμmoles EDTA, 0.1 μmole of ^3H-ribonucleoside triphosphate (20 μc/μmole), 50 μg of RNA, 2 μmoles phosphocreatine, 20 μg phosphocreatine kinase and up to 1 mg. of microsomal protein in a total volume of 0.5 ml.

Where indicated 0.1 μmole each of the specified ribonucleoside triphosphates were included.

Incubations at 37°C for 10 minutes.

TABLE 12

^{3}H-nucleotide	Additions	nmole ^{3}H nucleotide incorporated per mg protein on two different occasions	
		I	II
UTP	NIL	0.134	0.022
	ATP	0.456	0.269
	GTP	0.252	0.120
	CTP	0.307	0.037
	ATP, GTP, CTP	0.222	0.204
GTP	NIL	0.148	0.032
	ATP	0.308	0.265
	GTP	0.267	0.085
	UTP	0.157	0.055
	ATP, GTP, UTP	0.178	0.137
ATP	NIL	0.187	0.148
	UTP	0.112	0.075
	GTP	0.284	0.301
	CTP	0.218	0.120
	UTP, GTP, CTP	0.100	0.058
CTP	NIL	0.040	0.000
	ATP	0.090	0.081
	UTP	0.020	0.009
	GTP	0.060	0.009
	ATP, UTP, GTP	0.000	0.000

3 H-ribonucleoside triphosphate from phosphatase activity, which would render the substrate inactive. This was tested by an experiment in which the effect of ATP, and ATP, GTP and CTP on the ratio of UTP to other uridine nucleotides produced during incubation was measured. Microsomes were incubated with ^{14}C-UTP under normal conditions and then acid insoluble material precipitated. Ribonucleoside triphosphates were separated from the diphosphates and monophosphates from the acid soluble fraction, by descending chromatography on paper, as described under Experimental. The chromatogram was eluted with 0.001M formic acid and the radioactivity in the triphosphate spot, and the diphosphate and monophosphate region, measured in the scintillation spectrometer. The results are presented in Table 13. After 10 min approximately 50% of the UTP has been degraded to UDP or UMP. Whereas the presence of ATP alone barely effects the ratio, in the presence of ATP, GTP and CTP approximately 70% of the uridine nucleotides were recovered as UTP. That the experimental approach is valid is suggested by an unincubated sample, from which greater than 90% of the uridine nucleotides were recovered as UTP. The results suggest that the stimulation of UTP uptake observed on addition of ATP, GTP and CTP may be due to protection of the UTP. However, it can be seen from Table 11 that ATP alone stimulates the reaction to a greater extent than ATP, GTP and CTP, and this result has been obtained in a number of independant experiments not shown here. It is obvious from Table 13 that this cannot be due to protection of UTP, and no explanation for this result can be offered at present. The

TABLE 13

The effect of ATP, GTP and CTP on the hydrolysis of the phosphate groups of ^{14}C-UTP by rat liver microsomes

Reaction mixtures contained 50 μmoles tris-HCl pH 8.0, 4 μmoles $MgCl_2$, 1 μmole EDTA., 0.1 μmole ^{14}C-UTP, (1μc/μmole), 50 μg RNA., 2 μmoles phosphocreatine, 20 μg phosphocreatine kinase, 0.476 mg of microsomal protein per total volume of 0.5 ml.

Where indicated 0.1 μmole each of ATP, GTP and CTP were included.

Incubations were at 37°C for 10 minutes.

After incubation, the tubes were chilled in crushed ice and 0.5 ml of 0.3$\bar{M}$ ice-cold PCA added. Insoluble material was removed by centrifugation at 600 x *g* for 10 minutes at 2°C. The supernatant was neutralised with 0.3$\bar{M}$ KOH and insoluble salts removed by centrifugation at 600 x *g* for 20 minutes at 2°C.

0.05 ml aliquots of the supernatant were subjected to paper chromatography to separate UTP, UDP and UMP, as described under Experimental. The spots were located, cut out and eluted and the radioactivity estimated in the Nuclear Chicago scintillation spectrometer using the dioxay-based scintillation fluid already described.

TABLE 13

Additions	Ratio of radioactivity $\frac{\text{UTP}}{\text{UDP + UMP}}$
NIL (unincubated sample)	10.76
NIL	1.04
+ ATP	1.31
ATP + GTP + CTP	2.24

The pattern of incorporation of the ribonucleoside triphosphates remains obscure.

The microsomes are known to be a highly heterogenous fraction and it was decided to fractionate them with a view to further characterisation of the activity.

4. The activities of sub-fractions of rat liver microsomes

Rat liver microsomes were fractionated into rough surfaced vesicles (RSV), smooth surfaced vesicles, (SSV) and "free" ribosomes (FR) as described under Experimental. Total microsomes were isolated from the same homogenate by centrifuging a portion of the 18,000 x g supernatant at 150,000 x g for two hours at 2°C. The ability of each of the fractions to incorporate ^{3}H-UTP into acid-insoluble material is shown in Table 14. In experiment I the total RSV fraction was used, but in experiment II the RSV were fractionated into a heavy and a light fraction as under Experimental. Only the light fraction is shown. The heavy fraction exhibited absolutely no incorporating ability. Thereafter, only the light fraction of the RSV was used.

From Table 14 it is apparent that the fraction with the highest specific activity was the F.R. The SSV fraction contains very little activity, and was not further investigated. The characteristics of the RSV resembled that of the total microsomes, while uptake of UTP by the F.R. was consistently inhibited slightly by the presence of ATP, GTP and CTP. Despite the high specific activity of the F.R. approximately 30% of the total activity was recovered in this fraction and 70% in the RSV. The activity of each of these two subfractions was then investigated in greater detail.

TABLE 14

Ability of subfractions of rat liver microsomes to incorporate ^{3}H-UTP into acid insoluble material

Reaction mixtures contained 50 µmoles tris-HCl buffer pH 8.0, 4 µmoles $MgCl_2$, 0.1 µmole ^{3}H-UTP, (20 µc/µmole), 2 µmoles phosphocreatine, 20 µg phosphocreatine kinase and up to 1 mg of protein in a total volume of 0.5 ml.

Where indicated 50 µg of highly polymerised yeast RNA and 0.1 µmole each of ATP, GTP and CTP were included.

Incubations were at 37°C for 10 minutes.

T A B L E 1 4

Cell Fraction	Additions	mμmoles UMP per mg. protein on different occasions	
		I	II
Total microsomes	NIL	0.	0.
	ATP, GTP, CTP	0.321	0.150
	RNA	0.249	0.014
	RNA, ATP, GTP, CTP.	0.229	0.189
RSV	NIL	0.	0.
	ATP, GTP, CTP	0.166	0.410
	RNA	0	0.045
	RNA, ATP, GTP, CTP.	0.203	0.396
SSV	NIL	0.002	
	ATP, GTP CTP	0	----
	RNA	0.05	
	ATP, GTP, CTP, RNA	0.07	
F.R.	NIL	0.73	1.44
	ATP, GTP, CTP	0.684	1.362
	RNA	1.108	1.426
	RNA, ATP, GTP, CTP.	0.864	1.320

4. (i) The RSV fraction

The time course of the uptake of UTP by the RSV fraction is shown in Figure 19. The reason for the peculiar biphasic nature of the curve is not known but it has been observed on many occasions, and appears to be real. The most obvious explanation would be that different enzymes contribute to the overall activity.

Since the light RSV fraction was difficult to prepare in a pure state (see Section 3(iii) under Experimental) and was always contaminated to some extent with 78,000 x g supernatant fraction, experiments were designed to test the true nature of the activity. Figure 20 shows that the specific activity of the RSV drops considerably following resuspension in the homogenising medium and recentrifugation. After two washes virtually no activity remains. This strongly suggests that the activity found in the RSV fraction is due to contamination with the 78,000 x g supernatant fractions. However, the characteristics of UTP uptake by the two fractions are quite different (compare Tables 14 and 15). Therefore experiments were carried out whereby the characteristics of UTP uptake by the unwashed RSV, washed RSV, the washings and the 78,000 x g supernatant were prepared (Table 16). Unfortunately the interpretation of the results is confused by the shifting pattern of incorporation in the RSV fraction from experiment to experiment. There is no doubt that the washings have substantial activity with specific activities close to that of the 78,000 x g supernatant. Indeed the washings have a tendency to mimic the incorporation pattern of

FIGURE 19

Time course of UTP uptake by the RSV fraction

Reaction mixtures contain 50 μmoles tris-HCl buffer, pH 8.0, 4 μmoles $MgCl_2$, 10 mμmoles EDTA, 0.1 μmole ^3H-UTP (20 μc per μmole), 0.1 μmole each of ATP, GTP and CTP, 50 μg RNA, 2 μmoles phosphocreatine, 20 μg phosphocreatine kinase and 0.75 mg protein in a total volume of 0.5 ml.

Incubations were at 37°C.

Figure 19.

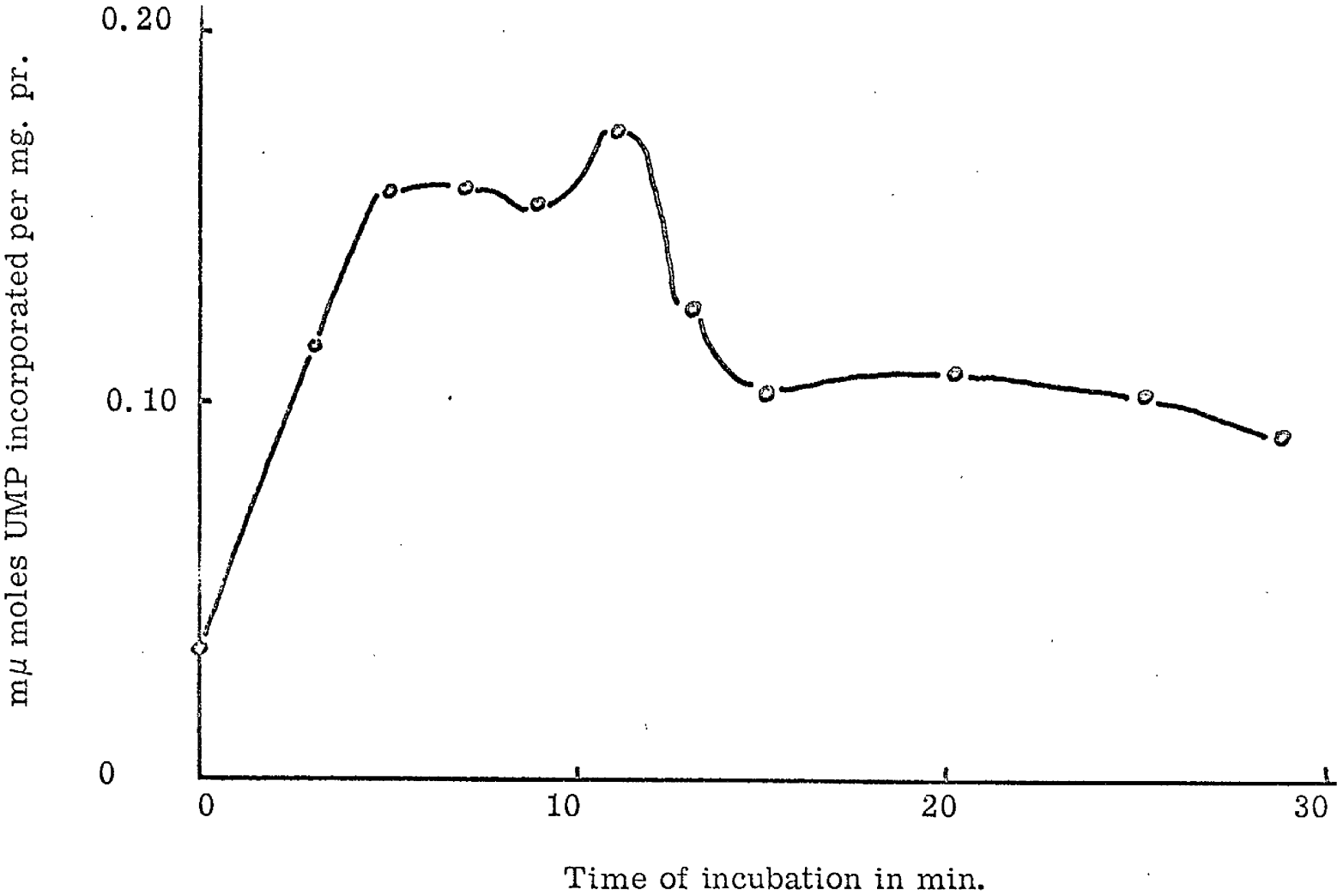

0.20
0.10
0
mμ moles UMP incorporated per mg. pr.
0
10
20
30
Time of incubation in min.

FIGURE 20

Effect of washing the RSV fraction

Reaction mixtures as in Figure 19 except that 5 μmoles of mercaptoethanol were included.

Precipitates obtained on centrifugation at 78,000 x g for one hour were resuspended in 10 ml of ice-cold homogenising medium (30% sucrose, 0.01$\bar{M}$ tris-HCl pH 8.0 and 0.003 $\bar{M}$ $MgCl_2$), and re-centrifuged. The protein concentration in each case adjusted to 0.3 mg protein per tube.

———Δ——— unwashed

———×——— one wash

———•——— two washes

Figure 20.

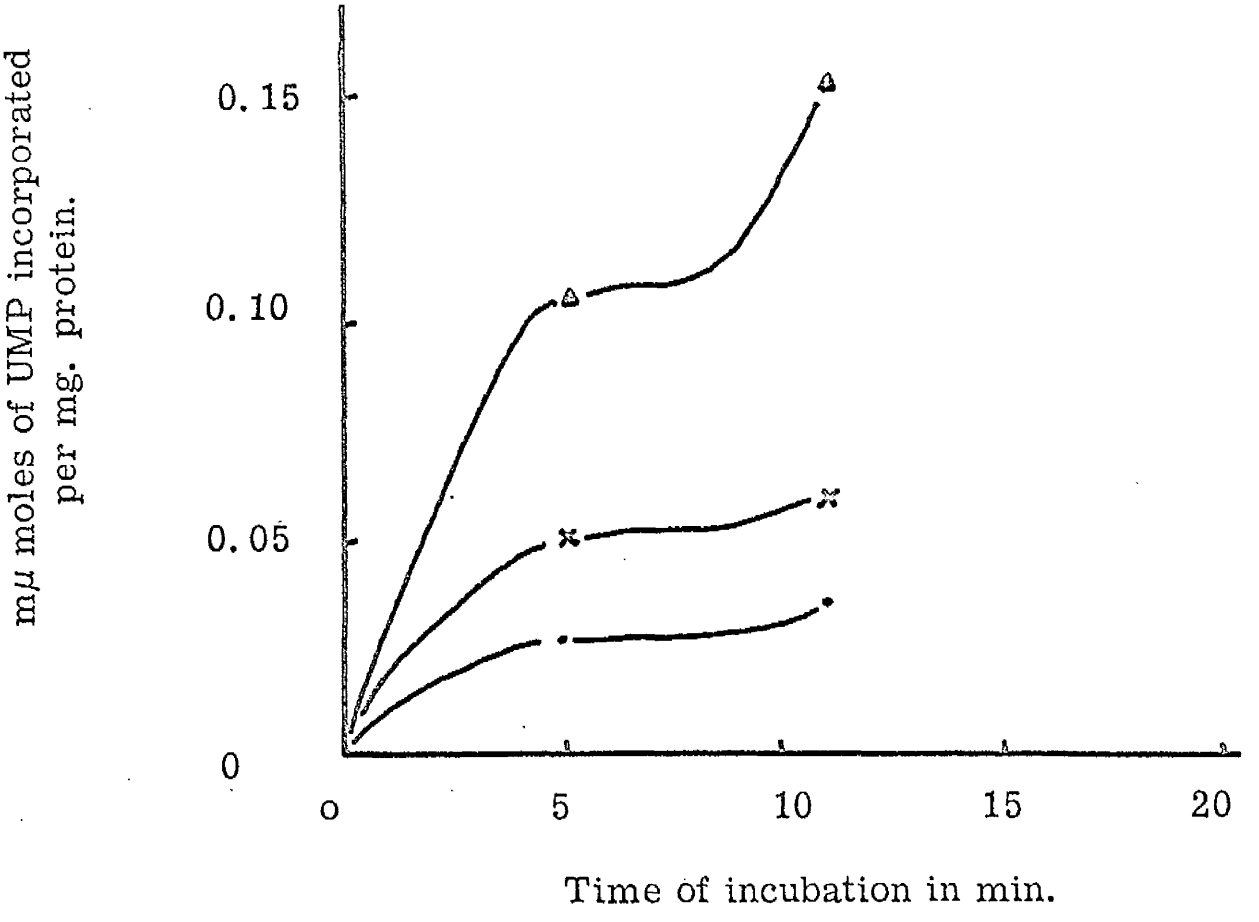

mμ moles of UMP incorporated per mg. protein.
0.15
0.10
0.05
0
o
5
10
15
20
Time of incubation in min.

TABLE 15

Characteristics of the uptake of UTP

by the 78,000 x g supernatant of rat liver cells

Reaction mixtures were as in Table 13, and contained 0.2 mg of protein per 0.5 ml.

Incubations were at 37°C for 8 minutes.

TABLE 15

Additions	mμmoles UMP per mg. protein
NIL	0.08
ATP, GTP, CTP	0.048
RNA	0.398
RNA, ATP, GTP, CTP.	0.351

TABLE 16

Characteristics of the uptake of UTP by unwashed RSV, washed RSV, washings from the RSV and the 78,000 x g supernatant of rat liver cells

Reaction mixtures were identical to those in Table 13. All fractions were adjusted so that reaction mixtures contained the same amount of protein in each case (0.2 mg to 0.7 mg).

After centrifugation all particulate fractions were suspended in the homogenising medium.

Incubations were at 37°C for 8 minutes (I), 9 minutes (II) and 12 minutes (III).

TABLE 16

Fractions	Additions	mμmoles UMP incorporated per mg. protein on different occasions.		
		I	II	III
unwashed RSV	NIL	0.122	0.	0.009
	ATP, GTP, CTP	0.082	0.052	0.052
	RNA	0.279	0.044	0.028
	ATP, GTP, CTP, RNA.	0.225	0.153	0.157
1 x washed RSV	NIL	0.074	0.	0.002
	ATP, GTP, CTP	0.043	0.027	0.068
	RNA	0.171	0.	0.009
	ATP, GTP, CTP, RNA	0.170	0.076	0.149
washings from RSV	NIL	0.090	0.	0.047
	ATP, GTP, CTP	0.036	0.	0.049
	RNA	0.307	0.207	0.017
	ATP, GTP, CTP, RNA.	0.310	0.221	0.189
78,000 x g supernatant	NIL	0.071	0.022	0.012
	ATP, GTP, CTP	0.048	0.	0.019
	RNA	0.398	0.255	0.127
	ATP, GTP, CTP, RNA.	0.351	0.196	0.117

the supernatant enzymes. In addition, the characteristics of the RSV remain unchanged after washing, and this provides further evidence that the RSV activity may be attributed to 78,000 x g enzymes whose pattern of activity has been distorted by the presence of other RSV enzymes.

Nonetheless, since there was some doubt about the matter, the RSV activity was characterised more fully. Using the experimental approach already described, the distribution of radioactivity in UMP, GMP, AMP and CMP was measured following alkaline hydrolysis of the product of (α-^{32}P)UTP uptake by the RSV. The time course of the activity is given in Figure 21 and the ^{32}P distributions in Table 17. The results for 7 min and 11 min are almost identical. Less than half the radioactivity was recovered in UMP. Significant incorporation of UMP adjacent to AMP, GMP and especially CMP is indicated. This is very reminiscent of the results obtained for the total microsome fraction (see Table 10). The interpretation of the results, of course, hinges on the length of the newly synthesised chains. If long chains are formed the ^{32}P distributions would suggest the formation of heteropolymers. If, on the other hand, very short chains are formed, the results could indicate terminal addition to the ends of different chains ending in all four ribonucleotides.

Accordingly the length of the newly synthesised chains was determined by an experiment identical to that previously described for the 150,000 x g supernatant. The time course of the experiment is shown in Figure 22 and the chain lengths in Table 18. The new chains are very short, only 1 - 2 nucleotides being incorporated

FIGURE 21

Time course of the uptake of UTP by the RSV fraction of rat liver cells

Reaction tubes contained 50 μmoles tris-HCl buffer pH 8.0 4 μmoles $MgCl_2$, 0.2 μmole EDTA, 0.1 μmole (α-^{32}P) UTP, (2×10^6 cpm per μmole), 0.1 μmole each of ATP, GTP + CTP, 20 μg phosphocreatine kinase and 0.623 mg of protein in a total volume of 0.5 ml.

Incubations at 37°C.

At the times indicated by the arrows, the distribution of ^{32}P in the four ribonucleoside monophosphates obtained on alkaline hydrolysis of the product was determined as before and the results are given in Table 15.

Figure 21.

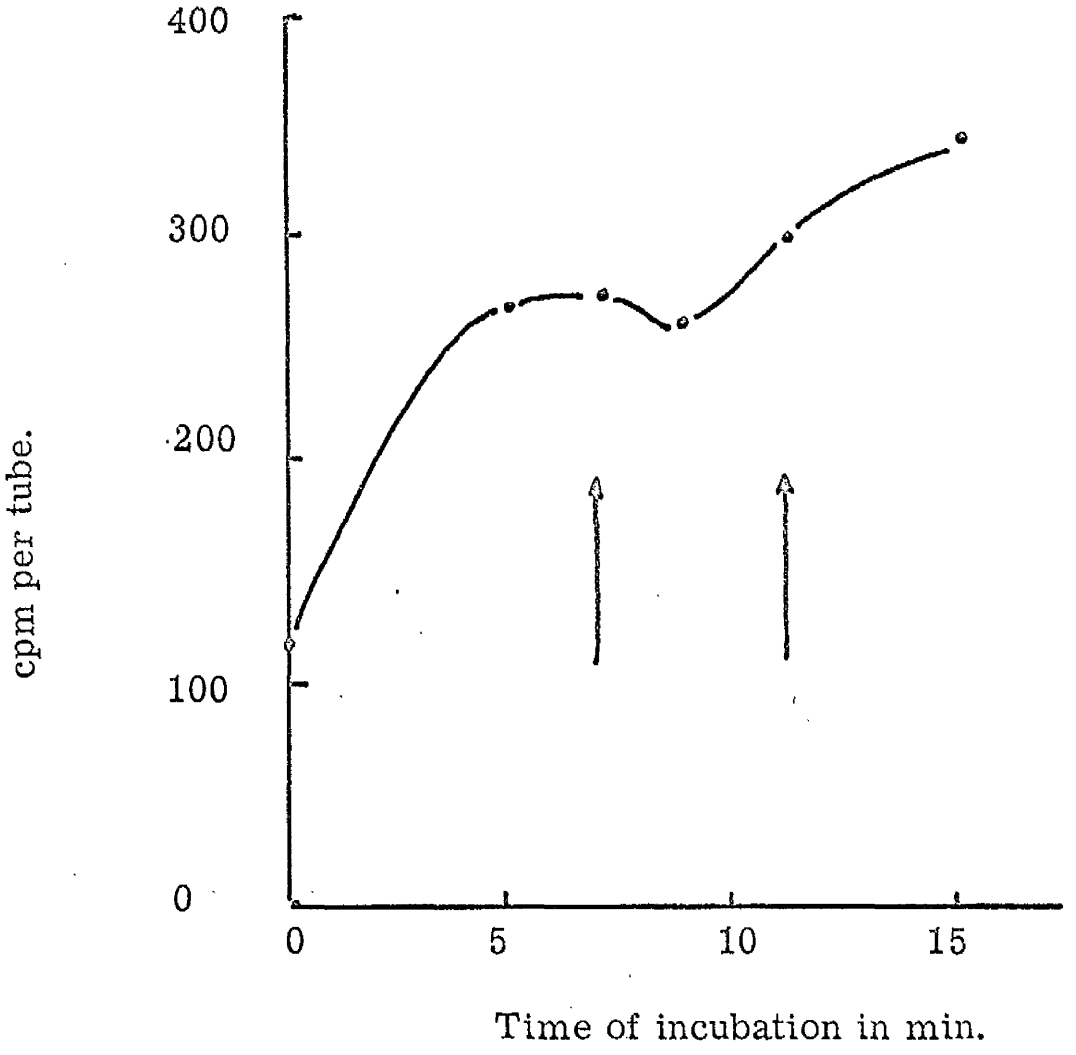

400
300
200
100
0
cpm per tube.
0
5
10
15
Time of incubation in min.

TABLE 17

Distribution of ^{32}P in the ribonucleoside monophosphates obtained by alkaline hydrolysis of the product of (α-^{32}P)UTP uptake into the RSV fraction of rat liver microsomes

Reaction mixtures identical to those in Figure 21 except that the specific activity of the UTP was 20 x 10^6 cpm per micromole, and all quantities were scaled up by a factor of 10.

Incubations were at 37°C for the indicated times.

The figures are corrected for the radioactivity due to unincorporated UTP.

TABLE 17

Time of incubation	nucleotide	% recovery of radioactivity
7 minutes	UMP	48.9
	GMP	24.7
	AMP	13.4
	GMP	13.0
11 minutes	UMP	41.7
	GMP	29.6
	AMP	13.2
	GMP	15.5

FIGURE 22

Time course of UTP uptake by the RSV fraction

Reaction mixtures identical to those in Table 13 and contained 0.48 mg protein in a total volume of 0.5 ml.

Incubations at 37°C for the time indicated.

The average lengths of the newly synthesized chains were estimated at the times indicated by the arrows, and are presented in Table 17.

FIGURE 23

Phosphodiesterase activity of the RSV fraction of rat liver microsomes

Reaction mixtures contained 50 μmoles tris-HCl buffer pH 8.0, 4 μmoles $MgCl_2$, 10 mμmoles EDTA, 0.1 μmole each of UTP, ATP, GTP and CTP, 2 μmoles phosphocreatine, 20 μg phosphocreatine kinase, 2 μmoles p-nitrophenyl-pT and 0.252 mg of RSV protein in a total volume of 0.5 ml.

Incubations at 37°C.

The amount of p-nitrophenol released was measured as described in Section under Experimental.

Figure 22.

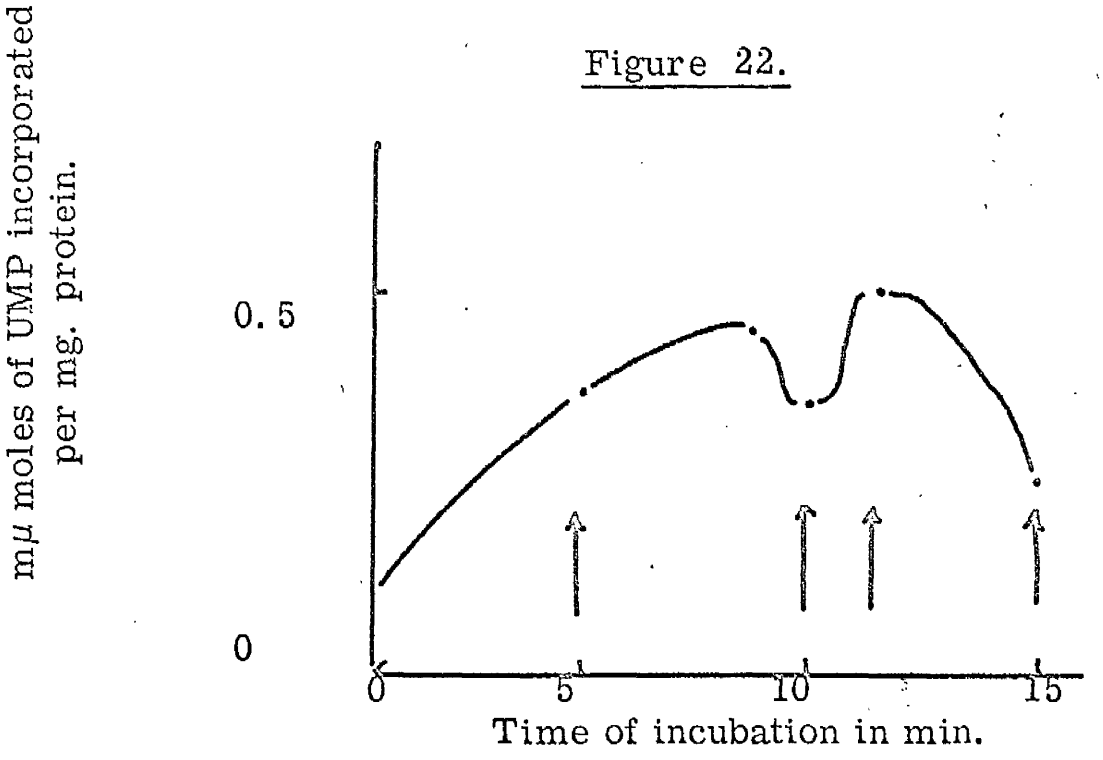


Figure 23.

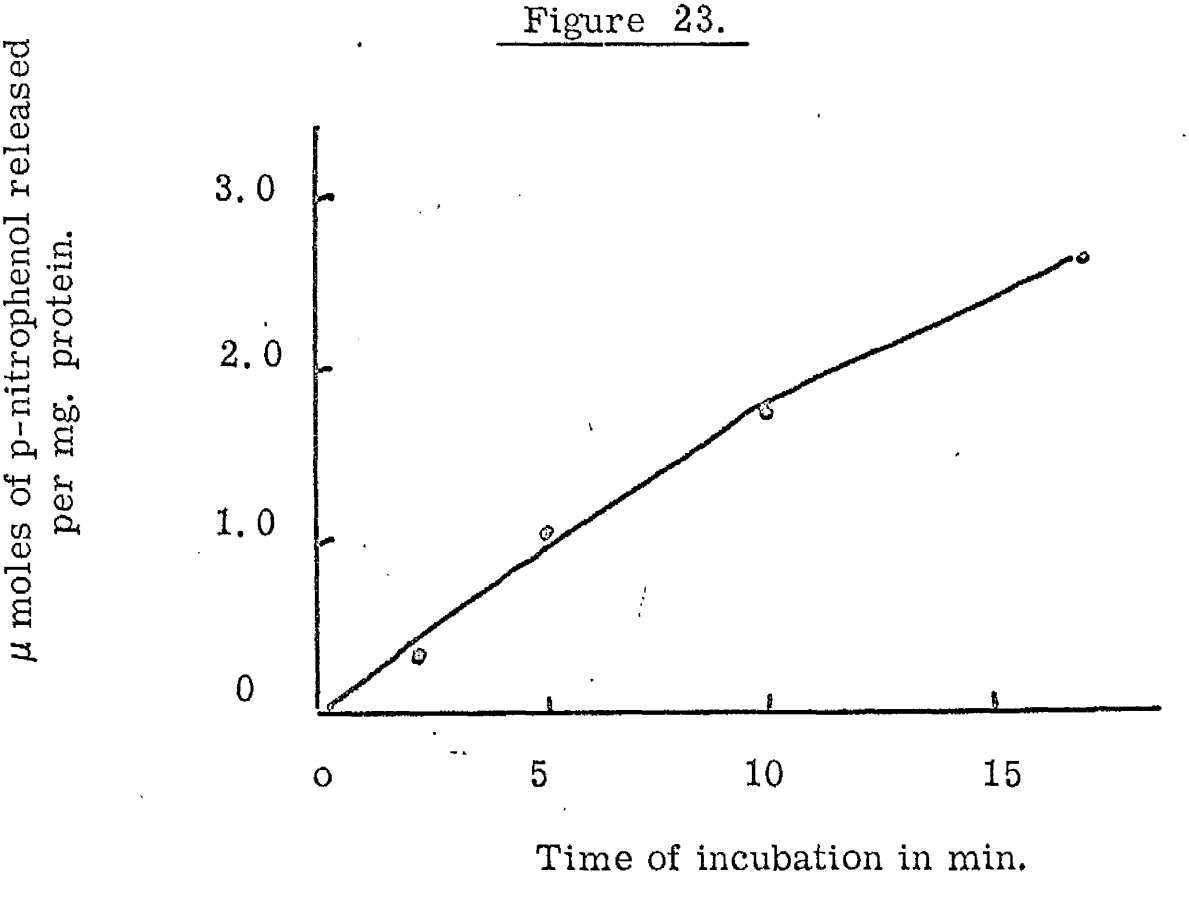


TABLE 18

Average length of the newly synthesised chains formed by the uptake of UTP by the RSV fraction of rat liver

Reaction mixtures as in Figure 22, except that the specific radioactivity of the UTP was 200 μc/μmole.

Incubations were at 37°C for 5, 10, 12 and 15 minutes. The time course of the reaction is given in Figure 22.

The average chain lengths were estimated by the method described previously for the 150,000 x g supernatant fraction.

T A B L E 1 8

Time of incubation in minutes	Ratio of radioactivities; $\frac{\text{nucleotide}}{\text{nucleoside}}$	average chain length
5	0.57	1 - 2
10	0.40	1 - 2
12	0.73	2
15	0.2	1

into terminal positions. The results of the ^{32}P distribution experiment are entirely consistent with such terminal addition.

The shortness of the chain lengths suggested that this fraction might exhibit high diesterase activity, and this is borne out by the experiment illustrated in Figure 23. Since the nucleotides are added to the 3′ terminal ends of the primer (see below), 3′ phosphodiesterase activity was estimated by measuring the release of p-nitrophenol from p-nitrophenyl thymidine 5′ phosphate. As can be seen the phosphodiesterase activity was extremely high, 0.172 μmoles of substrate being hydrolysed per min. per mg. protein. It is considered that the high levels of phosphodiesterase activity found explains the low levels of incorporation and the short chain lengths synthesised by the RSV fraction.

Experiments were carried out to demonstrate that UMP is incorporated into the 3′ terminal end of the primer chain. Highly polymerised yeast RNA was hydrolysed in alkali as before to produce new 2′, 3′ phosphorylated ends. A portion of this RNA was treated with alkaline phosphatase and the enzyme inactivated as previously described. The ability of these two RNA primers to support the uptake of UTP by the RSV fraction is shown in Figures 24 a and b. The dephosphorylated RNA was a much more effective primer than the RNA carrying predominantly 3′ phosphorylated ends at the two time intervals selected. The variations in the points in Figure 24b, may be because the experiment was performed in the biphasic region of the time curve (see Figure 19). The priming ability of the untreated short RNA was approximately 20 - 25% of that of the dephosphorylated short RNA. This is in good agreement with the

FIGURE 24 a

Effect of adding increasing amounts of 3 hydroxyl or 3 -phosphorylated RNA primers on the uptake of UTP by the RSV fraction of rat liver cells

Reaction mixtures contained 50 μmoles tris-HCl, 4 μmoles $MgCl_2$, 10 mμmoles EDTA, 5 μmoles mercaptoethanol, 0.1 μmoles 3-H UTP (20 μc/μmole), 0.1 μmole each of ATP, GTP and CTP, 2 μmoles phosphocreatine, 20 μg phosphocreatine kinase and 0.62 mg of protein in a total volume of 0.5 ml.

Increasing amounts of shortened RNA primer were included as indicated.

Incubations at 37°C for 2.5 minutes.

FIGURE 24 b

As for Figure 24 a; 0.9 mg of protein per tube.

Incubations were at 37°C for 12 minutes.

——●—— dephosphorylated, short primer

- - - -△- - - - untreated short primer

Figure 24a.

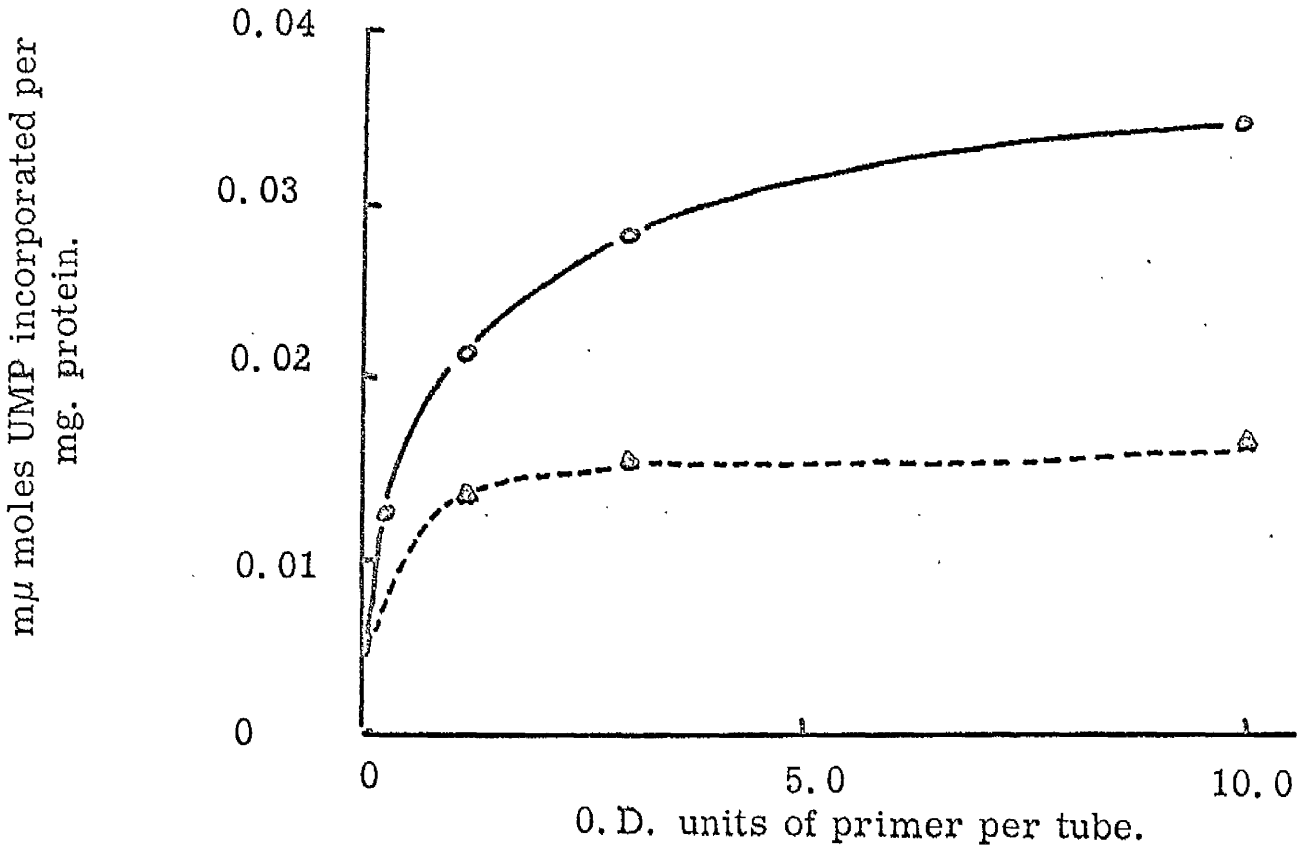
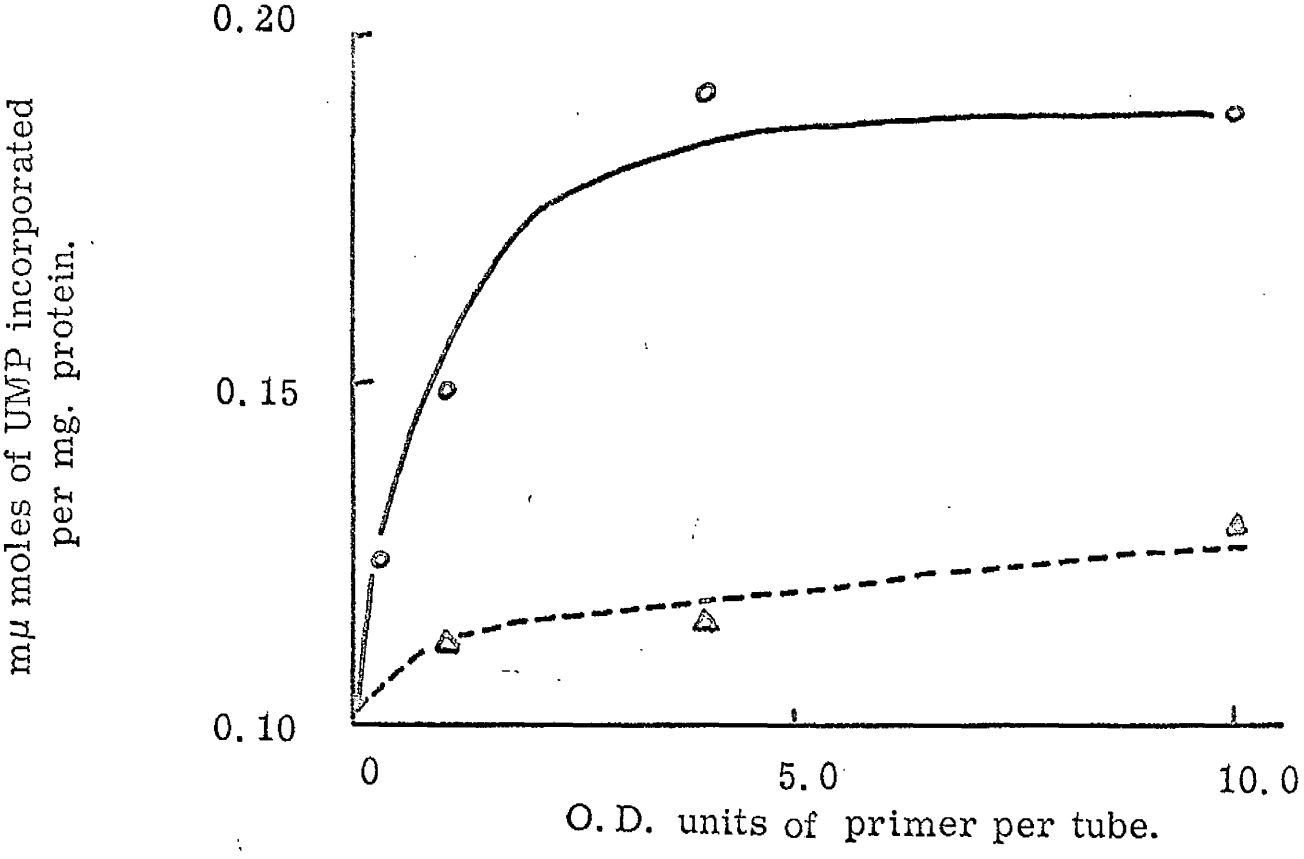


Figure 24b.



estimated amount of 3 hydroxyl ends remaining after alkaline hydrolysis of the yeast RNA (see 2 of this section).

It was concluded, therefore, that the RSV enzyme requires free 3 hydroxyl ends in the primer and that the activity of the RSV fraction can be adequately described as terminal addition of a few ribonucleotide residues on to the 3 end of primer RNA chains.

4. (ii) Free Ribosomes

The time course of the uptake of UTP by the F.R. in the presence and absence of ATP, GTP and CTP is shown in Figure 25. The reaction is fairly extensive and incorporation increases linearly for 10 mins and has its maximum value at 20 mins. Following this radioactivity is lost, presumably due to nuclease activity. The high levels of incorporation observed, 3.5 mumoles of UMP per mg.of protein at 20 min. are quite remarkable for such an unpurified fraction from mammalian cells which must contain an excess of non-enzyme protein. High levels of incorporation of particulate fractions of mammalion cells have also been described by Burdon (1963) and Wykes and Smellie (1966).

The location of the enzyme and the physical characteristics of the fractions were investigated in more detail. It is known that in the presence of Mg^{++}, ribosomes can absorb exogenous enzyme protein exposed to them. Treatment of such ribosomes with low Mg^{++} solutions or with EDTA can lead to release of such enzyme proteins (Tal and Elson, 1961; Neu and Heppel, 1964). Accordingly, experiments were carried out to find the effect of washing the ribosomes and also of preparing them in the presence of high levels

FIGURE 25

Time course of the uptake of ^{3}H-UTP by the F.H. fraction of rat liver cells

Reaction mixtures contained 50 μmoles tris-HCl buffer pH 8.0, 4 μmoles $MgCl_2$, 10 mμmoles EDTA, 0.1 μmole ^{3}H-UTP, (20 μc per μmole), 50 μg of RNA, 2 μmoles phosphocreatine, 20 μg phosphocreatine kinase and 0.608 mg of protein in a total volume of 0.5 ml.

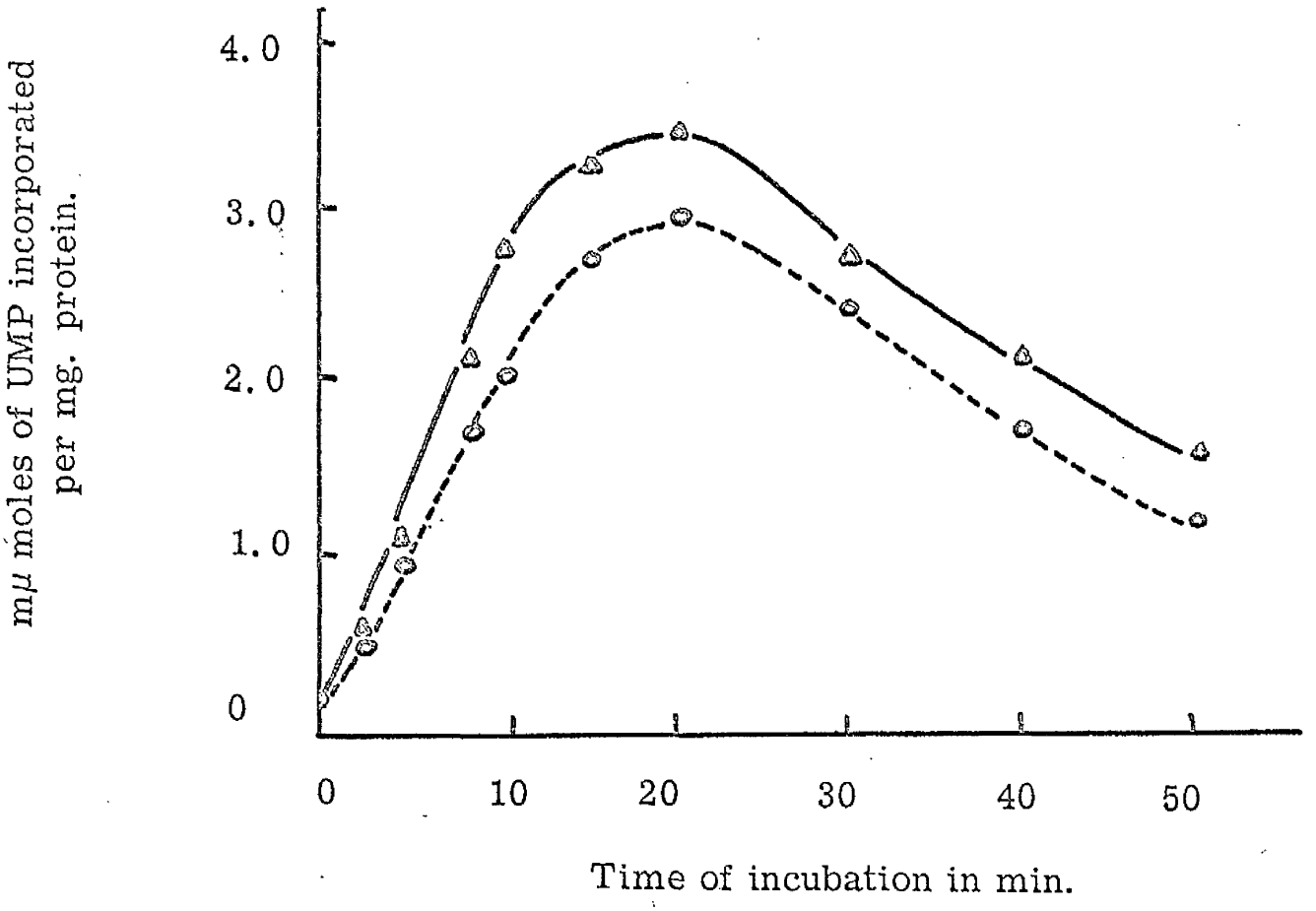
Where indicated 0.1 μmole each of ATP, GTP and CTP were included.

Incubations at 37°C.

———Δ——— ^{3}H-UTP only

- - - -o- - - - ^{3}H- UTP + ATP, GTP + CTP

Figure 25.


mμ moles of UMP incorporated per mg. protein.
4.0
3.0
2.0
1.0
0
0
10
20
30
40
50
Time of incubation in min.

of EDTA. Table 19 shows that resuspension of the F.R. fraction and recentrifugation in 0.01$\bar{M}$ tris HCl pH 8.0, 0.0025 $\bar{M}$ $MgCl_2$ and 0.25 $\bar{M}$ sucrose, had virtually no effect on the activity of the F.R. fraction. This suggests that the enzyme actually sediments with the particles and is not due to contamination of the supernatant fraction. In another experiment F.R. fractions were prepared by the normal procedure from two samples of the same liver. One sample was homogenised in 30% sucrose, 0.01$\bar{M}$ tris HCl pH 8.0 + 0.005$\bar{M}$ $MgCl_2$, and the other in 30% sucrose, 0.01$\bar{M}$ tris-HCl pH 8.0 and 0.0125 $\bar{M}$ EDTA. The 150,000 x g sediments were suspended in 0.01$\bar{M}$ tris-HCl pH 8.0 gently, and assayed for the ability to incorporate UTP into an acid-insoluble product. From Table 20 it can be seen that preparation of the F.R. fraction in 0.01$\bar{M}$ EDTA (final conc.) did not diminish its ability to incorporate UTP but rather enhanced it. This provides convincing evidence that the enzyme is not absorbed, and therefore concentrated from the supernatant by the ribosomes, but is in fact either attached tightly to the ribosome structure, or large enough to be sedimented at 150,000 x g.

Figures 26 a and b show the profile obtained on sucrose gradient analysis of the free ribosomes. At 260 mμ five distinct peaks can be distinguished sedimenting through the gradient. Figure 26 b shows the profile obtained on reading an identical gradient at 320 mμ. A single peak, which coincided with peak II and which represents Ferritin was observed (see Section 9(i)2. under Experimental). The total amount of absorbing material under these two peaks was estimated by cutting out the area under the peaks and

TABLE 19

The effect of washing the F.R. fraction on the uptake of UTP

Reaction mixtures contained 50 μmoles tris-HCl buffer pH 8.0, 4 μmoles $MgCl_2$, 0.1 μmole ^{3}H-UTP (20 μc/μmole), 10 mμmoles EDTA and 0.53 mg of protein in a total volume of 0.5 ml.

Incubations were at 37°C for 5.0 minutes. F.R. were prepared as described. A portion was resuspended in 0.25 $\bar{M}$ Sucrose, 0.01$\bar{M}$ tris-HCl buffer, 0.0025 $\bar{M}$ $MgCl_2$ and recentrifuged at 150,000 x g for two hours.

TABLE 20

Effect of preparing the F.R. fraction in a medium containing 0.01$\bar{M}$ EDTA

Reaction mixtures were as in Table 18. There was 0.8 mg of enzyme protein per tube.

The fractions were prepared as described in the text.

Incubations were at 37°C for 5.0 minutes.

TABLE 19

Treatment	mμmoles UMP incorporated per mg. pr.
unwashed	4.11
washed	4.41

TABLE 20

Homogenised in	mμmoles UMP incorporated per mg.pr.
Mg^{++}	0.750
EDTA	0.950

FIGURE 26 a

Sucrose gradient centrifugation analysis of the F.R. fraction from rat liver cells

The F.R. fraction was prepared from the liver of one animal by the normal method. The 150,000 x g pellet was suspended in 0.45 ml 0.01 $\bar{M}$ tris-HCl, pH 7.0, 0.0025$\bar{M}$ $MgCl_2$.

0.06 ml of this suspension was layered on top of a 5.0 ml 5-20% Sucrose gradient buffered with 0.01$\bar{M}$ tris HCl pH 7.0, 0.0025 $\bar{M}$ $MgCl_2$. The gradient was centrifuged in the SW 39 head of the M.S.E. 50 centrifuge at 38,000 rpm for 1 hour, 30 min. The gradient was then read at 260 mμ as described under Experimental.

FIGURE 26 b

Exactly as for 26 a. The gradient was analysed at 320 mμ.

The direction of sedimentation is indicated by the arrows.

Figure 26a

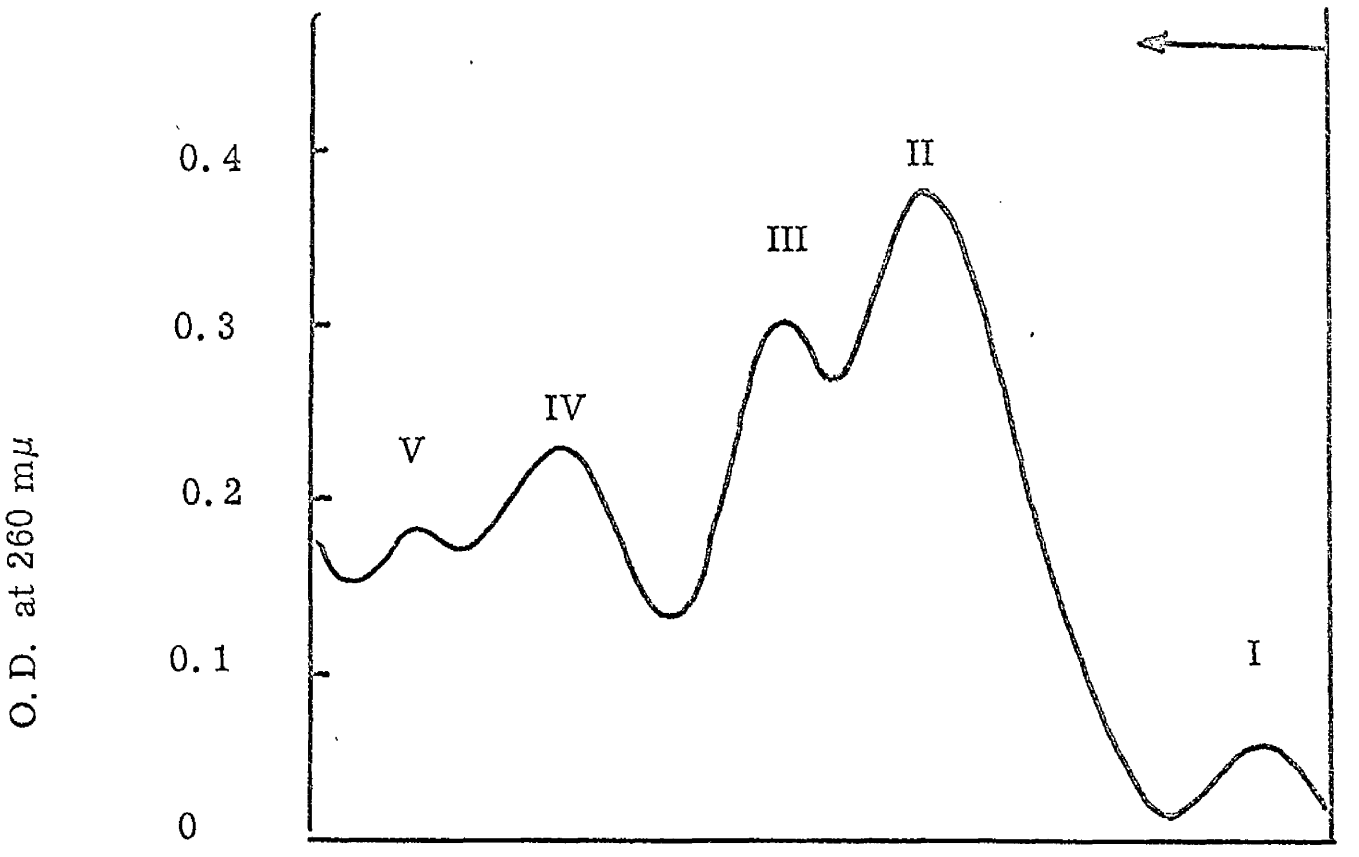
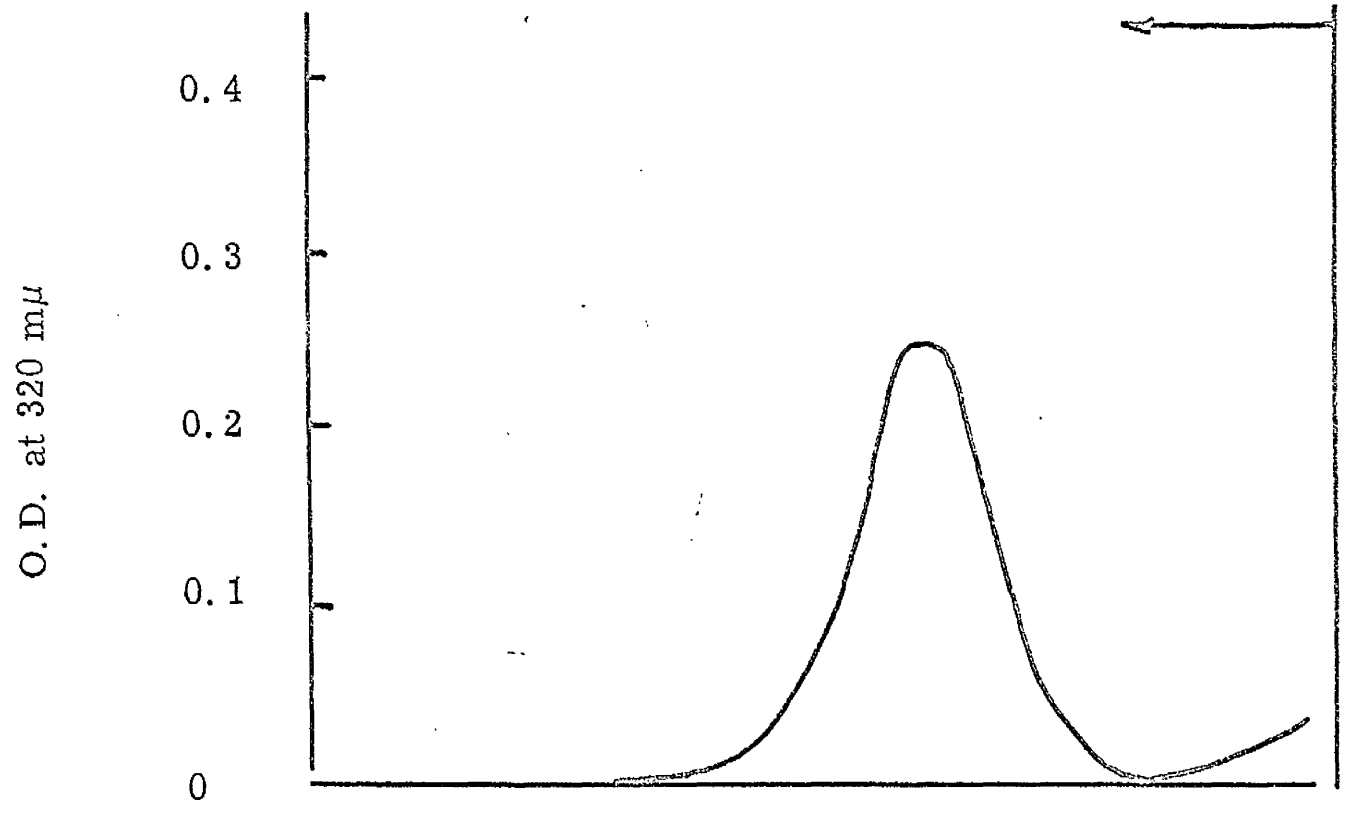


Figure 26b



weighing the paper. The total absorbance of the peak in 26b at 260 mμ was estimated by applying the correction factor of 1.5, (Munro, Jackson and Kerner, 1964) and it was found to represent 89.5% of the material in peak II. It was concluded therefore that peak II is Ferritin. Analytical centrifugations of material existing almost entirely of Ferritin indicated that the molecule as obtained by these procedures sedimented at about 56S. It was concluded therefore that peak III was the monomeric ribosomes, peak IV dimers and peak V trimers. The small peak sedimenting at the top of the gradient probably represents degraded RNA and protein. Therefore the free ribosomes as obtained by these procedures consists of monomeric ribosomes, dimers, trimers and probably small amounts of oligomeric ribosomes and a substantial amount of Ferritin.

The effect of preparing the free ribosomes in medium containing 0.01$\bar{M}$ EDTA was then studied. The O.D. profiles of sucrose gradients of EDTA-ribosomes and Mg^{++} ribosomes is shown in Figure 27. Isolating the ribosomes from 0.01$\bar{M}$ EDTA had no effect on the patterns the ribosomes gave on the gradient, but it can be seen that the amount of ribosomes relative to Ferritin has increased. This probably accounts for the higher activity observed in EDTA ribosomes in Table 19, and suggests that the activity is probably a function of the ribosomes rather than of Ferritin.

The response of the reaction to increasing amounts of F.R's is shown in Figure 28. A linear response was observed from 0 to 1.00 mg pr. per tube and work was within this range thereafter. The reaction, again was dependant on added $MgCl_2$ (Figure 29). Figure 30 shows that the presence of phosphocreatine and phospho-

FIGURE 27

Effect of preparing the free ribosomes in medium containing 0.01M EDTA on the sucrose gradient pattern

Free ribosomes were prepared as before from either 30% sucrose, 0.01M tris-HCl, pH 8.0, 0.0025M $MgCl_2$ (final concentration), or 30% sucrose, 0.01M tris-HCl pH 8.0, 0.01M EDTA (final concentration)

The pellets were suspended in 0.01M tris-HCl pH 7.0 and layered onto 5 ml gradients, 5-20% sucrose, 0.01M tris-HCl pH 7.0, 0.0025M $MgCl_2$. Centrifugation was for 1 hour 30 min at 38,000 x g in the SW 39 roter of the M.S.E. 50 ultracentrifuge.

The gradients were analysed at 260 mμ.

a - Mg^{++} ribosomes

b - EDTA ribosomes

Figure 27a

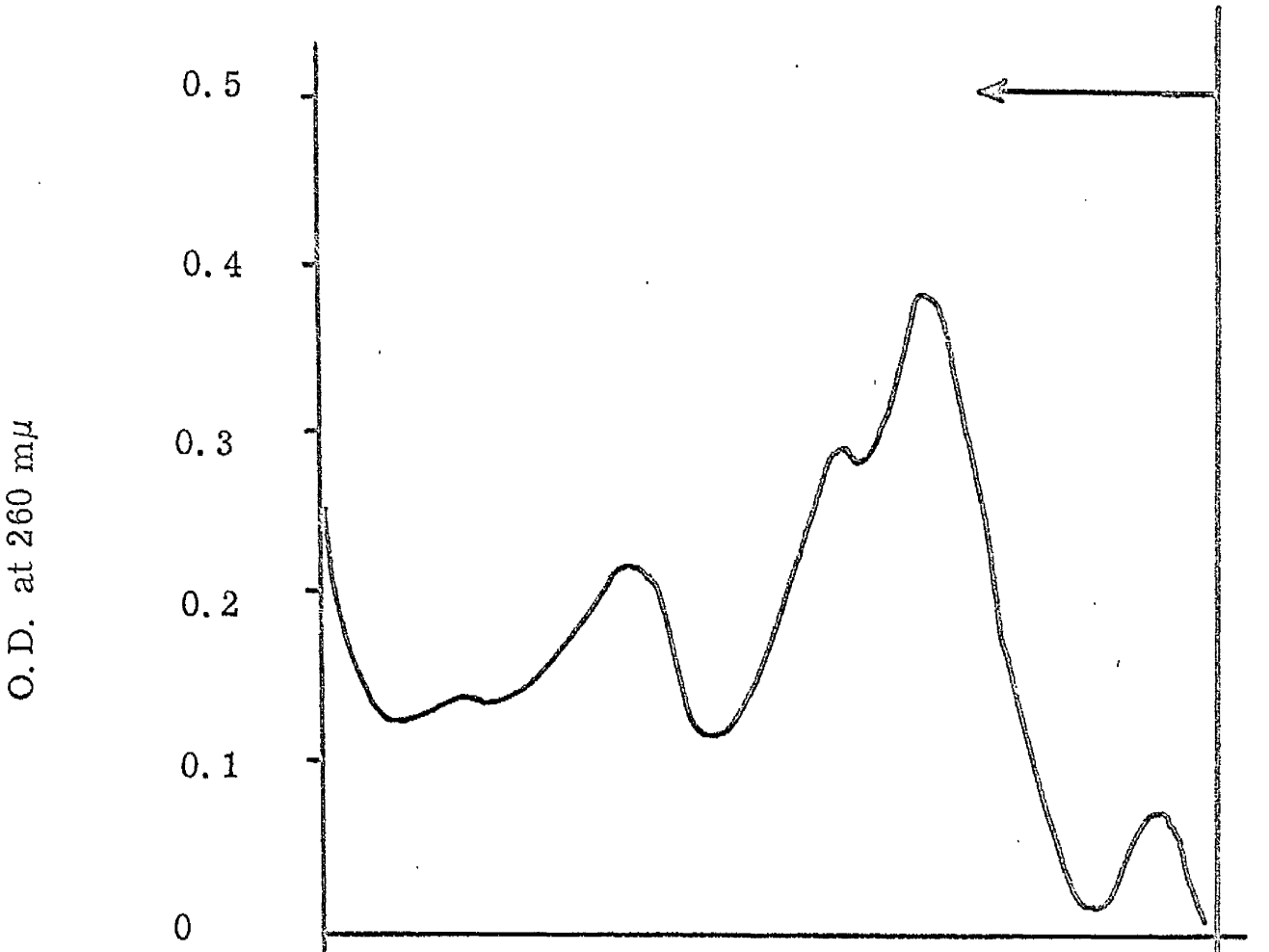


Figure 27b

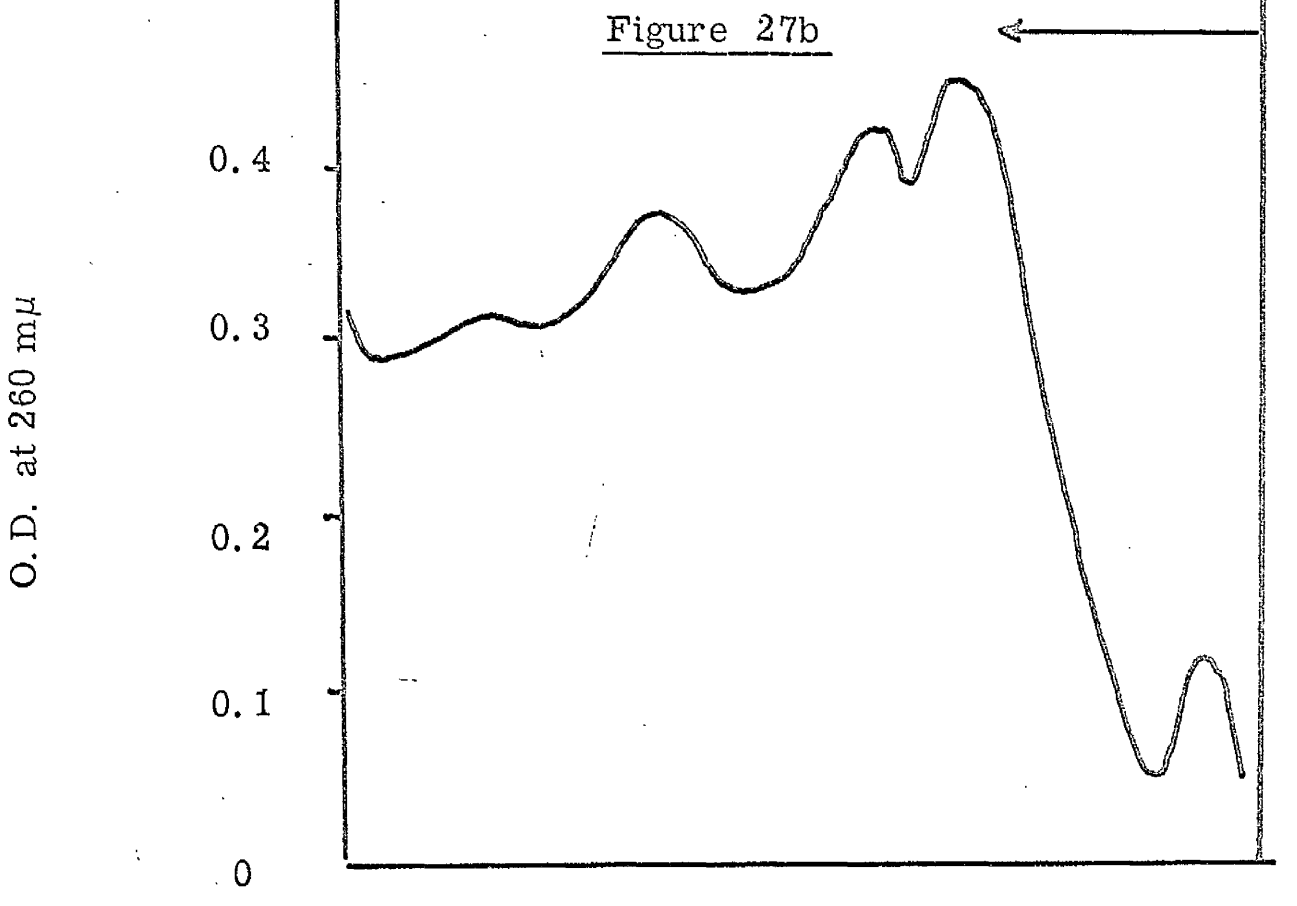


FIGURE 28

Effect of increasing amounts of enzyme protein on the uptake of UTP by the F.R. fraction

Reaction mixtures contained 50 μmoles tris-HCl buffer, pH 8.0, 4 μmoles $MgCl_2$, 10 mμmoles EDTA, 0.1 μmole (α-^{32}P) UTP, 50 μg of RNA, 2 μmoles phosphocreatine, and 20 μg phosphocreatine kinase in a total volume of 0.5 ml.

Incubations were at 37°C for 10 mins.

FIGURE 29

Effect of increasing concentrations of $MgCl_2$ on the uptake of UTP by the F.R. fraction

Reaction mixtures contained 50 μmoles of tris-HCl buffer pH 8.0, 10 mμmoles EDTA, 0.1 μmole (α-^{32}P) UTP, (5×10^6 cpm per μmole), 50 μg of RNA and 0.47 mg of protein in a total volume of 0.5 ml.

Incubations at 37°C.

Figure 28

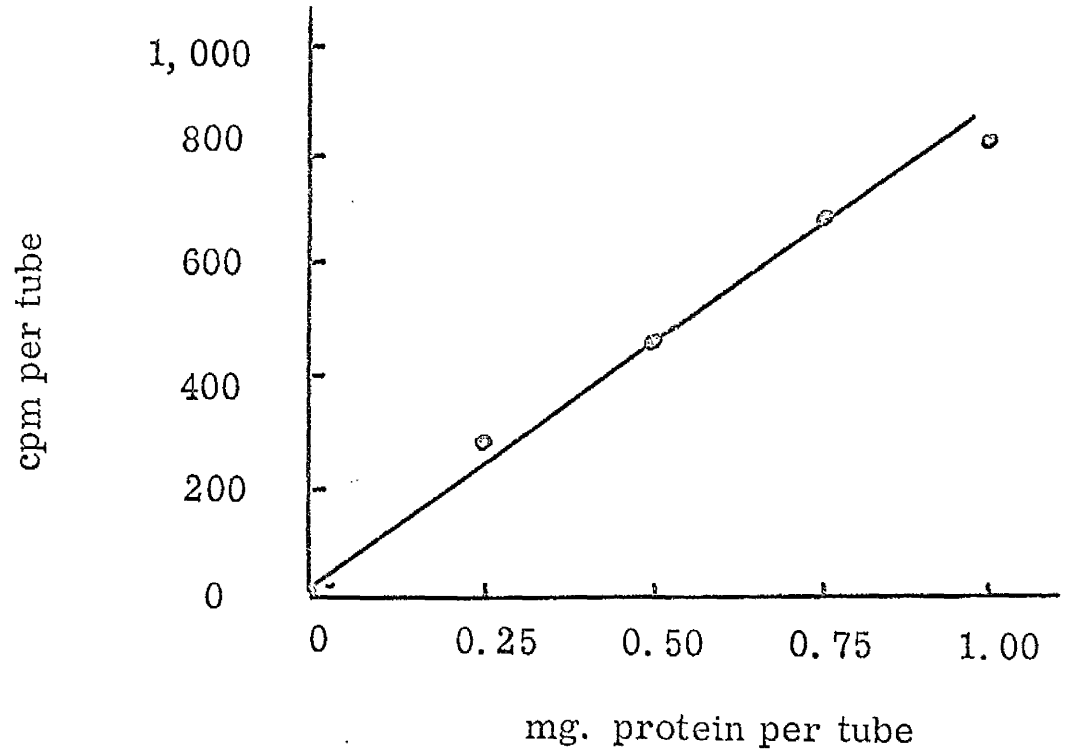
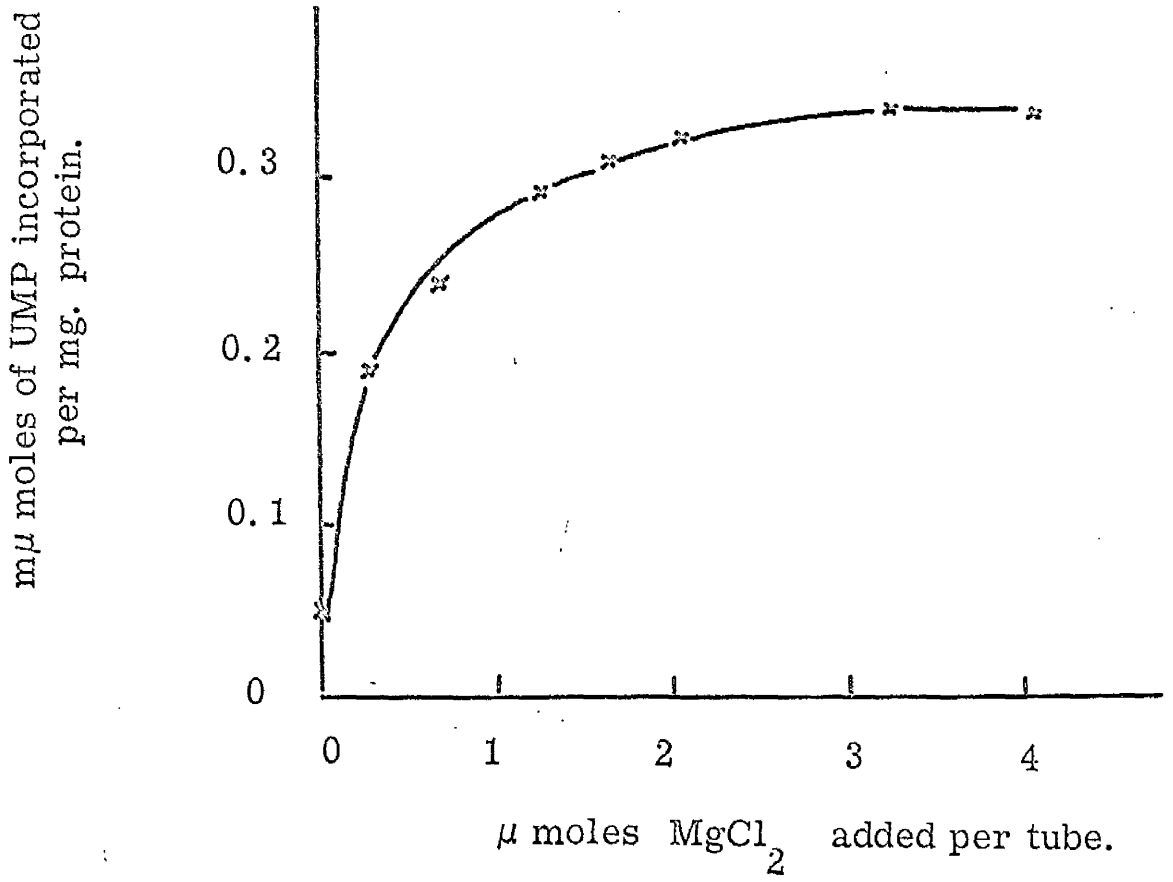


Figure 29.



creatine kinase has virtually no effect on the reaction, and they were subsequently omitted from the reaction mixture. This might be taken to mean that UTP was not the true substrate for the reaction. However Figure 31 shows that UTP was the uridine nucleotide to be incorporated, both UDP and UMP being completely inactive. Substrate concentration curves for UTP and ATP are shown in Figure 32 and as can be seen both curves are similar. The ability of the free ribosomes to incorporate UTP, ATP, GTP into acid insoluble products is shown in Figure 33. ATP and GTP were incorporated to a lesser extent than UTP. In each case the presence of the other ribonucleoside triphosphates inhibited incorporation slightly. No GTP incorporation was observed. The dependence of UTP uptake on added RNA is shown in Figure 34. The reaction is only partly dependant on added RNA. There is a sharp rise in the incorporation of UTP which reaches a plateau at 50 µg per tube of added RNA. Obviously the F.R. fraction contains endogenous RNA primers.

The results indicated that the F.R. fraction catalysed the synthesis of homopolymers sequences of UMP, AMP and to a lesser extent GMP. This was borne out by the results presented in Figures 35 and 36 and Tables 21 and 22. Table 22 shows the recovery of radioactivity in UMP, CMP, AMP and GMP. Following alkaline hydrolysis of the product of (α-^{32}P) UTP incorporation. Table 22 gives the average lengths of the newly synthesised chains, assuming mainly homopolymer formation occurs. The experimental approach was as previously described and the time course of incorporation for the two experiments are shown in

FIGURE 30

The effect of phosphocreatine and phosphocreatine kinase at the uptake of UTP by the F.R. fraction

Reaction mixtures contained 50 μmoles tris-HCl, pH 8.0, 3 μmoles $MgCl_2$, 10 μmoles EDTA, 0.1 μmole ^{3}H-UTP, (20 μc per μmole), 50 μg RNA and 0.34 mg of F.R. protein in a total volume of 0.5 ml.

Where indicated 0.1 μmole each of ATP, GTP and CTP and 2 μmoles of phosphocreatine and 20 μg of phosphocreatine kinase were included.

Incubations at 37°C.

———Δ——— ^{3}H-UTP only

———•——— ^{3}H-UTP + ATP, GTP + CTP

- - - -▲- - - - ^{3}H-UTP + PC/PCK

- - - -•- - - - ^{3}H-UTP + PC/PCK + ATP, GTP and CTP

FIGURE 31

Uptake of UTP, UDP and UMP by the F.R. fraction

Reaction mixtures as above except that 0.1 μmole of radioactive labelled UTP, UDP or UMP were included where indicated. 0.52 mg protein per tube.

———Δ——— ^{3}H-UTP (30 μc/μmole)

- - - -x- - - - ^{14}C-UDP (5μc/μmole)

- - - -•- - - - ^{3}H-UMP (30 μc/μmole)

Figure 30.

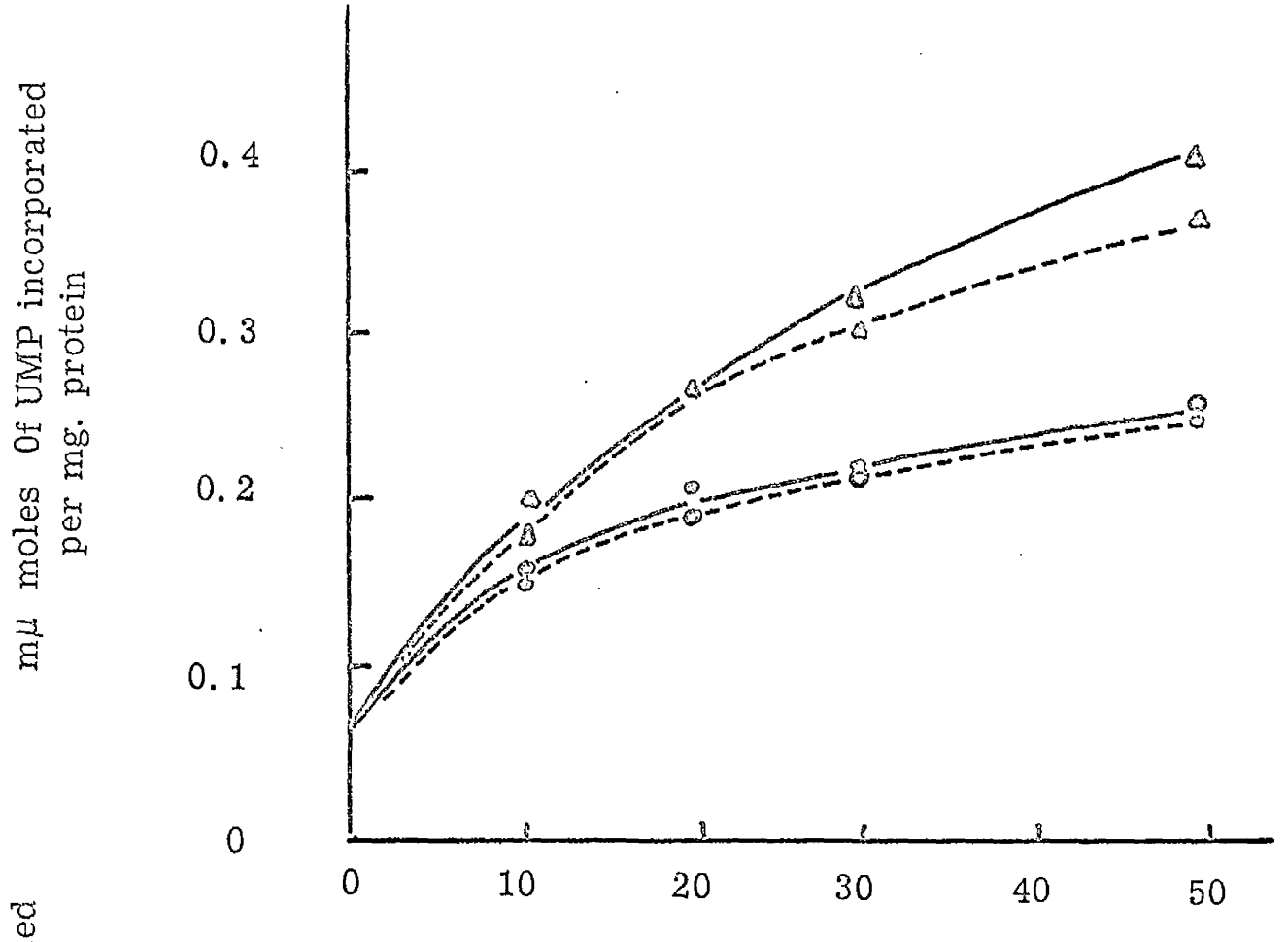


Figure 31

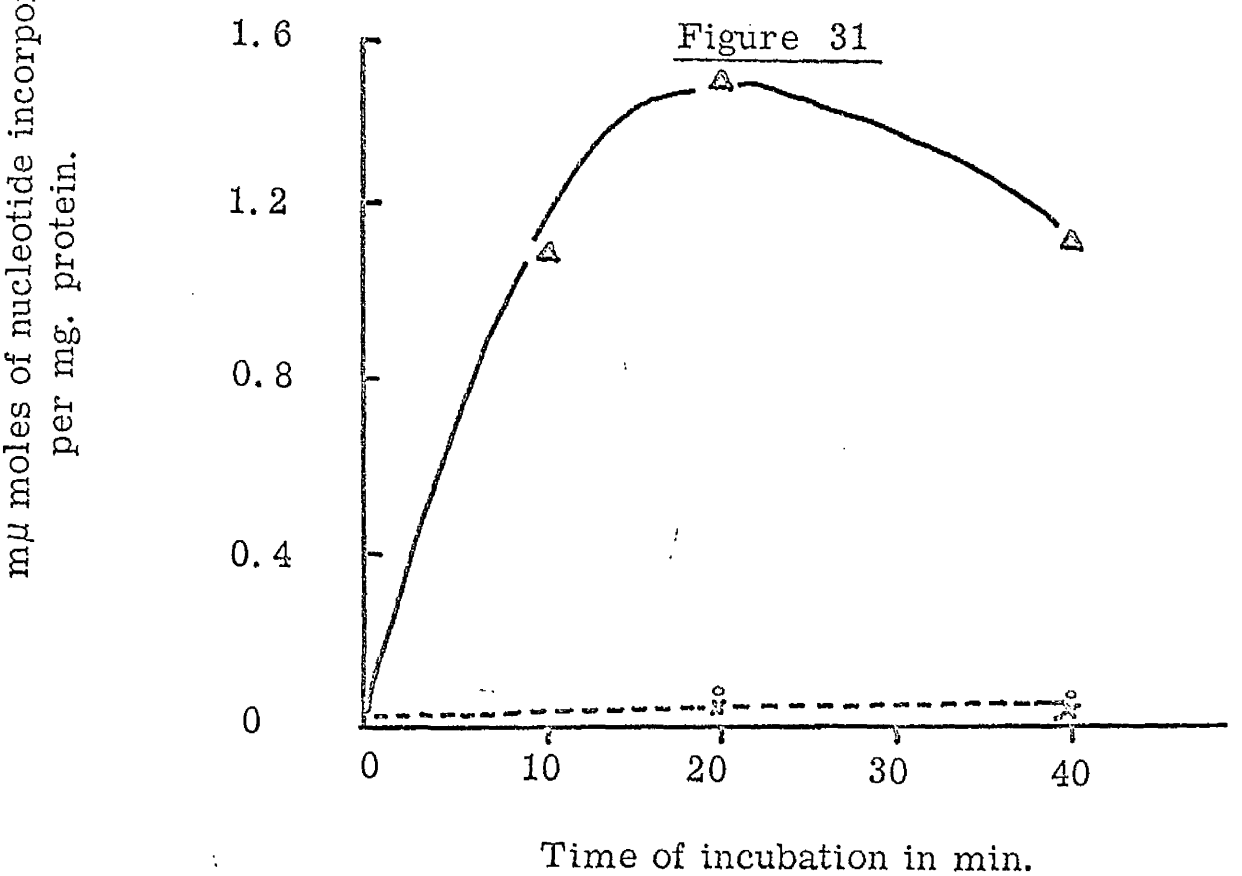


FIGURE 32

Effect of increasing concentrations of substrate on the uptake of ATP and UTP by the F.R. fraction

Reaction mixtures contained 50 μmoles tris-HCl buffer, pH 8.0, 4 μmoles $MgCl_2$, 10 mμmoles EDTA, 50 μg RNA, and 0.56 mg of protein in a total volume of 0.5 ml.

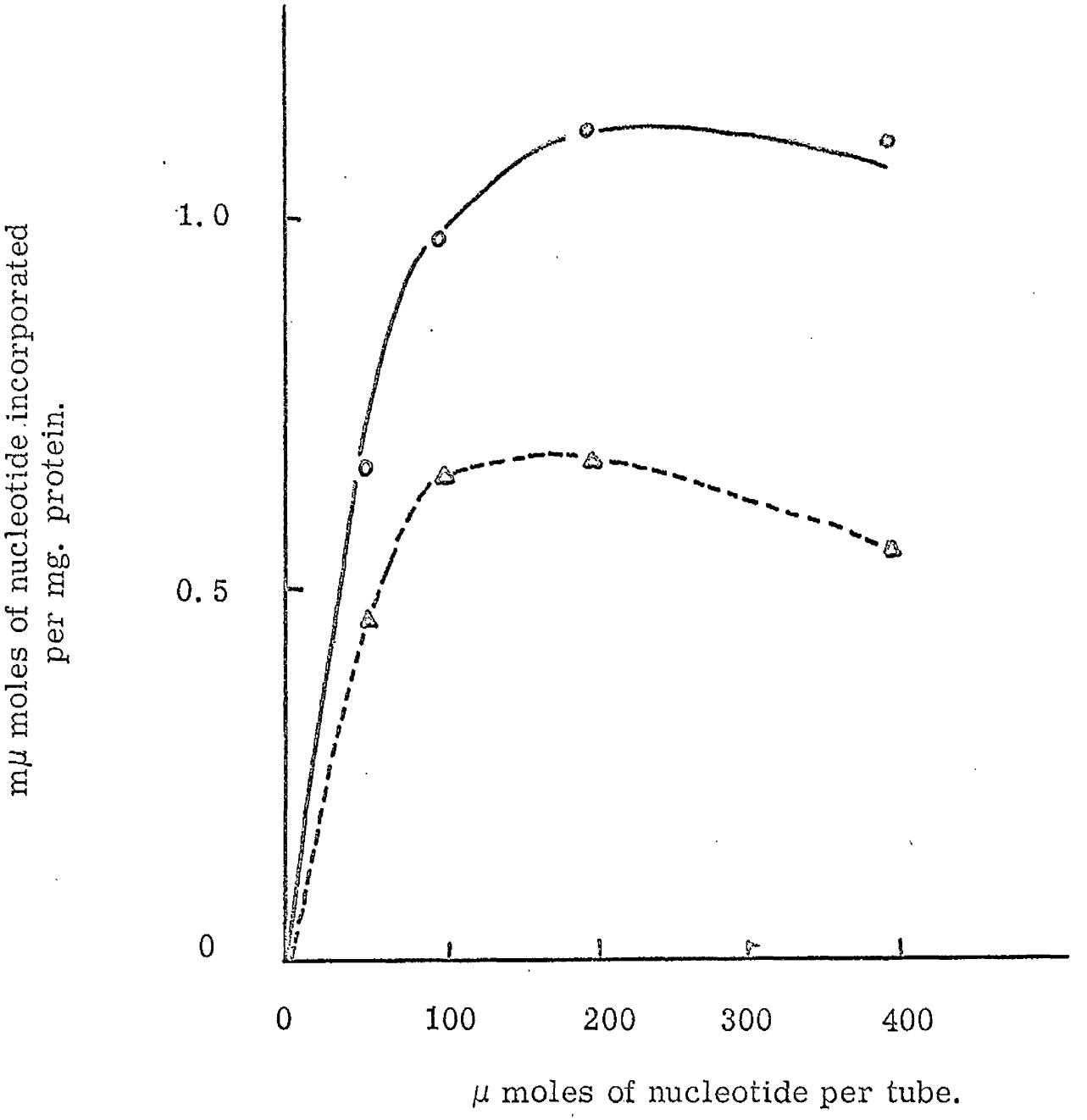
Various amounts of ^{3}H-ATP or ^{3}H-UTP were included as indicated (20 μc/μmole).

Incubations at 37°C for 10 minutes.

——— o ——— ^{3}H-UTP

- - - - Δ - - - - ^{3}H-ATP

Figure 32


mμ moles of nucleotide incorporated per mg. protein.
1.0
0.5
0
0
100
200
300
400
μ moles of nucleotide per tube.

F I G U R E 33

Uptake of UTP, CTP and ATP by the F.R. fraction

Reaction mixtures contained 50 μmoles tris-HCl buffer pH 8.0, 4 μmoles $MgCl_2$, 10 mμmoles EDTA, 50 μg RNA and 0.52 mg protein in a total volume of 0.5 ml.

Where indicated 0.1 μmole of ^{3}H-UTP, ^{3}H-CTP, ^{3}H-ATP (20 μc per μmole) and 0.1 μmole of each of the other three ribonucleoside triphosphates were included.

Incubations at 37°C.

—●— ^{3}H-UTP only

—●— ^{3}H-ATP

- - -▲- - - ^{3}H-ATP + UTP, CTP + GTP

—x— ^{3}H-CTP

- - -□- - - ^{3}H-CTP + UTP, ATP + GTP

F I G U R E 34

Effect of increasing concentrations of yeast RNA on the uptake of UTP by the F.R. fraction

Reaction mixtures contained 50 μmoles tris-HCl buffer pH 8.0, 4 μmoles $MgCl_2$, 10 mμmoles EDTA, 0.1 μmole of ^{3}H-UTP (20 μc/μmole), and 0.433 mg of protein in a total volume of 0.5 ml.

Where indicated increasing amounts of highly polymerised yeast RNA were included.

Incubations were at 37°C for 10 minutes.

Figure 33.

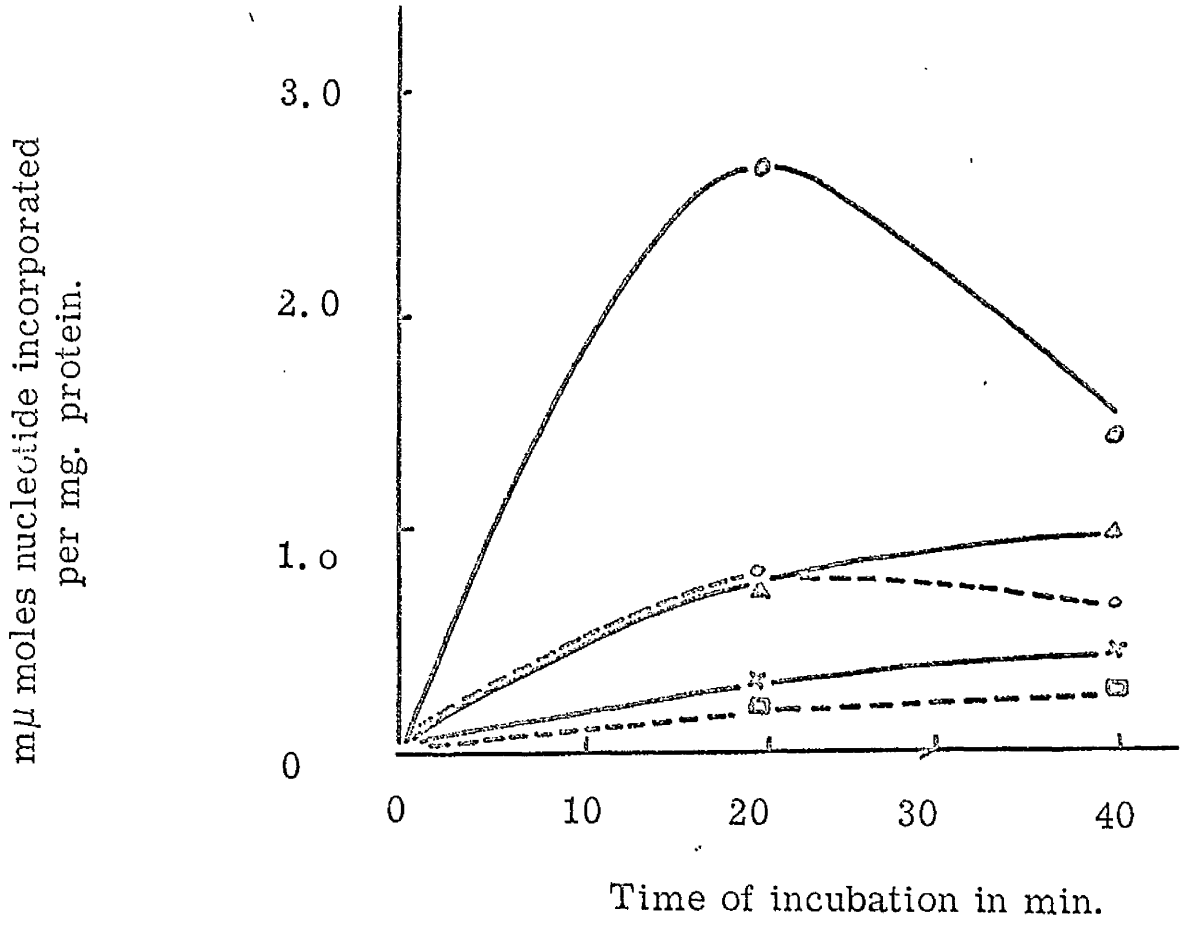


Figure 34.

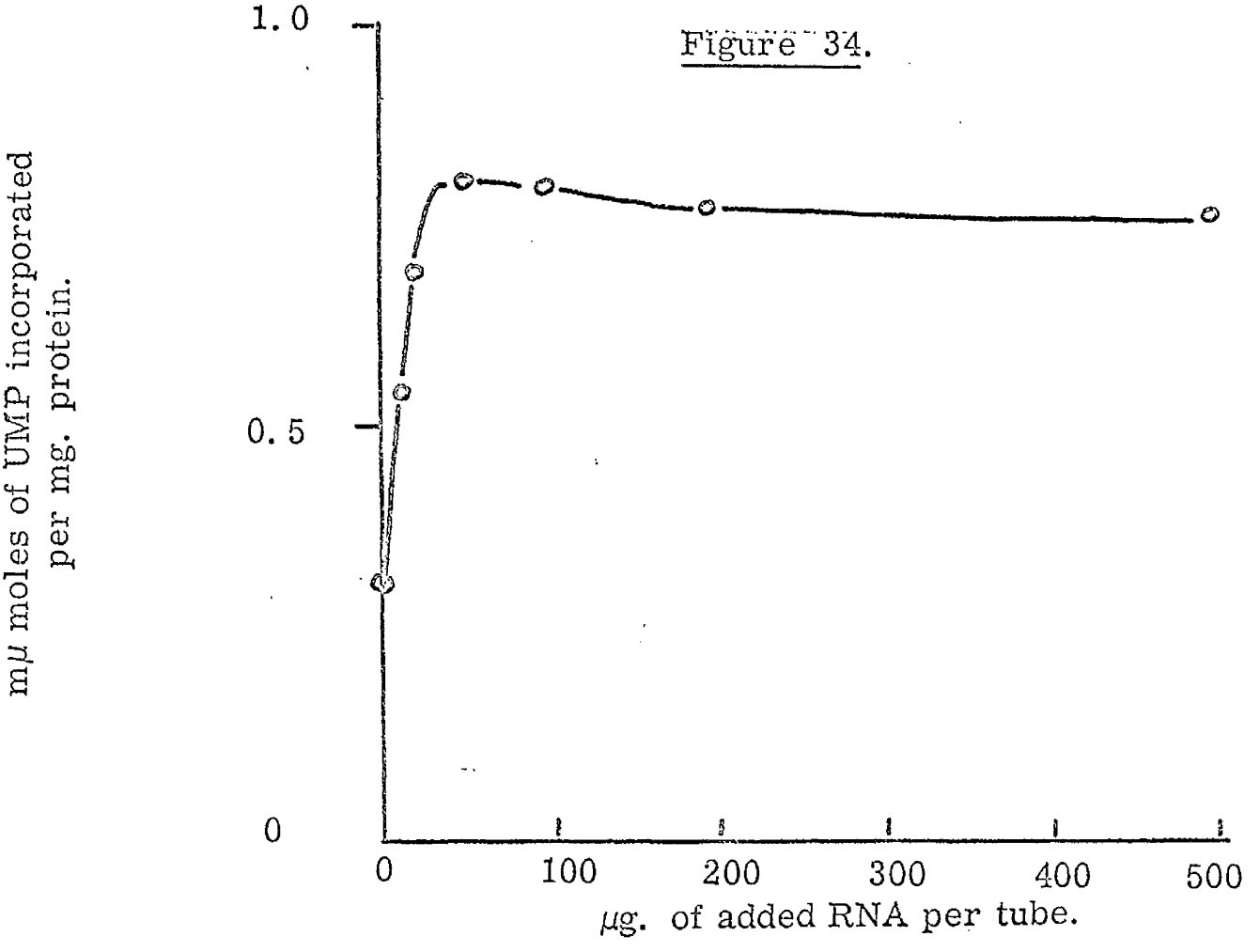


FIGURE 35

Time course of UTP uptake by F.R. Fraction

Reaction mixtures contained 50 μmoles tris-HCl, pH 8.0, 4 μmoles $MgCl_2$, 10 mμmoles EDTA, 0.1 μmole (α-^{32}P) UTP, (4×10^6 cpm per μmole), 50 μg RNA and 0.472 mg protein in a total volume of 0.5 ml.

Where indicated 0.1 μmole each of ATP, GTP and CTP were included. Incubations at 37°C.

———Δ——— UTP only

———●——— UTP + ATP, GTP + CTP

FIGURE 36

Time course of the UTP uptake by the F.R. fraction

As for Figure 35 except that ^{3}H-UTP (20 μc/μmole) was used; 0.344 mg protein per tube.

Figure 35.

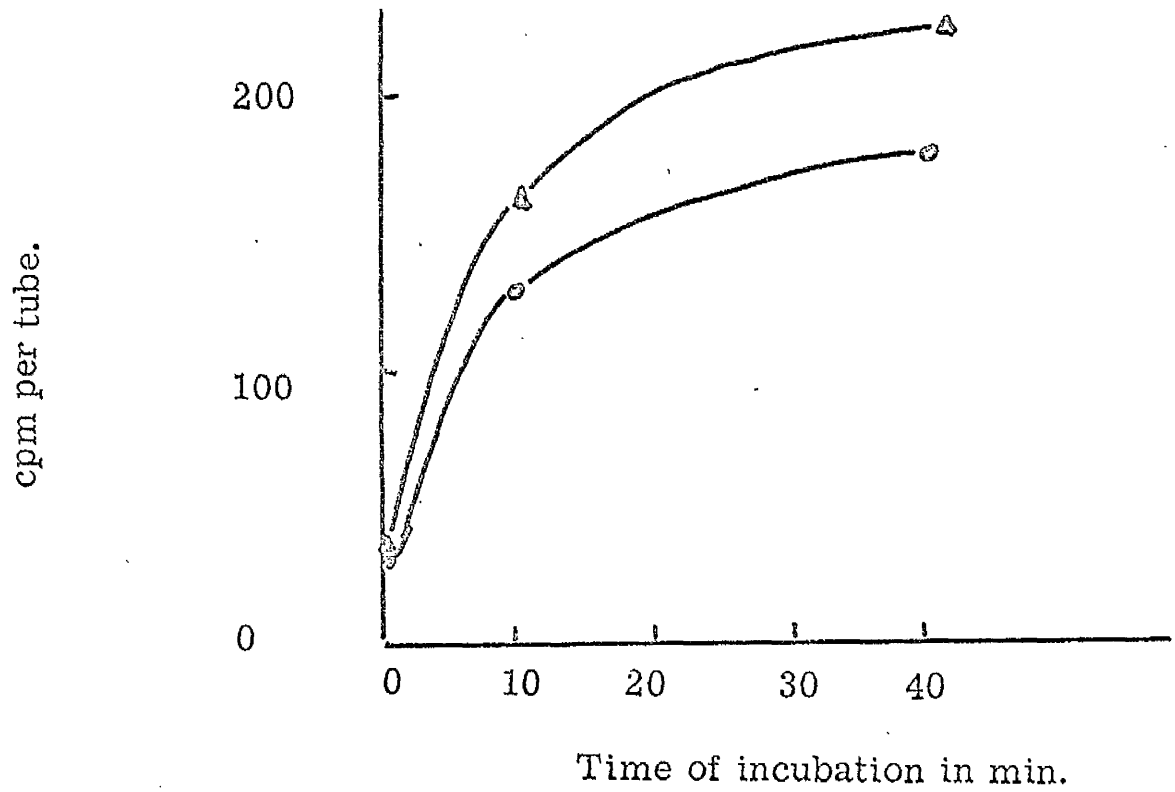


Figure 36.

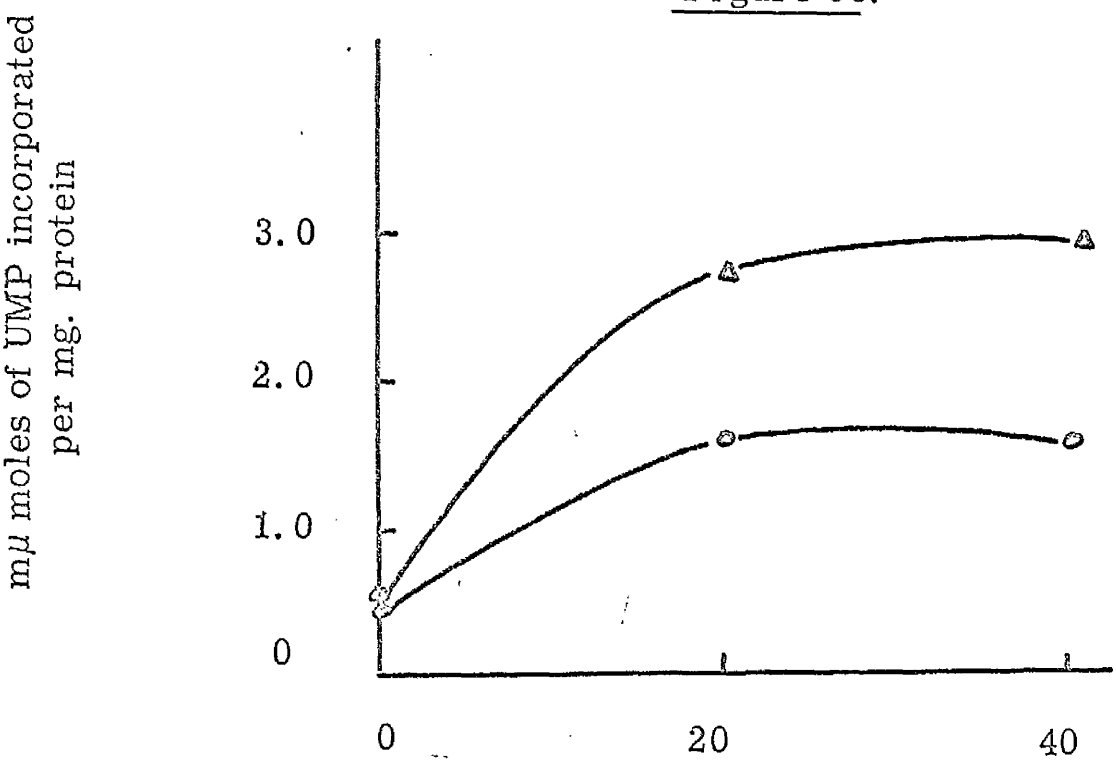


TABLE 21

Distribution of ^{32}P in the ribonucleoside monophosphates obtained on alkaline hydrolysis of the product of (α-^{32}P)UTP uptake of the P.R. fraction

Reaction mixtures contained 200 μmoles tris-HCl buffer, pH 8.0, 12 μmoles $MgCl_2$, 40 mμmoles EDTA., 0.4 μmole (α-^{32}P) UTP, (20×10^6 cpm per μmole), 200 μg RNA and 1.9 mg of protein in a total volume of 2.0 ml.

Incubations at 37°C.

T A B L E 21

Additions	% recovery of ^{32}P in			
	UMP	GMP	AMP	CMP
10 minutes	92.	2.3	2.7	3.0
40 minutes	92.5	1.8	2.4	3.3
10 minutes + ATP, GTP, and CTP.	78	10.9	5.4	5.7
40 minutes + ATP, GTP, and CTP	77.4	11.6	5.8	5.2

TABLE 22

Average chain length of the product of UTP uptake by the F.R. fraction

Reaction mixtures as in Figure 36 except that the specific radioactivity of the UTP was 200 μc per μmole.

Incubations at 37°C for 40 minutes.

T A B L E 2 2

Additions	Ratio of radioactivity $\frac{\text{nucleotide}}{\text{nucleoside}}$	∴ average chain length.
NIL	7.10	8
ATP GTP CTP	3.90	5

Figures 35 and 36. In the presence of UTP only, almost all the radioactivity was recovered in UMP. In the presence of ATP, GTP and CTP the proportions dropped a little but still almost 80% of the ^{32}P was found in UMP. The average length of the newly incorporated UMP residues in the presence and absence of ATP, GTP and CTP was 5 and 8 respectively. Thus the slightly lower percentage of radioactivity recorded in UMP in the presence of ATP GTP and CTP may only reflect slightly shorter chains being formed.

It was of some interest to determine the 3′ phosphodiesterase activity of the F.R. and this is shown in Figure 37 using p-nitrophenyl thymidine 5′-phosphate as substrate. Again, high levels of diesterase activity were found, but were some fivefold lower than those found for the RSV fraction (see Figure 23). The experiment was carried out using the F.R. fraction from the livers of the same animals as those in Figure 23 so that the results are strictly comparable. It is of interest to note that the levels of UTP uptake and the chain length of the product of the RSV fraction are approximately 5 times smaller than that of the F.R. fraction. It is possible that the phosphodiesterase activity in the two fractions may account for these differences.

The results are strongly in favour of a process of chain elongation. This was proved conclusively in the following experiments. Polyuridylic acid, which originally was completely excluded from the gel particles of a 35 x 2.4 cm column of G-100 Sephadex was hydrolysised in a 0.1M KOH as previously described for yeast RNA, and the hydrolysate partially purified on the G-100

F I G U R E 37

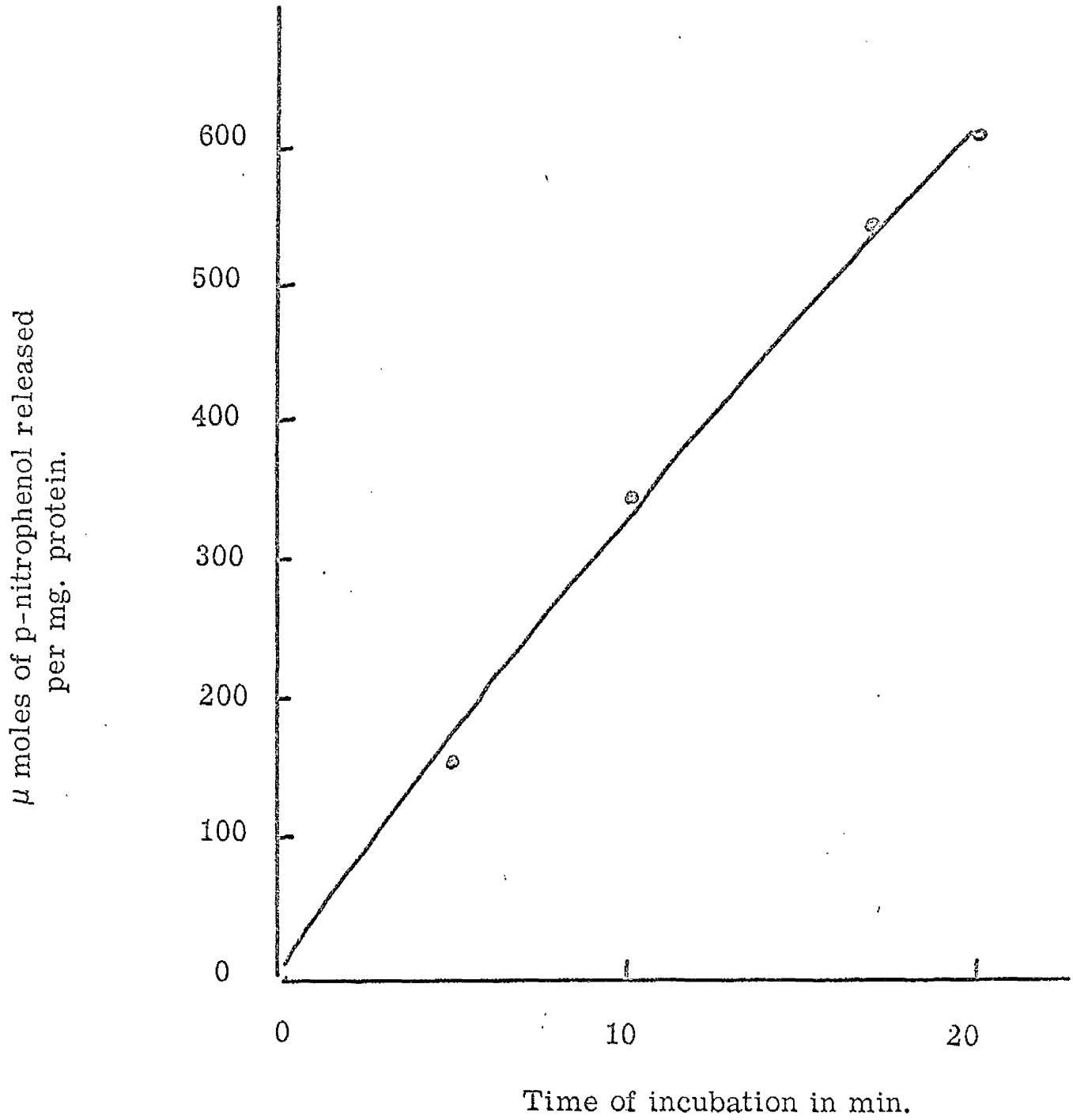
3′ phosphodiesterase activity of the free ribosomes

Reaction mixtures contained 50 μmoles tris-HCl buffer, pH 8.0, 4 μmoles $MgCl_2$, 10 μmoles EDTA, 0.1 μmole UTP, 2 μmoles p-nitrophenyl thymidine 5′ phosphate and 0.252 mg of protein in a total volume of 0.5 ml.

Incubations at 37°C.

The reaction was terminated by cooling the reaction tubes in crushed ice. The amount of p-nitrophenol released was then estimated as described under Experimental.

Figure 37.


600
500
400
300
200
100
0
μ moles of p-nitrophenol released per mg. protein.
0
10
20
Time of incubation in min.

Sephadex column. The poly U was precipitated from 66% ethanol, 1% potassium acetate, and subjected to the action of alkaline phosphatase as previously described. The material was then filtered through the column once again and the poly U from tubes 27 - 54 collected (Figure 38a). This yielded a preparation of poly U which was capable of stimulating UTP uptake by the F.R. fraction (see Figure 39). It was also completely distinguishable from the free ribosomal RNA, which was completely excluded from the gel particles of the G-100 Sephadex column (Figure 38b).

A large preparation of free ribosomes was then isolated. The ability of the poly U to stimulate UTP uptake was measured (Figure 39). In the presence of 1 optical density unit (at 260 mμ) of primer per tube, the poly U primed activity was responsible for 45.4% of the total activity, and the endogenous primers for 54.6%. Using the same F.R. preparation the enzyme was allowed to incorporate ^{3}H-UTP in the presence and absence of the same preparation of poly U primer. The RNA was then extracted from the two reaction mixtures and the optical density and radioactivity elution profiles from column of G-100 Sephadex determined. The results are presented in Figures 40 a and b. The poly U was obviously extracted by the phenol and is clearly separated from the endogenous RNA of the free ribosomes. Radioactivity has been incorporated into poly U. The total radioactivity in each peak was estimated by cutting out the area under the peaks and weighing the paper. 48% of the radioactivity was recovered in endogenous RNA and 52% in the region of poly U. This corresponds rather well to the results obtained from Figure 39. It was concluded that UMP

FIGURE 38

Column chromatography on G-100 Sephadex.

A 35 cm x 2.5 cm column of G-100 Sephadex was equilibrated with 0.01M tris-HCl, pH 7.0 at room temperature. The samples were applied to the surface of the gel in a small volume, and elution carried out with 0.01M tris-HCl pH 8.0. 2.0 ml fractions were collected and the absorbance at 260 mμ read. The hatched peak is the polysaccharide Blue Dextran, which is completely excluded from the gel particles.

a. 40 mg of poly U were hydrolysed in 0.1M KOH at 37°C for 9.0 min. The hydrolysate was neutralised with PCA as before, and the hydrolysed material partially purified on the same column of G-100 Sephadex. The poly U was dissolved in 0.01M tris pH 8.0 and treated with alkaline phosphatase for 10 min at 37°C. The alkaline phosphatase was sufficient to hydrolyse 1.5 μmoles of p-nitrophenyl phosphate per min. The enzyme was inactivated and the poly U recovered and passed through the column once again. The material from tubes 27-34 were pooled and the poly U precipitated from 66% ethanol, 1% potassium acetate, dissolved in 0.01M tris-HCl pH 8.0 and stored at -17°C.

b. RNA from a preparation of free ribosomes was extracted using the phenol method as described under experimental. The RNA was dissolved in 0.01M tris-HCl pH 7.0 and 1.0 ml (20 o.D. units) applied to the column and eluted as above.

Figure 38a.

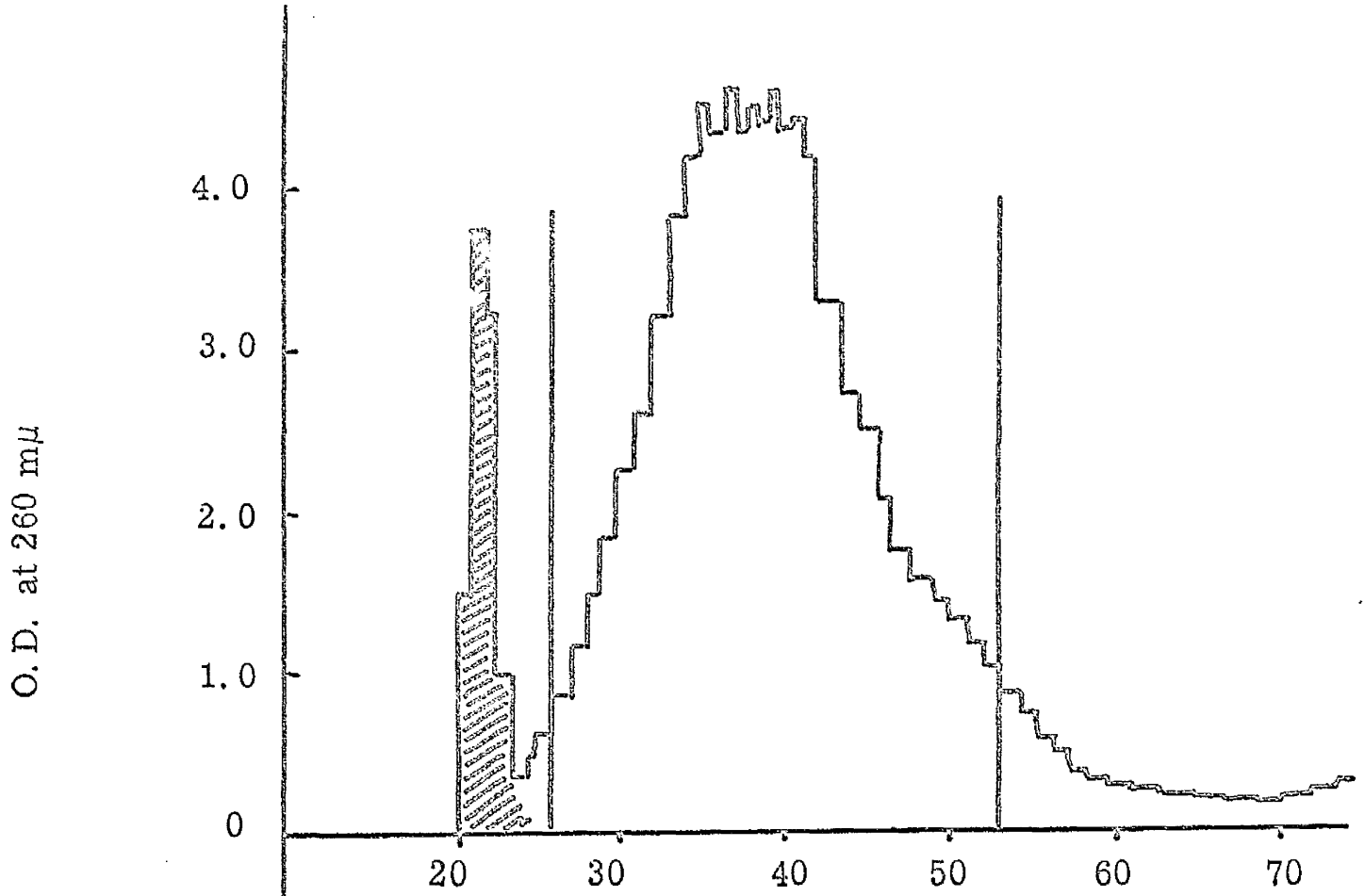


Figure 38b.

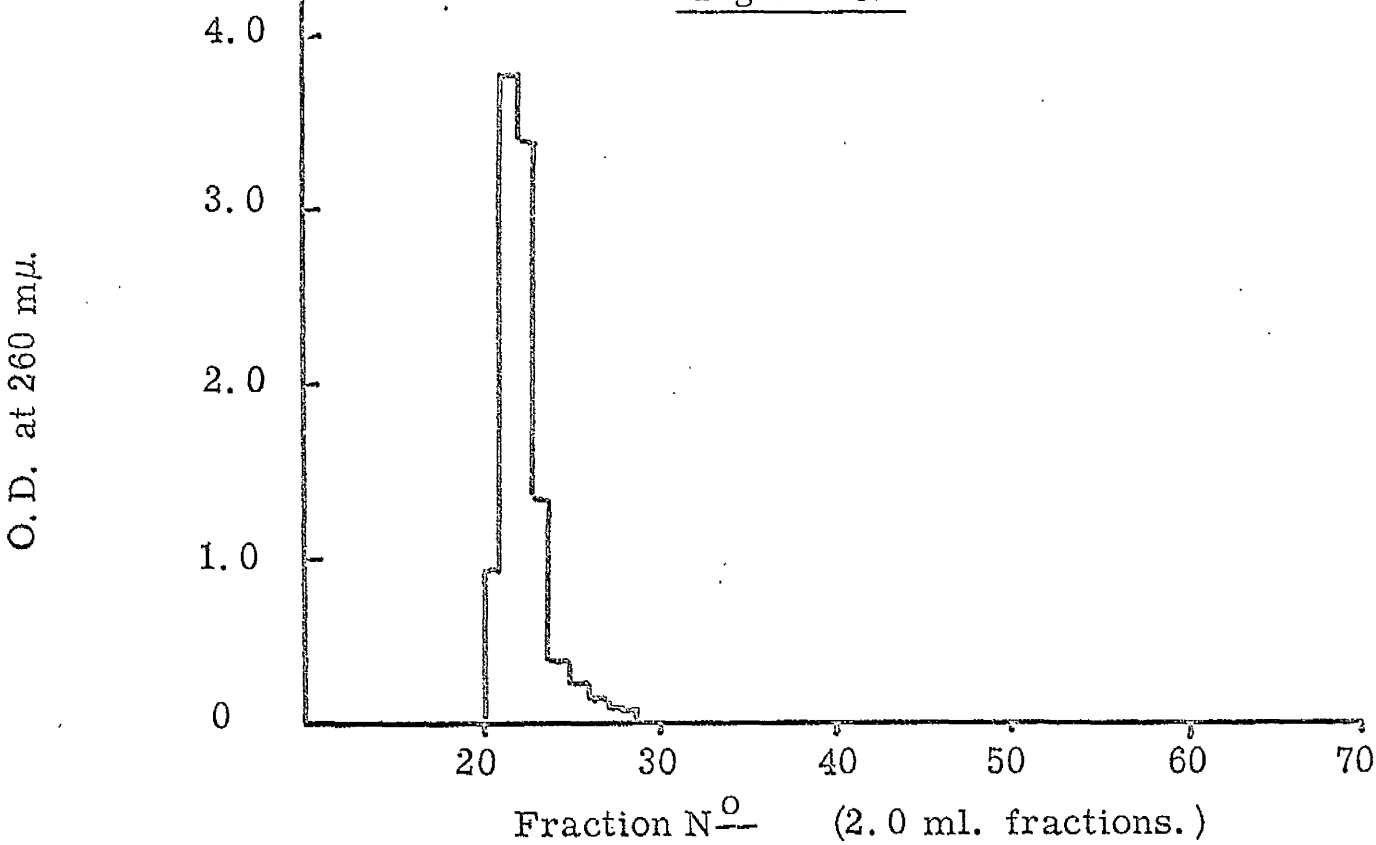


FIGURE 39

Effect of increasing amounts of shortened poly U on the uptake of UTP by the P.R. fraction

Reaction mixtures contained 50 μmoles tris-HCl buffer pH 8.0, 4 μmoles $MgCl_2$, 10μmoles DTA, 0.1 μmole ^{3}H-UTP (20 μc/μmole) and 0.465 mg of protein in a total volume of 0.5 ml.

Where indicated various amounts of poly U (Figure 37b) were included.

Incubations at 37°C for 5.0 min.

Figure 39.

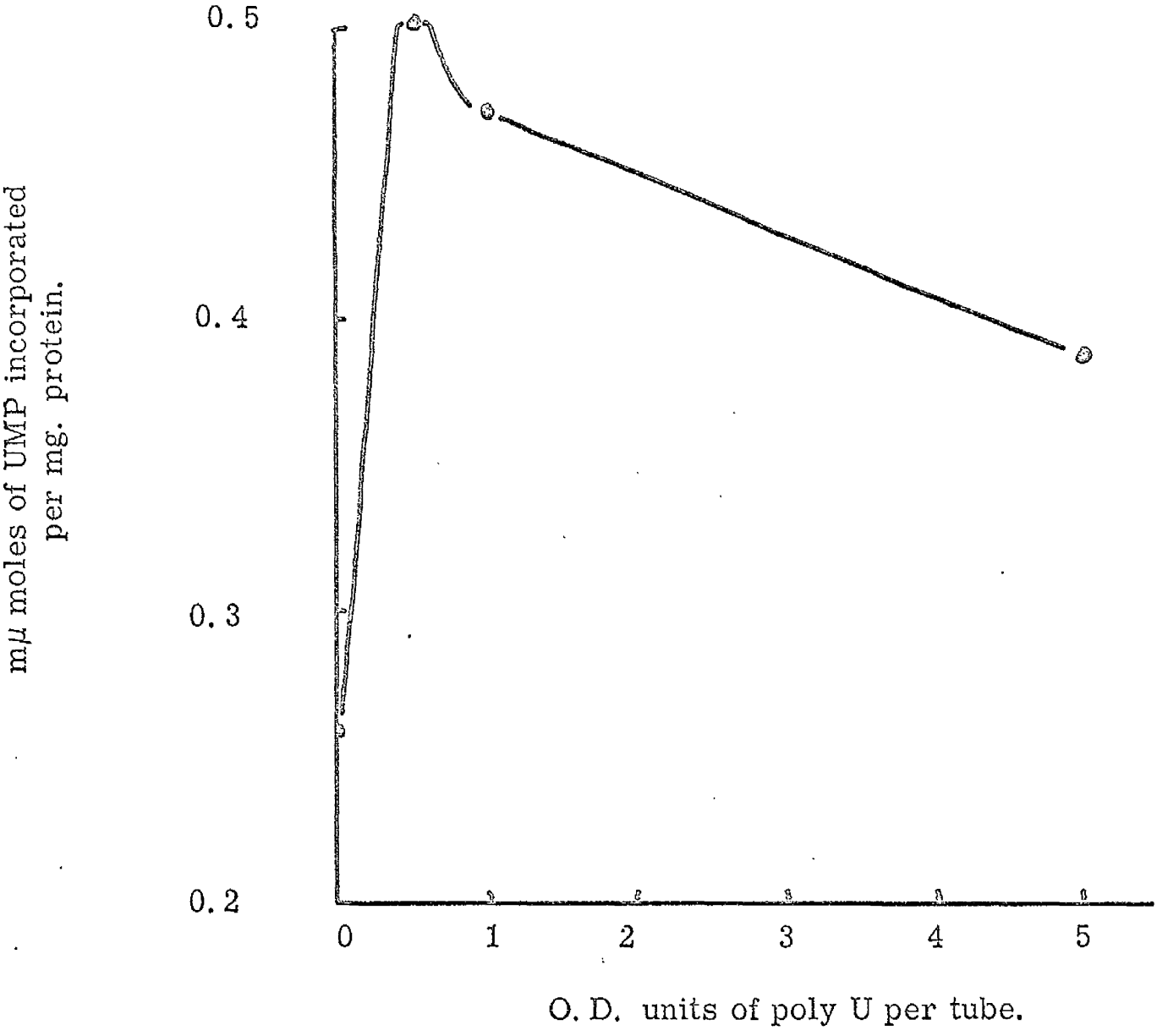

0.5
0.4
0.3
0.2
mμ moles of UMP incorporated per mg. protein.
0
1
2
3
4
5
O. D. units of poly U per tube.

FIGURE 40a

Chromatography of ^{3}H-UTP labelled free-ribosomal RNA on G-100 Sephadex : no added primer

Reaction mixture contained 750 μmoles tris-HCl pH 8.0, 60 μmoles $MgCl_2$, 0.15 μmole EDTA, 1.5 μmoles ^{3}H-UTP (66.6 μc per μmole) and 7.0 mg of protein in a total volume of 7.5 ml. Incubation was at 37°C for 5.0 min. The reaction was cooled in crushed ice and the RNA extracted by the method previously described. The RNA was dissolved in 0.01M tris-HCl pH 7.0 and chromatographed on a 35 x 2.5 cm column of G-100 Sephadex as previously described. Two ml fractions were collected and the absorbance at 260 mμ measured. The RNA in the tubes was then precipitated with 5.0 ml of ice-cold 5% TCA and the precipitate collected by centrifugation. The acid insoluble was washed a further three times in TCA and the radioactivity in the precipitates counted in the Packard scintillation spectrometer as before.

———— o.d. at 260 mμ

- - - - - - - - acid insoluble radioactivity (cpm)

Figure 40a

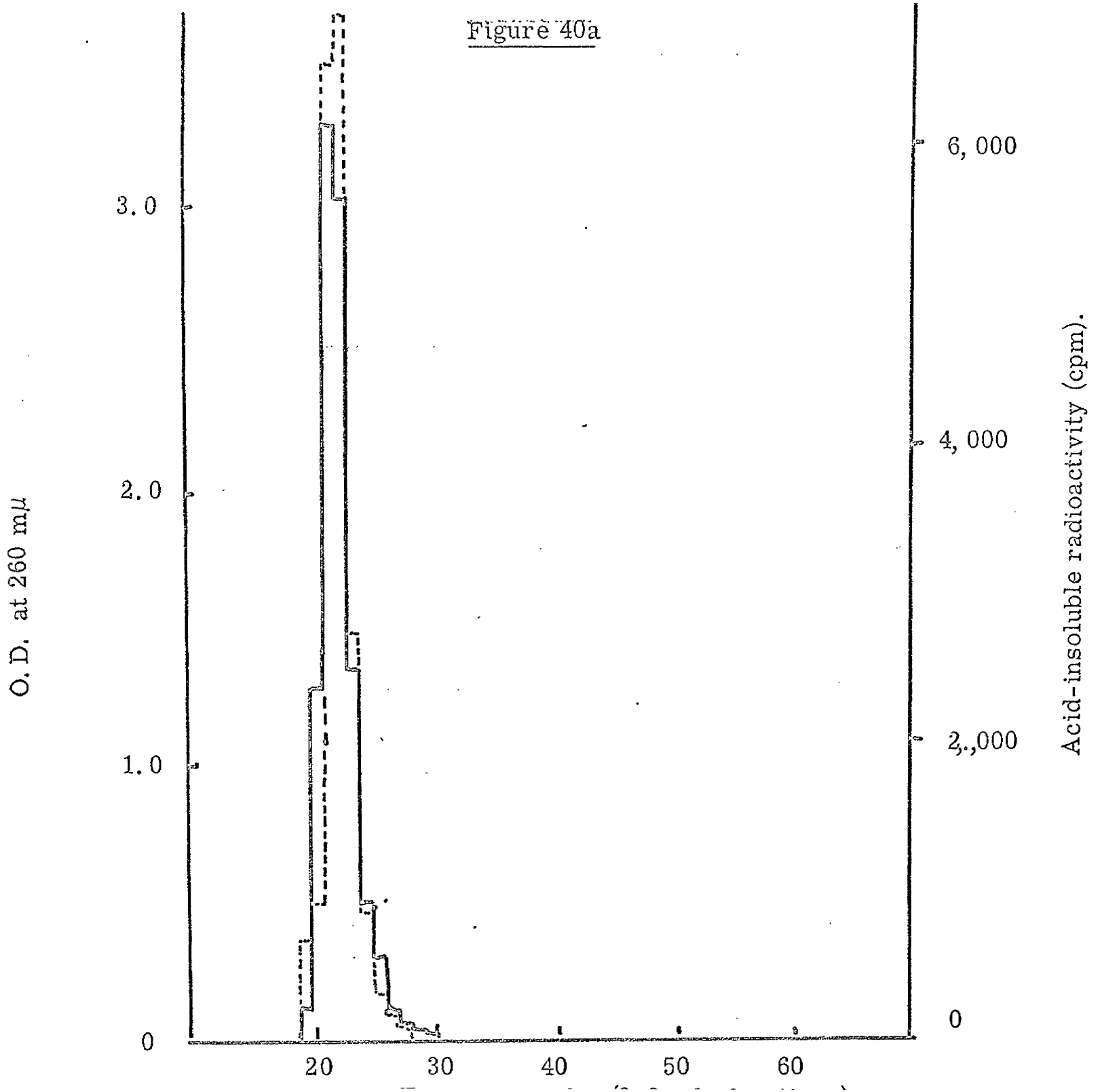
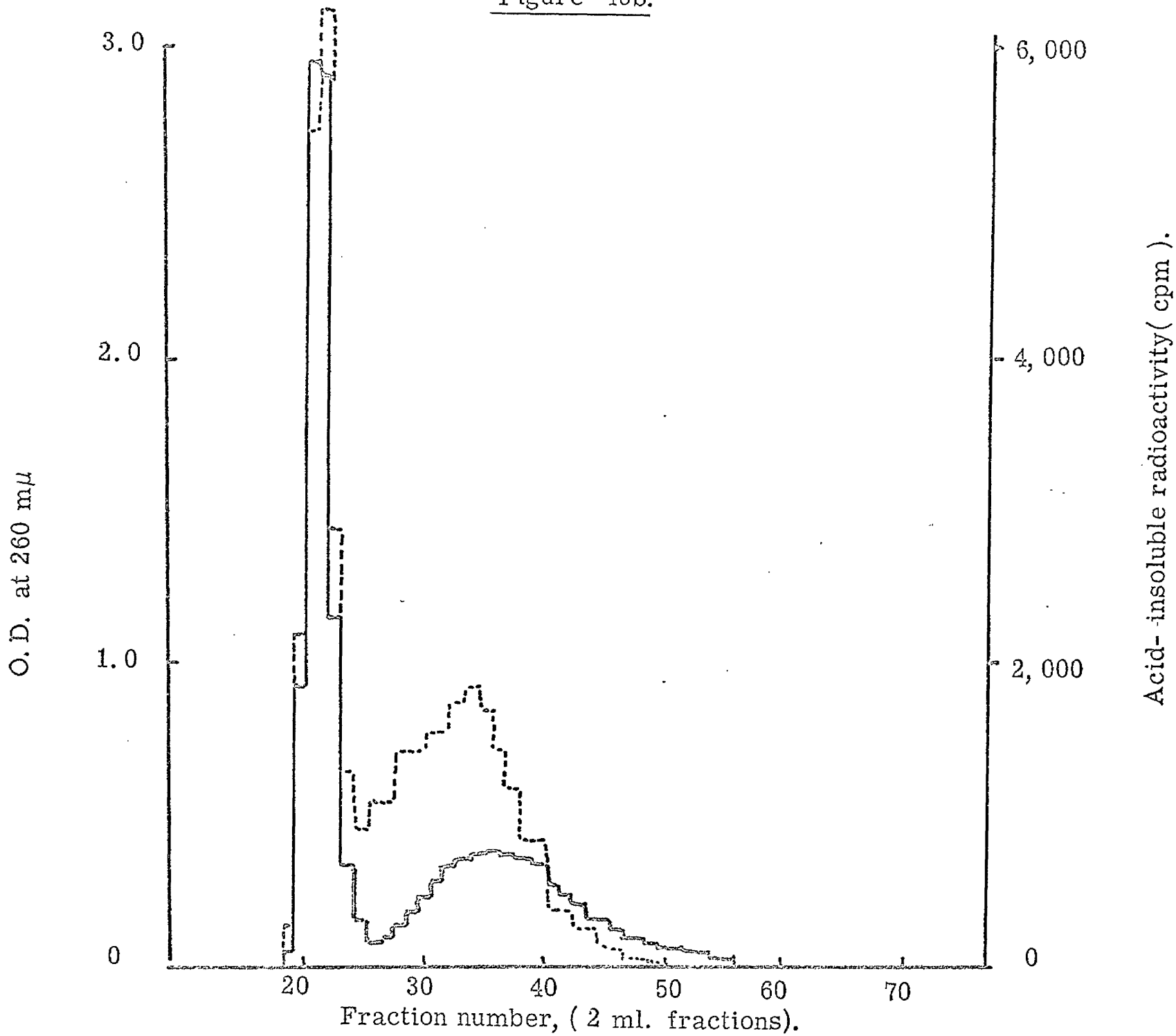

O. D. at 260 mμ
3.0
2.0
1.0
0
20
30
40
50
60
6, 000
4, 000
2,,000
0
Acid-insoluble radioactivity (cpm).

FIGURE 40b

Chromatography of ^{3}H-UTP labelled free-ribosomal RNA on G-100 Sephadex : poly U primer added

As for Figure 39a. Reaction mixture contained 15 o.D. units (at 260 mμ) of the shortened poly U primer (see Figure 37a).

Figure 40b.


3.0
2.0
1.0
0
O. D. at 260 mμ
6, 000
4, 000
2, 000
0
Acid- insoluble radioactivity(cpm).
20
30
40
50
60
70
Fraction number, (2 ml. fractions).

was incorporated into the primer chains.

It was possible that incorporation was by some form of hydrogen bonding attaching nucleotide residues to complementary sequences in internal positions of primer chains. Accordingly the characteristics of stimulation of ATP and UTP by poly A and poly U were studied. From Figures 41 a and b, it can be seen that while poly U acts as an efficient primer for UTP uptake, poly A inhibits the reaction. Unfortunately in the experiments performed, ATP uptake was rather low, possibly due to the short incubation time. Poly A was not a very efficient primer for ATP uptake. It did however stimulate the reaction by about 20% while poly U inhibited ATP incorporation. From the results it is clear that a copying mechanism, such an observed with RNA polymerase, is not involved in the reaction.

It is not possible to demonstrate the dependance of the reaction on free 3′ hydroxyl ends on the primer as for the previous fractions. Short yeast RNA was a very poor primer in the F.E. system, although in most of the experiments attempted the dephosphoxylated RNA gave higher levels of incorporation than the untreated shortened RNA. However, since UMP is incorporated from uridine 5′ triphosphate it is assumed that the mechanism involves nucleotide addition to the 3′ hydroxyl of the terminal nucleotide of the primer RNA.

There is no doubt therefore the free ribosomal enzyme catalyses chain extensions of pre-existing RNA primers by the addition of homopolymer sequences, probably to the 3′ end of the primer chain.

The question then arose, which molecular species of RNA in

FIGURE 41a

Effect of increasing amounts of poly U and poly A on the uptake of UTP by the F.R. fraction

Reaction mixtures as in Figure 38; 0.494 mg of protein per tube.

Incubations at 37°C for 5 min. Where indicated untreated poly U and poly A were included.

———o——— poly U added

- - - -Δ- - - - poly A added

FIGURE 41b

Effect of increasing amounts of poly U and poly A on the uptake of ATP by the F.R. fraction

As above except that 0.1 μmole of ^{3}H-ATP (20 μc per μmole) replaced UTP.

———●——— poly A added

- - - -▲- - - - poly U added

Figure 41a.

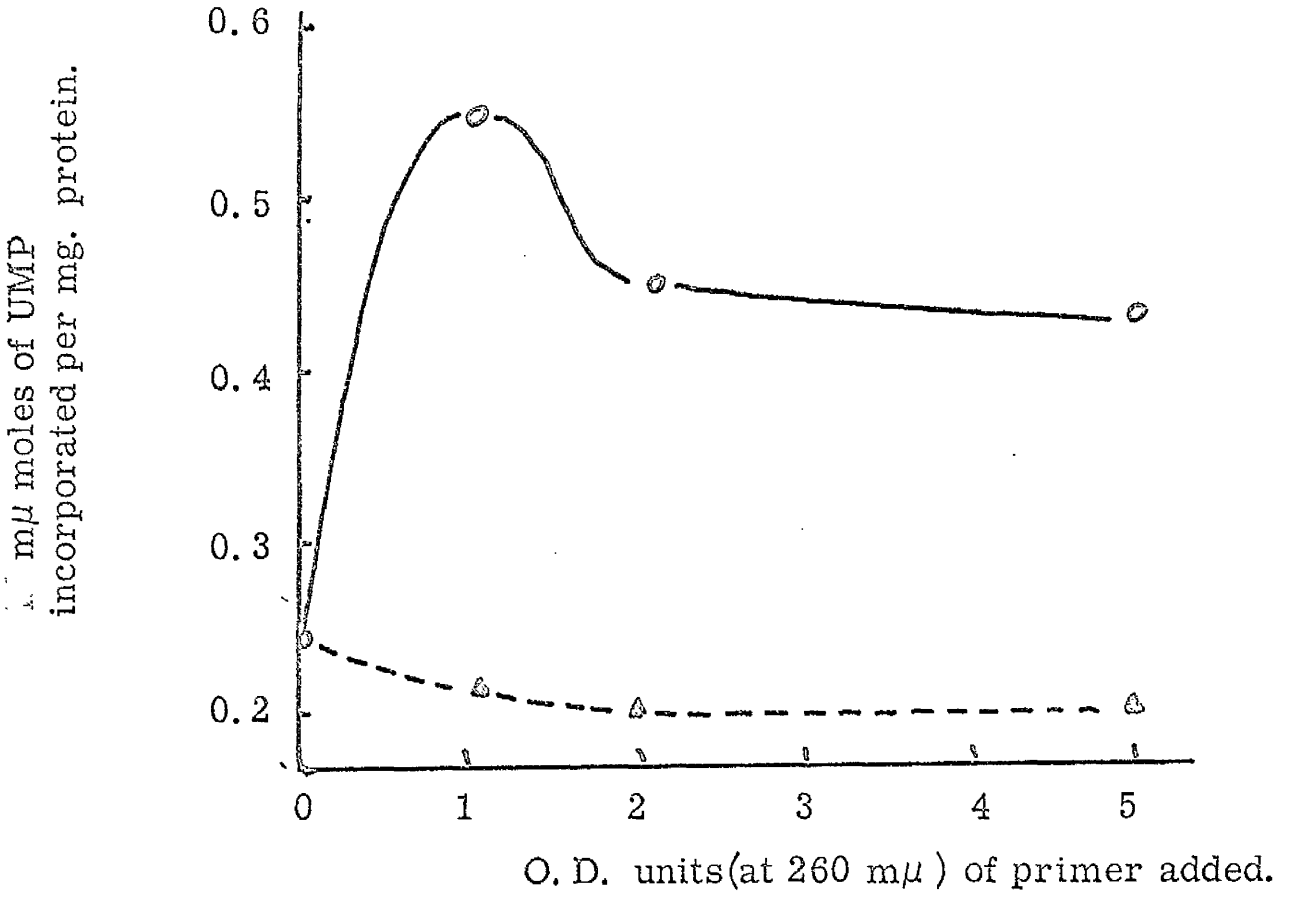
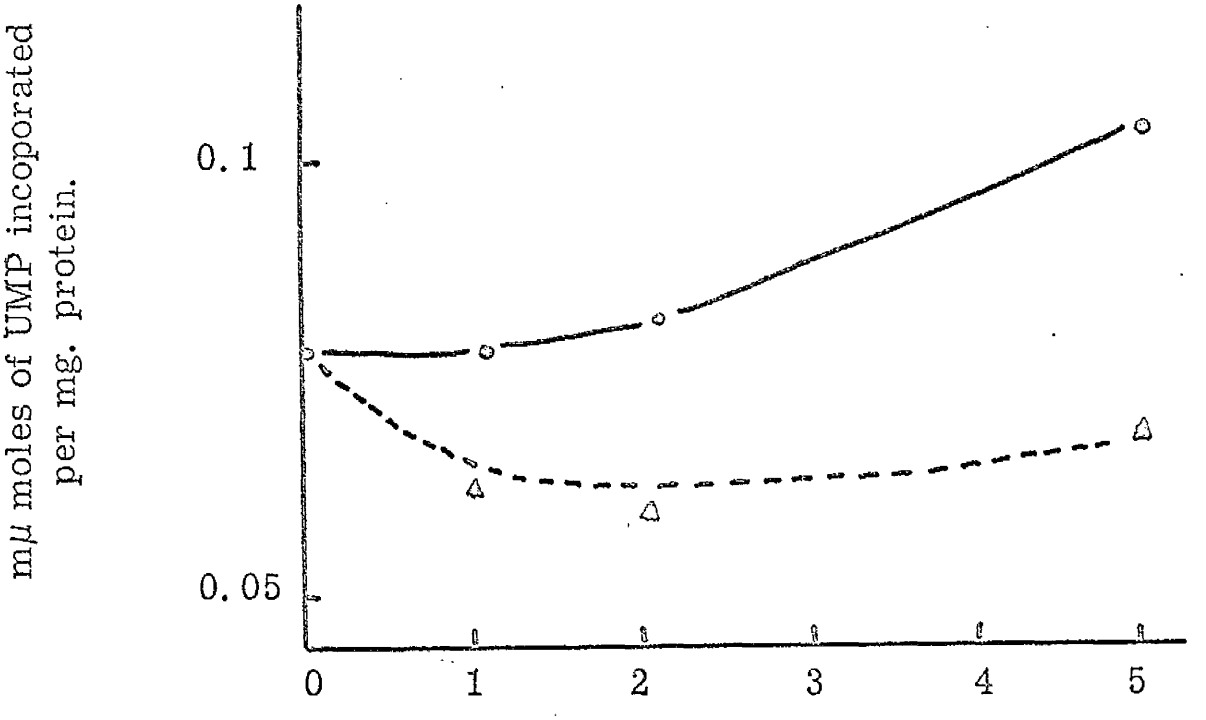


Figure 41b



the free ribosomal preparation was responsible for priming the reaction? In an attempt to answer this question the RNA was labelled *in vitro* with ^{3}H-UTP and extracted with phenol as above. Fractionation was first attempted on sucrose gradients, and the results are shown in Figure 42. The RNA has been separated into 3 main fractions which appear to correspond to the two large ribosomal RNA species (28S and 18S) and lighter material which sediments at the top of the gradient. As can be seen, ^{3}H-UMP has not been incorporated into ribosomal RNA, but all the radioactivity was associated with the slowly sedimenting material. It should be mentioned that unincubated samples gave similar patterns for the distribution of ultraviolet-absorbing material but no acid-insoluble radioactivity was recovered whatsoever.

The results obviously suggest that either t-RNA, which is known to be attached to ribosomes (Galibert *et al*, 1965) or 5S-ribosomal RNA were acting as primers. Recently the same workers (1965) have described a method of chromatography on large columns of G-100 Sephadex which resolves t-RNA and 5s-RNA from the bulk RNA. This method appeared to be well suited to the investigation of the problem and it was decided to utilise it. 94 x 2.5 cm columns of G-100 Sephadex were prepared as previously described and equilibrated with 0.05M potassium acetate buffer pH 5.1. In order to minimise degradation of RNA during separation, the column was prepared and used with a water jacket which contained circulating fluid at $1^{\circ}C - 3^{\circ}C$.

The free ribosomal RNA was labelled *in vitro* for 5.0 min with ^{3}H-UTP as previously described and then extracted with phenol. The

FIGURE 42

Sucrose density gradient analysis of ^{3}H-UTP labelled RNA from free ribosomes

The P.R. RNA was labelled in a reaction mixture which contained 1.5 m moles tris-HCl buffer, pH 8.0, 120 μmoles $MgCl_2$, 0.3 μmole EDTA, 3 μmoles ^{3}H-UTP (33.3 μc/μmole) and 21.6 mg of protein in a total volume of 15 ml.

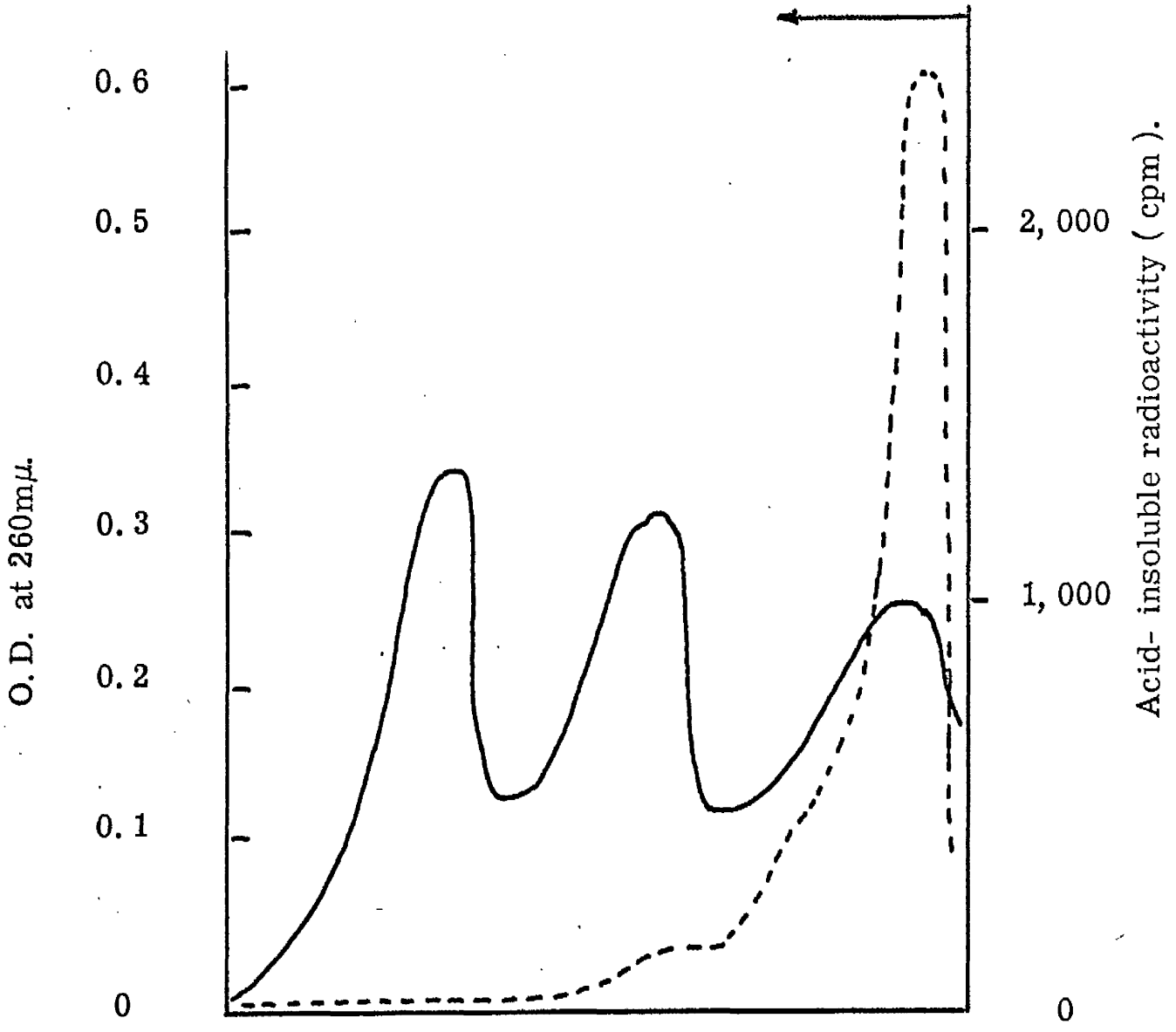
Incubations at 37°C for 5.0 min, and then the reaction mixture cooled in crushed ice. The RNA extracted with phenol as previously described and dissolved in 0.01$\bar{M}$ potassium acetate pH 5.2, 0.1$\bar{M}$ NaCl, 0.0004$\bar{M}$ $MgCl_2$.

0.04 ml of this RNA solution (containing 2.44 o.D. units at 260 mμ) was layered on top of a 5-20% sucrose gradient containing the same buffer. The gradient was centrifuged at 39,000 rpm in the SW 39 rotor of the Spinco Model L ultracentrifuge for three hours 30 mins. at 2°C. The o.D. profile at 260 mμ of the gradient was read by the continuous flow method already described, and the eluate collected in 28 fractions. 0.5 mg of BSA was added to each fraction followed by 5.0 ml of ice-cold 5% TCA. Acid precipitable material was washed four times in TCA and then the radioactivity measured as before.

——————— o.D. at 260 mμ

– – – – – – – – acid-insoluble radioactivity (cpm)

Figure 42.


0.6
0.5
0.4
0.3
0.2
0.1
0
O.D. at 260mμ.
2,000
1,000
0
Acid- insoluble radioactivity (cpm).

RNA was then applied to the large column of G-100 Sephadex and eluted with 0.05M potassium acetate pH 5.1. Two ml. fractions were collected and the O.D. at 260 mμ read. The acid-insoluble radioactivity contained in the tubes was then measured as before. The separation obtained is shown in Figure 43. 3 peaks were obtained. When Figure 43 is compared with Figure 44, which shows the elution profile of s-RNA isolated from the 150,000 x g supernatant, it is clear that peak III represents t-RNA and peak II 5S-RNA, as previously reported (Galibert et al, 1965). The full O.D. profile and acid-insoluble radioactivity is shown in Figure 45. It is obvious that only peak I contains the incorporated ^{3}H-UMP. It can be concluded therefore, that neither t-RNA, nor 5S-RNA acts as the endogenous primer for the F.R. enzyme.

The other species of RNA which might be present in the free ribosomal preparations is m-RNA. If m-RNA chains are labelled it might be expected that the label would remain with the ribosomes. Therefore the free ribosomes were allowed to incorporate ^{3}H-UTP, and then subjected to sucrose gradient centrifugation and the acid-insoluble radioactivity determined. It was hoped that this experiment might also provide some more information about the location of the enzyme. However, none of the many experiments attempted were conclusive. It was found that after exposure to the conditions of the reaction mixture, the ribosomes tended to aggregate, leaving virtually only Ferritin observable on the gradient. This happened even if the ribosomes were re-pelleted and resuspended in Mg^{++}-free buffer prior to sucrose gradient

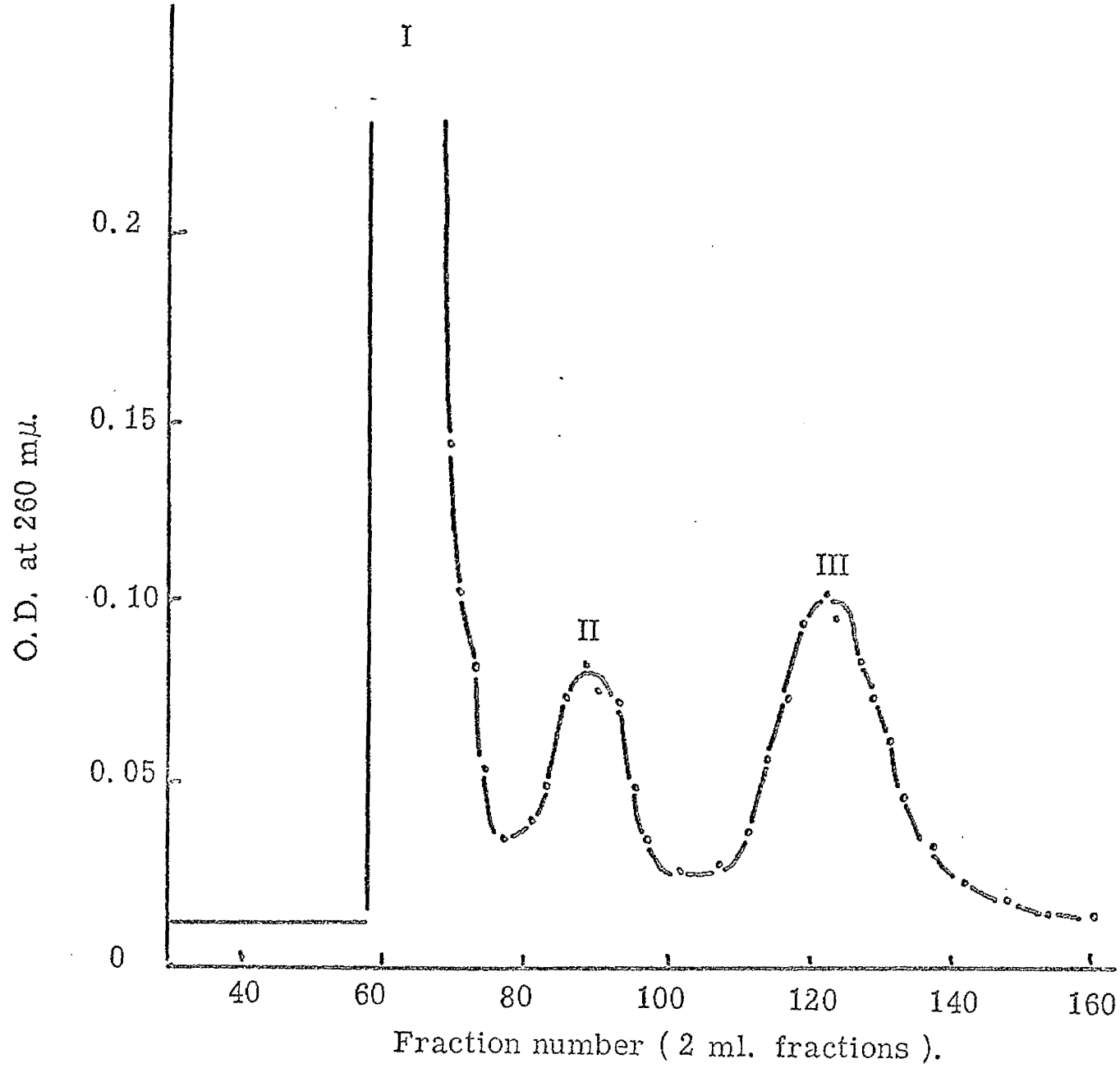
FIGURE 43

Sephadex column chromatography of the RNA from the F.R. fraction

Free ribosomes were incubated for 5.0 min at 37°C in a reaction mixture containing 2.5 m moles tris-HCl buffer pH 8.0, 200 μmoles $MgCl_2$, 0.5 μmole EDTA, 5 μmoles ^{3}H-UTP (a total of 200 μc), and 25.6 mg of protein in a total volume of 25 ml.

The RNA was then extracted as before and applied to a 94 x 2.5 cm column of G-100 Sephadex, which had been equilibrated at 1°- 3°C with 0.05M potassium acetate pH 5.1. Elution was with the same buffer and 2.0 ml fractions were collected. The o.D at 260 mμ and the acid insoluble radioactivity were determined as before. The o.D profile is shown here. The full o.D profile and radioactivity are given in Figure 44.

Figure 43.


I
II
III
0.2
0.15
0.10
0.05
0
O. D. at 260 mμ.
40
60
80
100
120
140
160
Fraction number (2 ml. fractions).

F I G U R E 44

G-100 Sephadex chromatography of the RNA from the 150,000 x g supernatant fraction

The RNA was extracted from the 150,000 x g supernatant by the method described previously and passed through a 94 x 2.5 cm column of G-100 Sephadex as described in Figure 42. Only the o.D profile was determined, of course.

Figure 44.

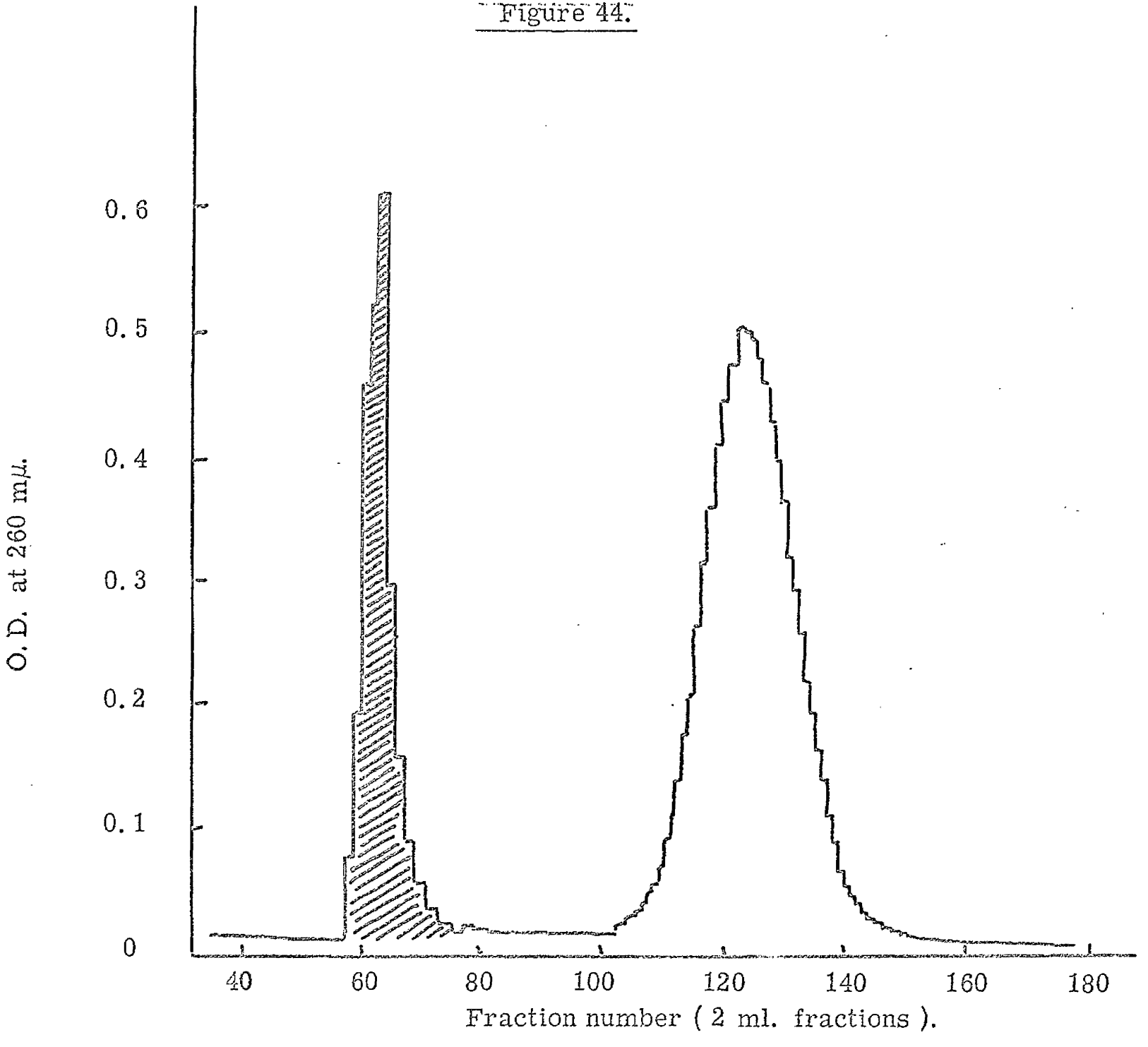

0. 6
0. 5
0. 4
0. 3
0. 2
0. 1
0
O. D. at 260 mμ.
40
60
80
100
120
140
160
180
Fraction number (2 ml. fractions).

FIGURE 45

G-100 Sephadex chromatography of ^{3}H-UTP labelled RNA from the F.R. fraction

For details see Figure 42.

_____ o.D. at 260 mμ

- - - - - - - - - acid-insoluble radioactivity (cpm)

Figure 45.

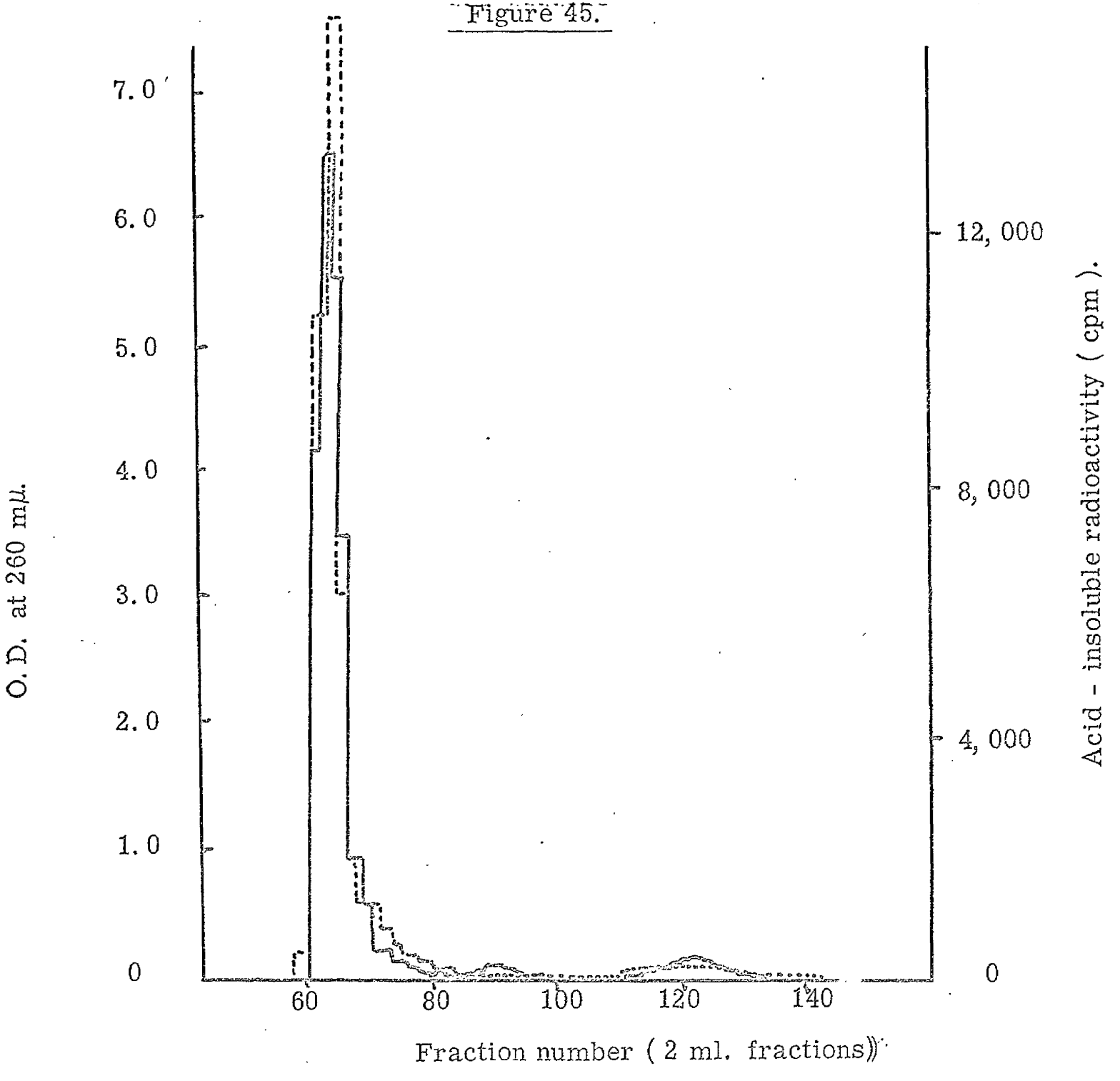

7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
O. D. at 260 mμ.
12, 000
8, 000
4, 000
0
Acid - insoluble radioactivity (cpm).
60
80
100
120
140
Fraction number (2 ml. fractions)

FIGURE 46

Sucrose gradient analysis of the F.R. fraction after incubation at 37°C under normal reaction conditions

9.2 mg of protein were incubated in reaction mixture containing 500 μmoles tris-HCl buffer pH 8.0, 30 μmoles $MgCl_2$, 100 mμmoles EDTA, 1 μmole ^{3}H-UTP (100 μc) in a total volume of 5.0 ml.

Incubations at 37°C for 5.0 min.

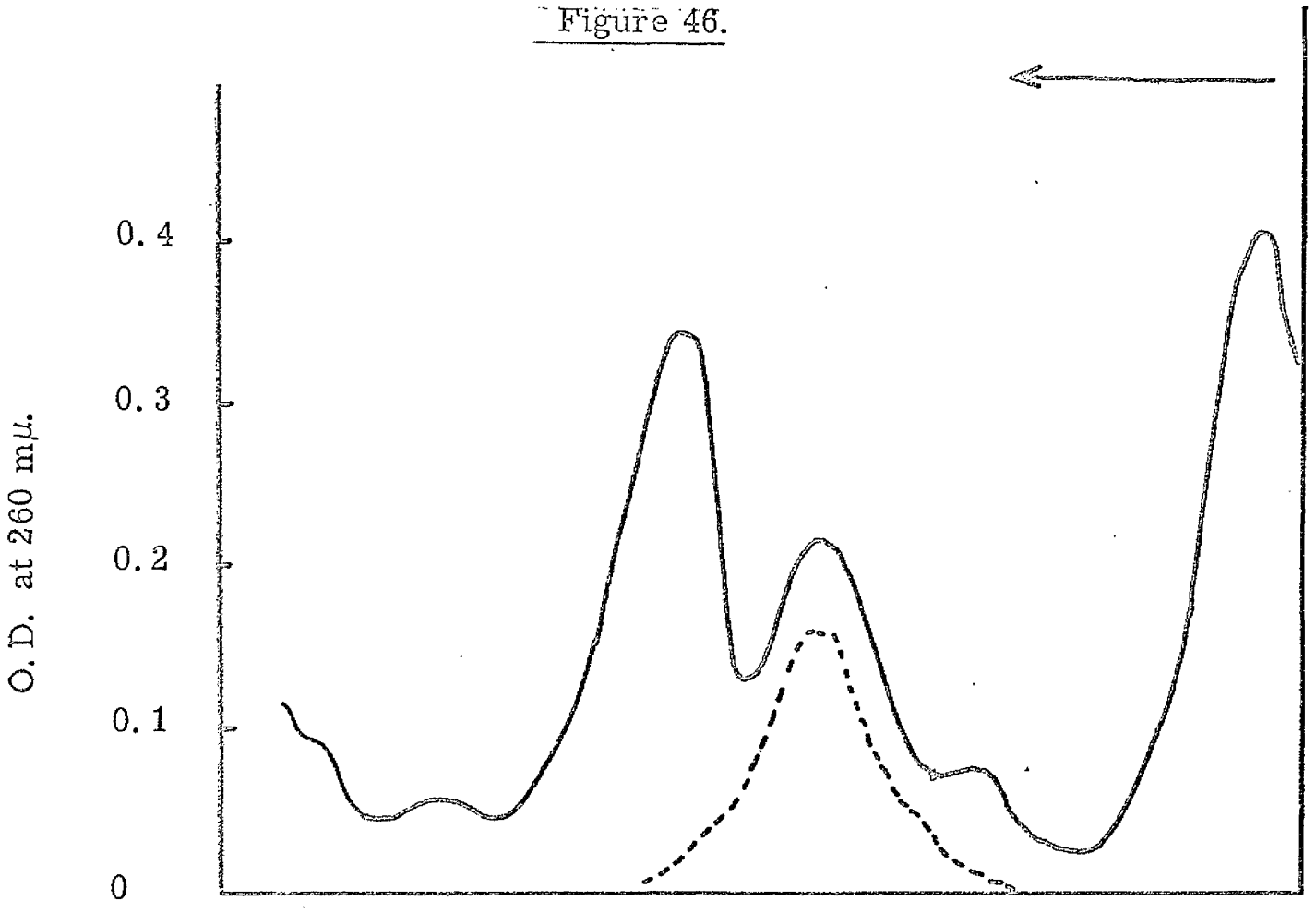
The reaction vessel was then chilled in crushed ice and 0.17 ml layered on top of a 5.0 ml 5 - 15% sucrose gradient containing 0.01$\bar{M}$ tris-HCl, pH 7.0, 0.0025$\bar{M}$ $MgCl_2$. The gradient was centrifuged at 39,000 rpm in the SW 39 rotor of the Griffin-Christ ultracentrifuge for 1.5 hours at 2°C.

The gradient was then passed through a continuous flow recording spectrophotometer and the profile at 260 mμ noted. 28 fractions were then collected and the o.D at 340 mμ measured.

———— o.D. at 260 mμ

- - - - - - o.D. at 340 mμ (Ferritin)

Figure 46.



analysis. In the most successful experiment the free ribosomes were analysed straight from the reaction mixture. From Figure 46, it can be seen that the Ferritin and the ribosomes were clearly separated. However when the acid-insoluble radioactivity was assayed, no counts higher than those of zero time (Unincubated) samples could be observed. It was concluded that radioactive material had aggregated once again, and sedimented to the bottom of the gradient.

DISCUSSION

1. The supernatant fraction

1.(i) The characteristics of the supernatant fraction enzyme

The supernatant fraction was investigated in much less detail than the microsome fraction and microsome subfractions. Nevertheless the results are sufficient to categorise the enzyme activity with some certainty.

UTP uptake required the addition of Mg^{++}, ATP, GTP and CTP, and an RNA primer. The enzyme probably requires nucleoside triphosphates as substrate since the uptake of UTP is stimulated by the presence of an ATP generating system (Table 4). Phosphocreatine and phosphocreatine kinase probably protect UTP from degradation to UDP by a transphosphorylation mechanism involving catalytic amounts of ATP, ADP and ribonucleoside diphosphate kinases present in the supernatant fraction (see Section 2 under Results). The stimulation observed on the addition of ATP, GTP and CTP may be due to a similar protection.

Alkaline hydrolysis of the reaction products after incorporation of (α-^{32}P)UTP followed by measurements of the distribution of the ribonucleoside 2′ (3′)- monophosphates so obtained, show that approximately 80% of the UMP residues were incorporated adjacent to UMP. When the average length of the newly synthesised chains was determined, it was found to be only 2 - 3. (Tables 6a and b). Now, if the observed reaction represented end addition of 3 UMP residues to primer chains ending in a random manner (i.e. all four ribonucleotides) about 76% of the radioactivity would be recovered in 2′(3′) UMP on alkaline hydrolysis, and about 8% each in AMP, GMP

and GMP. This is rather close to the observed results. In fact, the actual results might mean that incorporation of UMP showed a slight preference for UMP ends in the primer RNA. In any event, end addition to pre-existing primer RNA chains is obviously suggested.

This was confirmed by experiments using primer RNA molecules with either 2′(3′) hydroxyl or 2′(3′) phosphate ends. The RNA with free 3′(2′) hydroxyl ends was a much more effective primer than the phosphorylated RNA (Fig. 13). As discussed in Section 2 under Results, residual activity in the phosphylated primer is probably due to the presence of an estimated 20 - 25% of free 2′(3′) hydroxyl ends. Two main points emerge from this experiment. Firstly, the enzyme obviously requires free 2′(3′) hydroxyl ends in the primer RNA chains. This suggests that the mechanism of incorporation is similar to other RNA synthesising enzymes, such as RNA polymerase, and involves ester formation between the α 5′-phosphate of the ribonucleoside triphosphate substrate and the 2′(3′) hydroxyl of the terminal nucleotide of the RNA primer, with concommitant release of inorganic pyrophosphate. No naturally occurring RNA molecules containing a 2′- 5′ diester bond have been reported; and so it is probably the 3′- 5′ diester bond which is formed. In the second place, the dependance on free 3′-OH confirms that nucleotides are incorporated into terminal positions in the actual RNA primer, rather than into short new RNA sequences.

It can be concluded, therefore, that the observed incorporation of ribonucleotides does not represent synthesis of new RNA molecules, but rather terminal addition to pre-existing RNA chains.

The nature of the endogenous primers for the reaction was not investigated. The results with the incorporation of CTP and ATP (Figure 9) and the fact that only one adenylate residue is incorporated per primer RNA chain suggest that turnover of the CCA terminal sequence of S-RNA may contribute to the activity.

1.(ii) The biological significance of the supernatent fraction

1.(ii) The biological significance of the supernatent fraction

Various nucleotide sequences on the ends of pre-existing RNA primers have been reported in the supernatant fraction of rat liver. That the activity observed in this work is due to the contributions of different enzyme fractions is suggested by the biphasic nature of the response of the enzymes to increasing amounts of $MgCl_2$ and primer RNA.

Investigations of the *in vivo* turnover of the terminal CCA sequence of S-RNA in rat liver revealed that the adenylate residues had a far higher rate of turnover than the cytidylate residues. (Scholtissek, 1962). It can be deduced, therefore, that at any given time the S-RNA population in rat liver will have a greater ability to accept AMP from the CCA synthesising systems described by Heidelberger *et al* (1956), Hecht Zamecnik, Stephenson and Scott (1958) and Preiss, Dieckmann and Berg (1964)., than the ability to accept CMP. This is exactly what is found in the supernatant fraction under investigation. From Figure 9 it can be seen that ATP incorporation was some 4 - 5 times greater than CTP uptake. In addition only one adenylate residue was incorporated per primer RNA chain, exactly what one would expect for S-RNA. It is felt,

therefore,

therefore, that the synthesis of the terminal CCA sequence of S-RNA accounts for part or all observed incorporation of AMP and CMP residues.

Daniel and Littauer (1963) have reported a preparation from the 105,000 x g supernatant of rat liver which catalyses the uptake of UTP into s-RNA as well as the normal CCA synthesis. However, the incorporation of UTP by the supernatant fraction cannot be attributed to this enzyme. The main product of UTP uptake was s-RNA ... pXpCpU. Neither s-RNA ... pXpCpC nor s-RNA ... pXpCpCpA would accept uridylate residues. Since as discussed above, the most probable form of the s-RNA primer chains in the supernatant fraction under study would be s-RNA ... pXpCpC it is unlikely that UTP would be incorporated into s-RNA in the experiments reported here.

However, Klemperer (1963a), has described an enzyme which was partially purified from the pH 5 supernatant fraction of rat liver, and which incorporated UTP into 3′ terminal positions of various natural and synthetic polyribonucleotides. In the presence of Mg^{++}, only UTP was incorporated. When Mn^{++} was present the enzyme catalysed the uptake of ATP (Klemperer 1963b). Using Mn^{++} and r-RNA primer, UMP itself was the predominant terminal acceptor nucleotide to which labelled UMP was attached. Between 3 and 5 molecules of UMP were incorporated per molecule of acceptor. The results obtained during the studies on the supernatant fraction are obviously similar to those of Klemperer. The longer chain lengths synthesised by Klemperer's enzyme are probably best explained by the more purified state of his preparations. It

would have been of interest to have measured the incorporation of ATP and UTP in the presence of either Mg^{++} or Mn^{++}, but it is felt that under the conditions employed, UTP uptake can be attributed to the Klemperer enzyme. Similar enzymes have been reported in a soluble fraction of Ehrlich ascites cells (Burdon and Smellie, 1961).

Neither Klemperer, nor Burdon and Smellie, however, were able to reach any firm conclusion as to the biological significance of this reaction, and it must be considered to remain obscure.

In conclusion, the activity found in the supernatant fraction can be adequately described in terms of previously reported and well characterised enzymes.

2. The Microsome Fraction

The uptake of labelled UTP into acid-insoluble material by the microsome fraction requires a pH of 8.0 (Figure 15), the presence of Mg^{++} ions (Figure 16) and of ATP, GTP and CTP (Table 2).

UTP, rather than UDP produced by phosphatase activity, is the most probable substrate for the enzyme since the addition of inorganic pyrophosphate inhibits the reaction completely, while inorganic orthophosphate inhibits the reaction by only 50%. This would be expected if UMP was incorporated into RNA with the concommitant release of pyrophosphate. Under these circumstances the law of mass action predicts that excess pyrophosphate would inhibit the uptake of UTP. If UDP were the substrate and inorganic phosphate were released, then phosphate would be expected to inhibit the reaction. The observed 50% inhibition by inorganic phosphate may be attributed to binding of free Mg^{++} by the high levels of phosphate used in the experiment. It might be argued that phosphatases present in the microsome fraction could degrade N moles of pyrophosphate to 2N moles of phosphate resulting in the observed inhibition. However only 2.5 µmoles of pyrophosphate were added per tube so that even if complete hydrolysis occurred only 5 µmoles of phosphate could be produced, the exact amount of phosphate used in the other part of the experiment. In addition the presence of phosphocreatine and phosphocreatine kinase tended to stimulate the reaction, further suggesting that ribonucleoside triphosphates are the true substrates (Table 7).

The uptake of UTP showed no absolute dependance on added RNA, although the addition of 50 µgs of yeast highly polymerised RNA did

stimulate the reaction. From the results presented in Table 9, however, there can be no doubt that the reaction is not dependant on either added or endogenous DNA. Deoxyribonuclease and Act. D, over a wide conc. range (Section 4 (i) under Introduction) had no effect on the incorporation of UTP. It was concluded that the reaction was dependant on RNA chains, either added or already present in the enzyme fraction.

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 showed that UMP residues were incorporated mainly adjacent to UMP residues, but that 30 - 45% of the label appeared in AMP, GMP and CMP. However, in the absence of ATP, GTP and CTP, almost all the radioactivity was recovered in UMP. There are several possible explanations for such results. In the absence of ATP, GTP and CTP, it would appear that poly U sequences are formed. Addition of ATP, GTP and CTP, caused more random distribution of the incorporated UMP residues among all four ribonucleotides. This could be due to the formation of new heteropolymer RNA chains of ATP, GTP and CTP may be incorporated into short poly U sequences. Alternatively the addition of ATP, GTP and CTP could shorten the length of newly formed poly U sequences so that incorporation of UMP into the ends of RNA sequences ending in AMP, GMP and CMP would become relatively more prominent.

In fact all four ribonucleoside triphosphates were incorporated into polyribonucleotides by the microsome fraction (Table 11) so that the first two of these possibilities are certainly allowable.

However, GTP incorporation was relatively low in comparison to the uptake of ATP, UTP and CTP. This reduces the probability of the formation of new heteropolymer RNA chains. It would also be difficult to reconcile the stimulation of UTP uptake by ATP, GTP and CTP, with reductions in chain length, although this is not impossible. The ATP, GTP and CTP could have a multiple effect, reducing the chain length, but at the time protecting UTP from degradation so that incorporation was stimulated.

Several experiments were performed to determine the effect of addition of each ribonucleoside triphosphate, singly and together, on the incorporation of each ribonucleoside triphosphate in turn. (Table 12). No definite pattern emerged from the experiment, but it was significant that in no case was maximum incorporation observed in the presence of all four ribonucleoside triphosphates, further reducing the probability of heteropolymer formation. Although ATP stimulated UTP uptake to a greater extent than a mixture of ATP, GTP and CTP, in the latter case hydrolysis of the UTP to UDP or UMP was much less (Table 13). This result reduces the possibility that stimulation of the reaction by adding various combinations of the other ribonucleoside triphosphates can be explained solely on the basis of protection of the substrates from hydrolysis of phosphatases.

3. Sub Fractionation of the Microsomes

3.(i) The Rough Surfaced Vesicles

3.(i) 1. Location of the enzyme activity

There is serious doubt that the activity found in the RSV fraction is actually due to enzymes present in this fraction. In view of the observation that the observed activity can be washed out of the RSV (Figure 20), it seems possible that it represented contamination by the 78,000 x g supernatant fraction. Incorporating activity appeared in the washings and took on some of the characteristics of the 78,000 x g supernatant fraction (Table 16).

The most obvious explanation is that the activity is due to contamination by the 78,000 x g supernatant fraction. This consists of the unattached, unsedimented ribosomes, the smooth surfaced vesicles and the soluble enzymes (see Section 3.(i) 1. under Experimental). Although the smooth surfaced vesicle fraction was inactive, both the free ribosomes and of course the supernatant (soluble) fractions exhibited incorporating ability (Tables 2 and 14). While the characteristics of the RSV fraction and the 78,000 x g supernatant fraction were different (Tables 14, 15 and 16), the characteristics of the RSV fraction did not change on washing, although the washings tended to resemble the 78,000 x g supernatant fraction (Table 16). This suggests that the differences in the enzyme requirements might merely reflect different environments in the two fractions.

Other explanations are however available. The RSV activity may represent large ribosomal aggregates which are precipitated

at 78,000 x g but which are degraded after prolonged manipulation to a form which is not precipitated at 78,000 x g. Another possibility is that the light rough surfaced vesicle fraction merely represents a non-specific aggregate of particulate material formed under the particular ionic and osmotic conditions of isolation. Although the ionic conditions during washing were identical to those during isolation (see Figure 20), the protein concentration was smaller and other unknown factors may have been removed. Under these circumstances it is possible that such an aggregate could have broken down to a non precipitable form. If this were so, only the heavy RSV fraction, which shows very little activity, would represent rough surfaced vesicles.

However, although the short, dephosphorylated yeast RNA supported the uptake of UTP by the RSV fraction quite successfully (Figures 24a and b), it was unable to stimulate incorporation into the F.R. fraction. This could be taken to mean that different enzymes were involved. Therefore the RSV activity was characterised in more detail.

3.(i) 2. Characteristics of the RSV enzyme

For the optimum uptake of UTP the enzyme appeared to require the presence of all four ribonucleoside triphosphates (Table 14). As discussed in the previous section, the reasons for this may be multiple and do not necessarily reflect the synthesis of new heteropolymer RNA chains. Stimulation of UTP uptake was obtained when 5 µmoles of mercaptoethanol were included in the reaction mixture (not shown) and this was added in some of the experiments. The peculiar, biphasic nature of the time course of the reaction, which

was observed repeatedly, must have been due to the contribution of different enzymes to the final activity.

The distribution of radioactivity in the 2′(3′) ribonucleoside monophosphates obtained on alkaline hydrolysis of the products of (α-^{32}P) UTP uptake is shown in Table 17. Less than 50% of the radioactivity was recovered in UMP, almost 30% in GMP and 13-16% in AMP and CMP. No significant difference was obtained between the two regions of the time curve. As previously discussed, the interpretation of such an experiment depends on the length of the newly synthesised sequences. These are given in Table 18, and only 1-2 residues were incorporated per molecule of primer RNA.

The results are quite unequivocal. Synthesis of new heteropolymer RNA sequences is ruled out and limited terminal addition to pre-existing RNA chains is suggested. This is supported by the studies illustrated in Figures 24a and 24b, where the dependence of the enzyme(s) on the presence of free 2′(3′) hydroxyl residues is demonstrated. This suggests that incorporation is into the 3′ terminal ends of the actual RNA primers.

In this respect the RSV fraction is similar to the F.R. fraction (see below) and the 150,000 x g supernatant fraction (see 1 of this section). The short chain lengths and low activity observed may be due to the presence of extremely active phosphodiesterases which attack the 3′ ends of the RNA. The same activity was found to a smaller extent in the F.R. fraction (Figure 37). This strengthens the impression that the RSV activity can be described in terms of enzymes present in the 78,000 x g supernatant.

3.(ii) The Free Ribosomes

3.(ii) 1. Location of the enzyme activity

Although an extremely active enzyme(s) which catalysed the uptake of UTP into terminal positions of RNA primers was found in the F.R. fraction, it is known that ribosomes are capable of the non-specific uptake of protein. However, neither washing the F.R. fraction in 0.25M sucrose nor isolating the fraction from media containing 0.1M EDTA, (final concn.) reduced the activity. The results with EDTA are particularly striking since it is known that under these conditions Esch. coli ribosomes release all their bound ribonuclease into the supernatant fraction (Neu and Heppel, 1964). It is felt, therefore, that the activity represents an enzyme(s) either tightly bound to the particulate material, or else large enough to sediment by itself at 150.000 x g.

The preparation contained monomeric and oligomeric ribosomes, and Ferritin. Isolation of the F.R. fraction from a medium containing EDTA had the effect of decreasing the amount of Ferritin relative to the ribosomes (Figures 27a and b). However EDTA-ribosomes were found to be some 23-24% more active than Mg^{++}-ribosomes (Table 20). This could be taken to mean that the activity is a function of the ribosomes rather than the Ferritin. This is supported by experiments in which the ribosomes were analysed by sucrose gradient centrifugation after incubation with 3H-UTP in normal reaction mixtures. On many occasions it was found that the acid insoluble radioactivity and the bulk of the ribosomes had precipitated out of the gradient leaving mainly Ferritin. However, it should be emphasised that the ribosomal

location of the enzyme(s) was not demonstrated directly. It might have been possible to resolve the question by assaying the fractions obtained on sucrose gradient centrifugation of the F.R. fraction for the ability to incorporate UTP. Unfortunately pressure of time did not permit this experiment to be performed.

3.(ii) 2. The characteristics of the F.R. fraction

The F.R. fraction catalysed very high levels of UTP uptake. Although there was no requirement for phosphocreatine or phosphocreatine kinase (Figure 30) there was no doubt that UTP was the required substrate. Figure 31 shows that when radioactive UMP, UDP and UTP were exposed to the enzyme, only UTP was incorporated into acid-insoluble material. This confirmed the previous result obtained using inorganic orthophosphate and pyrophosphate. ATP and GTP were incorporated to a smaller extent than UTP, but no CTP uptake was observed. In each case the presence of the other ribonucleoside triphosphates inhibited the reaction slightly.

Alkaline hydrolysis of the reaction products after the incorporation of (α-^{32}P)UTP, followed by measurement of the radioactivity in the ribonucleoside 2′(3′) monophosphates so obtained showed that UMP was incorporated mainly adjacent to UMP residues when only UTP was present. This is indicative of homopolymer formation. When ATP, GTP and CTP were included the amount of radioactivity in AMP, GMP and CMP increased somewhat. The average length of the newly synthesised sequences are given in table 22. It can be seen that the addition of ATP, GTP and CTP reduced the average chain length from 7-8 to approximately 5. The most reasonable interpretation of the results is that short

poly U sequences can be formed on the ends of primer RNA chains. Addition of ATP, GTP and CTP reduced the length of the newly formed sequences so that incorporation of UMP adjacent to AMP, GMP or CMP in the terminal positions of primer chains became relatively more prominent. The actual figures agree well with this interpretation. However, limited incorporation of ATP and CTP into the poly U sequences cannot be eliminated, nor can the possibility that short sequences complementary to some region of the primer are formed and which remain associated with the primer during the washing procedure.

The free ribosome fraction contained approximately 30% RNA and no absolute requirement for RNA could be demonstrated. However 50 µg of added yeast RNA caused a 165% stimulation of UTP uptake (Figure 34). To obtain information about the role of RNA as a primer the RNA from reaction mixtures was isolated following incubation with ^{3}H-UTP in the presence and absence of poly U, and then analysed on columns of G-100 Sephadex (Figures 40a and b). There was no doubt that UMP was incorporated into the poly U primer. Particularly striking was the observation that almost the same proportion of radioactivity was found in the poly U peak as was due to the stimulation observed in the presence of poly U (Figure 39). Figures 41a and b show that poly U stimulated UTP uptake, while poly A stimulated only ATP uptake. When these results are considered together, the possibility of copying short regions of the primer can be excluded. The newly synthesised regions must have been incorporated into terminal positions of the RNA primer by a process of chain extension.

When Figure 41b is scrutinised carefully it can be seen that the radioactivity in the poly U peak is skew towards the heavy material. This may mean that the enzyme shows some specificity for the size of the RNA primer. The possible significance of this observation will be discussed later.

To investigate the nature of the endogenous RNA primer, RNA was extracted from incubation mixtures following the incorporation of ^{3}H-UTP and analysed by sucrose gradient centrifugation and on columns of G-100 Sephadex. No labelled UMP was incorporated into the r-RNA, 5S-RNA or t-RNA. The other main class of RNA likely to be present is m-RNA, and the results raise the intriguing possibility that m-RNA is the ribonucleotide acceptor. Unfortunately it was impossible to exclude the possibility that it was degraded r-RNA which was labelled. Indeed from the relative heights of the two r-RNA peaks on the sucrose gradient it can be deduced that some degradation had occurred. However it might be expected that even if the RNA in the ribosomes had been degraded, the ribosomal structure would have been maintained. It would therefore be necessary to postulate a mechanism whereby the enzyme could distinguish between the ends of degraded and intact RNA. This is not impossible, of course, but the very location of the enzyme in the P.R. fraction suggests a more specific role.

4. Comparison with other enzymes of polynucleotide metabolism

Several lines of evidence suggest that the activity found in the particulate fraction is not connected with DNA-primed RNA polymerase. For example, the addition of DNA to the microsomes had no effect on the uptake of UTP. It is known, however, that the purified RNA polymerase from bacteria can catalyse the RNA dependant uptake of ribonucleotides by a base pairing mechanism. This activity requires the addition of Mn^{++} ions rather than Mg^{++}. The characteristics of the enzyme(s) under study are quite distinct from this. Mg^{++} is required and ribonucleotides are incorporated into the ends of pre-existing RNA primers. In the presence of one ribonucleoside triphosphate the purified RNA polymerases can synthesise homopolymers (Fox and Weiss, 1964; Stevens, 1964). Again this requires Mn^{++} ions as opposed to the enzyme(s) in the present report. Provided mammalian RNA polymerases have the same properties in these respects it can be concluded that DNA-primed RNA polymerase as such does not contribute to the observed activity.

It is possible that the subunits of RNA polymerase are synthesised independently on cytoplasmic ribosomes and assembled to the complete enzyme at other locations in the cell. Such subunits could have radically different properties than the complete enzyme. The high levels of incorporation observed in the F.R. fraction would tend to argue against such an interpretation but the possibility cannot be rigorously excluded at present.

It might be argued that the activity could be associated with a virus specific RNA replicase. However, the characteristics of

UTP uptake by the enzyme(s) of the particulate fraction are quite different from those of RNA replicases which catalyse the incorporation of all four ribonucleoside triphosphates into internal positions in new heteropolymer RNA chains. In addition Dalgarno et al (1966) have demonstrated that the RNA replicase induced on the infection of Krebs 2 ascites cells with Encephalomyocarditis virus appears in a different cell fraction than the homopolymer synthesising enzyme found in the particulate fraction of uninfected cells. This confirmed the results previously reported by Eason, Cline and Smellie (1963) and Baltimore and Franklin (1963).

Another possibility is that an induction of the cells with a virus whose genome becomes incorporated into that of the cells, partial or faulty transcription of the virus genome could give rise to a faulty RNA replicase. Such an enzyme could conceivably have the characteristics of the enzyme(s) in this report. It is felt, however that the levels of incorporation observed in the F.R. fraction are much higher than one might expect under these circumstances.

Recently, the presence of polynucleotide phosphorylase has been demonstrated in animal cells (Siebert et al, 1966). It is known that polynucleotide phosphylase can catalyse the synthesis of polyribonucleotides by condensation of ribonucleoside diphosphates with release of inorganic phosphate (see Section 5(i) under Introduction). However from the results it is clear that UTP and not UDP is the substrate for the particulate enzyme(s), and polynucleotide phosphorylase can be considered to have no role in the incorporation of UMP into polyribonucleotides.

Since both the RSV fraction and the F.R. fraction have been

shown to be rich in phosphodiesterases which cleave nucleotides from the 3′ end of the RNA chains, it could be argued that incorporation was due to a reversal of this nuclease activity. Indeed synthesis of oligoribonucleotides from ribonucleoside 2′(3′)-monophosphates and from pyrimidine 2′(3′) cyclic nucleotides by ribonuclease has been reported (Heppel, Whitfield and Markham, 1955; Sato-Asano and Egani, 1958; Bernfield, 1965 and 1966). Two lines of evidence argue against this interpretation. Firstly although the phosphodiesterase activity in the RSV fraction is several times greater than the F.R. fraction, the ability to incorporate UTP is in the reverse order. In the second place it it clear that while phosphodiesterases should require ribonucleoside 5′ monophosphates, the incorporating enzyme(s) under study required UTP as the preferable substrate.

The possibility that the UTP uptake by the free ribosome fraction was into s-RNA, as in the systems described by Hecht et al (1958) and Daniel and Littauer (1963), can be dismissed by the results obtained by the chromatography of the labelled RNA on G-100 Sephadex. However, it is possible that the ATP uptake observed could be into fractions other than that shown for the uptake of UTP. Pressure of time did not allow this experiment to be performed. It seems doubtful if the activity discussed in this thesis is related to the enzyme which was partially purified from a particle-free preparation of chorio-allantoic membranes of eleven day old chick embryos, and which formed poly A sequences 8-10 residues long (Venkataraman and Mahler, 1963).

Recently polyadenylatic acid has been reported to be found in

rat liver microsomes (Hadjivassiliou and Braverman, 1966). This raises the interesting possibility that the homopolymer may be self-priming in the microsomes. Polyadenylate synthetase has been reported in the ribosomes of Mesh. coli (August, Orits and Hurwitz, 1962), and an enzyme has been reported from calf thymus nuclei which catalyses the synthesis of poly A, on an endogenous poly A primer (Edmonds and Abrams, 1962 and 1963). Although the ATP uptake appears to be fairly limited in the F.R. fraction, this may be because of diesterase activity present in the F.R. fraction but not present in the ribosomes in vivo. Certainly polyA did stimulate the ATP uptake when added to the F.R. fraction in vitro. Added poly A was, however, a rather inefficient primer. This may be because polyA can adopt an ordered secondary structure in solution (Steiner and Beers, 1961). It is possible that endogenous poly A could be held in a configuration such that the primer becomes available to the enzymes. To date, no natural poly U has been reported in vivo. Such a homopolymer would, however, be more difficult to detect than poly A, which is not susceptible to the action of pancreatic ribonuclease. In vitro, added poly U was a very effective primer for UTP uptake. However, the results from the ^{32}P distributions following uptake of ($\alpha^{32}P$)UTP by the F.R. fraction suggest that UMP residues could be incorporated into RNA chains which also ended in AMP, GMP or CMP. This may indicate that UTP uptake was into a proper RNA primer rather than into poly U. This is supported by the fact that yeast RNA, in which it is unlikely that each chain ends in a UMP residue, was just as an effective primer for UTP uptake as poly U. This question cannot

be properly resolved until more information is available regarding the endogenous primer.

It is not known what relationship the present activity bears to the soluble enzyme in rat liver which in the presence of Mg^{++}, incorporates UTP into terminal positions in RNA primers, and in the presence of Mn^{++} catalyses the uptake of ATP. (Klemperer, 1963 a; and 1963b). The location of the high specific activity enzyme in the F.R. fraction in the present studies suggests that the enzymes may be different. It would have been of interest to measure the ATP uptake in the presence of Mn^{++} ions. Pressure of time did not permit this experiment. However small oligonucleotide primers were utilised as efficient primers with Klemperer's enzyme (Klemperer, 1964). Yeast RNA digests did not stimulate UTP uptake into the F.R. fraction although they did with Klemperer's preparation. In addition, when UMP was incorporated into poly U by the F.R. fraction and the RNA analysed on columns of G-100 Sephadex, there was a noticeable tendancy for the UMP to be incorporated into heavy poly U. This may reflect a restriction on the size of primer which the F.R. fraction can utilize for priming purposes. These differences could, of course, be explained in terms of the specific location and environment of the enzyme(s).

The activity described in this thesis is similar to that reported in particulate fractions from several sources. The uptake of ribonucleoside triphosphates has been reported in cytoplasmic microsomes preparations from pigeon liver (Strausz, Goldwasser, 1961), Krebs 2 ascites cells (Cline, Mason and Smellie, 1963; Horton, Liu, Martin and Work. 1966) and Landschutz ascites cells,

(Wykes and Smellie, 1966). Similar activities have been found in nuclear ribosomes from Landschutz ascites cells (Burdon, 1963) and calf thymus nuclei (Naora, 1966).

Working with Landschutz ascites cells, Wykes and Smellie (1966) concluded that ribonucleotides were incorporated into short homopolymer sequences at the ends of primer RNA chains. It was not possible to demonstrate uptake of nucleotides into added primer, and from their results it appeared that either s-RNA or short oligonucleotides acted as primers.

In the present work it has been possible to extend these observations to the liver system, and exclude t-RNA, 5s-RNA and r-RNA as primers. In some of the column runs not shown here, small oligonucleotide material was observed which contained small amounts of acid insoluble radioactivity. It was felt that this had been produced by nuclease action during incubation.

In the Krebs 2 ascites cell system, Dalgarno, Martin, Liu and Work (1966) also concluded that their enzyme fraction catalysed the addition of labelled nucleotides on to the ends of the pre-existing RNA molecules. However, on sucrose gradient analysis of the labelled RNA, as well as a major peak of radioactivity in the 4-6S region, some acid-insoluble radioactivity was also associated with the 30S and 18S RNA. This is contrary to the results obtained by both Wykes and Smellie (1966) and in the present report. The difference is not readily explicable. The conditions of isolation of the RNA, and in the analysis of the sucrose gradients differed from those of the present studies. In addition, very low levels of radioactivity were recovered in Dalgarno et al's experiment in contrast to the present work.

5. The biological significance of the particulate enzyme(s)

To date no satisfactory explanation of the particulate enzymes in mammalian cells has been advanced. The recent finding of poly A with a sedimentation coefficient of 10S in rat liver microsomes obviously suggests that they may be involved in the synthesis of long chain homopolymers. Poly A has also been found in calf thymus nuclei (Edmonds and Abrams, 1963) and Hadjivassilion (1966) in his report mentions that RNA fractions rich in A have been observed in Euglena gracilis. The significance of poly A in the cell cytoplasm remains obscure. The apparent location of the molecule in the microsomes suggests a possible role in protein synthesis but the current concepts of RNA function provide no clue as to what this might be. The evidence of the present studies tends to rule out the synthesis of long chain homopolymers by the particulate enzymes, but this cannot be rigorously excluded. It is possible that the short chain lengths synthesised by the F.R. fraction might be due to the high levels of phosphodiesterase activity found in this fraction. It is not certain that this would be associated with the ribosomes in vivo. Under these circumstances, the lengths of the chains synthesised in vivo might be much longer than those observed in vitro.

The ribosomal location of the enzyme(s) suggests a role in protein synthesis. If r-RNA had been modified by the enzyme(s) it would have been possible to visualise this as a mechanism which could control the binding of sRNA or m-RNA to the ribosomes. However, the results quite clearly show that r-RNA as such does not act as ribonucleotide acceptor.

One of the most interesting ideas thrown up by the results is that the enzyme(s) may synthesise limited homopolymer sequences on the ends of m-RNA, attached to ribosomes. It is known that the secondary structure of a polyribonucleotide determines its affinity for the ribosomes (Okamoto and Takanami, 1963 a and b), which in vitro systems affects the ability of a given polyribonucleotide to code for various amino acids (Singer, Jones and Nirenberg, 1963). Limited homopolymer formation onto the ends of natural m-RNA might be necessary to modulate the helical content of the ends of the chains and facilitate interaction with the ribosomes.

However recent reports suggest that the direction of reading of m-RNA during protein synthesis is from the 5′ end to the 3′ (Salas, Smith, Stanley, Wahba and Ochoa, 1965; Thach, Cooper, Sundaranjan and Doty, 1965; Smith, Salas, Stanley, Wahba and Ochoa, 1966). Initiation of protein synthesis in Esch. coli appears to involve the incorporation of N-formyl-methionine into the N-terminal position of the peptide to be synthesised (Adams, and Capecchi, 1966; Nakamoto and Kolakofsky, 1966). The first step involves the interaction of methionine and a specific s-RNA to form methionyl-sRNA, which can be formylated to form N-formyl-Methionyl-sRNA. The sRNA in this reaction is different from the sRNA normally involved in the incorporation of methionine into internal sites in the peptide. The N-formyl-methionyl-sRNA then interacts with the ribosome-m-RNA complex, occupying the site normally filled with the peptidyl-sRNA (Noll, 1966; Clark and Marcker, 1966a; Dretzcher and Marcker, 1966). The sRNA

appears to be quite specific and aligns itself to the specific codon Ap(Gp)UpGp ... on the m-RNA (Clark and Marcker, 1966b). Therefore the m-RNA can be considered to have a specific initiation codon at the 5′ end of the molecule.

Taking these considerations into account (and assuming that the situation in animal cells is the same as in Esch. coli) it would be reasonable to expect that any modification of m-RNA would be necessarily, and firstly, at the 5′ end of the chain. The results of the present studies clearly demonstrate that the particulate enzyme(s) modify the 3′ ends of primer RNA chains. In this connection it would be of great interest to investigate the possibility that enzymes exist in the particulate fraction which can add nucleotides to the 5′ ends of primer RNA chains. If this were the case, one could visualize it as a mechanism for the protection of the initiation codon from nuclease activity, or as a mechanism for specifically tagging m-RNA. The latter suggestion would be worthy of serious consideration if ribosomes were shown to be functionally heterogenious.

As well as an initiation codon, it appears that there may be a "stop" or termination codon, at least in the Esch. coli system, (Brenner, Stretton, and Kaplan, 1965; Nirenberg, Leder, Bernfield, Brimacombe, Trupin, Rottman and O'Neal, 1965; Ganoza and Nakamoto, 1966). The termination codon appears to be either ... pUpApA or ... pUpApG. In the absence of this codon the newly formed peptide is not released, but remains attached to the m-RNA and the ribosome. If the termination sequence was damaged during protein synthesis it is possible that the m-RNA might remain stuck on the ribosome,

rendering the complex inactive. One possibility is that the particulate enzyme(s) protects the termination codon from nuclease activity by forming a "buffer" of ribonucleotides on the 3′ end of the chain.

Perhaps one of the most attractive possibilities is as follows. The most likely place for single chain breaks in m-RNA during protein synthesis would be between ribosomes. The fragment of mRNA containing the initiation codon would be unlikely to end in a termination codon. Accordingly all the ribosomes to the 5′ side of the break would be inactivated by remaining attached to the fragment of m-RNA. It is possible that the ribosomal enzyme could add ribonucleotides to the 3′ ends of the broken fragment. Since the enzyme is presumably firmly attached to the ribosome, the m-RNA molecule would move relative to the ribosome. This might release the peptidyl-sRNA from the ribosome and allow the release of the m-RNA fragment, rendering the ribosome potentially active once again. The known behaviour of homo-polymers in coding for peptide synthesis tends to be against this theory at first sight. For example, poly U codes for poly-phenylalanine and it is known that <u>in vitro</u> the product is poly-phenylalanyl-sRNA (Lengyel and Nakamoto, 1966). However it is possible that the processes of addition of ribonucleotides and peptide bond formation are mutually exclusive.

The fragments to the 3′ side of such breaks would be released from the ribosomes and could be degraded by the normal processes. In a recent paper, Futai, Aoyama and Hiramo (1966) provided evidence that degradation of m-RNA in cell free fractions of

Escherichia coli was brought about by the combined action of enzymes such as polynucleotide phosphorylase and phosphodiesterases which attack the 3′ end of RNA chains, and not by ribonuclease. It would, of course, also be desirable to degrade the fragments of m-RNA which contain initiation sites, since they could initiate the synthesis of new peptides and render more ribosomes inactive. It is possible that the enzyme under study, in addition to aiding the release of m-RNA fragments, might also label such fragments in a manner which accelerated their degradation from the 3′ end.

However, until more definite information is obtained, such considerations must be regarded merely as interesting speculations.

SUMMARY

1. Experiments were performed *in vitro* to detect enzymes capable of catalysing the nett synthesis of RNA in the cytoplasm of rat liver cells. Enzyme fractions were obtained by differential centrifugation of tissue homogenates.

2. The soluble fraction of the cells contained enzyme systems capable of the uptake of UTP, ATP and CTP into RNA. The system was dependant on added RNA and Mg^{++} ions. Rather than new chains being formed, ribonucleotides appeared to be incorporated into the ends of pre-existing RNA primers, approximately 2 - 3 UMP residues being added to each primer molecule. The enzymes appeared to require free 2'(3') hydroxyl residues on the primer chains. The biological significance of the activity is discussed.

3. Activity in the microsomes was quite different in character from that of the soluble fraction. The uptake of UTP was dependant on the presence of ATP, GTP and CTP.

4. The reaction showed a pH optimum of 7.5 - 8.0, and an absolute dependance on added Mg^{++}, but not Mn^{++} ions. Neither Actinomycin D, DNA or DNase had any real effect on UTP uptake. On the other hand added RNA stimulated the reaction, and RNase abolished the uptake of UTP completely. It is concluded that ribonucleotide uptake was by an RNA-dependant reaction.

5. 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 that the major portion of the ^{32}P was recovered in UMP. However, approximately 30% of the radioactivity could be recovered in AMP, GMP and CMP. Since all four ribonucleotide triphosphates were incorporated, synthesis of heteropolymer RNA chains could not be excluded. The main activity appeared to be the formation of homopolymer sequences.

6. Although the incorporation of a ribonucleoside triphosphate was stimulated by the addition of the other three ribonucleoside triphosphates, in no case was maximum incorporation achieved under the latter conditions. The highest levels of uptake were observed in the presence of only one additional ribonucleotide. Since GTP incorporation was very low, it is concluded that the formation of new heteropolymer RNA chains was unlikely.

7. The microsomes were sub-fractionated by a method which involved differential centrifugation of microsomal suspensions. The highest specific activity was found in the "free" ribosome fraction. A large part of the activity was recovered in a rough surfaced vesicle fraction. It was possible that the activity was due to contamination of the rough surfaced vesicle fraction with a supernatant fraction. No activity was recovered in a smooth surfaced vesicle fraction.

8. The activity in the rough surfaced vesicle fraction could be adequately described as terminal addition of one or two ribonucleotide residues to the 3′ ends of existing RNA chains. The enzyme(s) appeared to require free 2′(3′) hydroxyl residues on the primer RNA.

9. The activity of the free ribosome fraction was more thoroughly investigated. UTP, ATP and CTP were incorporated by the enzyme. It was possible to prove unequivocally that seven to eight UMP residues were incorporated into the ends of pre-existing RNA chains.

10. The nature of the endogenous primer was investigated. Using a combination of sucrose gradient centrifugation and chromatography on columns of G-100 Sephadex, it was possible to exclude r-RNA, 5S-RNA and t-RNA as the endogenous primers. This left the interesting possibility that m-RNA accepted the ribonucleotides into 3 terminal positions.

11. The possible biological significance of the enzyme(s) is discussed.

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STUDIES ON THE SYNTHESIS OF RIBONUCLEOTIDE HOMOPOLYMER

by N. M. Wilkie

Previous work has shown that cytoplasmic fractions of animal cells are capable of the incorporation of ribonucleoside triphosphates into RNA. The present investigation was carried out to elucidate the mechanism and properties of such enzymes in rat liver cells.

Female rats were anaesthetised, the abdomens opened, the livers perfused, and then removed. Enzyme fractions were prepared by differential centrifugation of liver homogenates. The incorporation of radioactive labelled ribonucleoside triphosphates into polyribonucleotides catalysed by such fractions was measured.

The microsomal fraction was most active in incorporating (α-^{32}P) UTP into polyribonucleotides. Such activity required a pH of 7.5-8.0 and the addition of Mg^{++} rather than Mn^{++} ions. The uptake of (α-^{32}P) UTP was promoted by the presence of ATP, GTP and CTP, and an ATP generating system. While the addition of RNA stimulated the reaction and ribonuclease completely abolished the reaction, the addition of DNA, deoxy-ribonuclease or actinomycin had no effect on the uptake of (α-^{32}P) UTP. It was concluded that incorporation of ribonucleoside triphosphates was by an RNA-, rather than a DNA- dependant reaction.

Alkaline hydrolysis of the reaction products after (α-^{32}P) UTP incorporation, and measurement of the radioactivity in the 2′(3′) monophosphates so obtained, indicated that the main product was sequences of uridylic acid. It was not possible, however, to exclude heteropolymer formation. All four ribonucleoside triphosphates were incorporated into acid insoluble products. In no case was maximum incorporation obtained in the presence of all four ribonucleoside triphosphates, reducing the probability of heteropolymer formation.

The microsomes were fractionated into rough surfaced vesicles, smooth surfaced vesicles and free ribosomes. No activity was recovered from the smooth surfaced vesicle fraction. Activity in the rough surfaced vesicle fraction, appeared to be due to contamination by a supernatant fraction.

An enzyme fraction with very high specific activity was recovered with the free ribosomes. Washing the ribosomes in dense sucrose solutions and isolation of the free ribosome fraction from media containing high concentrations of EDTA had no effect on the activity.

It could be demonstrated unequivocally that the incorporation of UTP represented addition of seven to eight residues of uridylic acid to the 2′(3′) ends of pre-existing RNA chains. ATP and CTP, but not GTP,

were also incorporated into polyribonucleotides. Following the uptake of ^{3}H-UTP, the nucleic acids were extracted from reaction mixtures. Using a combination of fractionation by sucrose gradient centrifugation and on columns of G-100 Sephadex, it was possible to exclude ribosomal RNA, 5S-RNA and transfer RNA as endogenous primers. This left the interesting possibility that messenger RNA acted as acceptors for the homopolymer sequences.

The biological significance, and the relationship of the free ribosomal enzyme(s) to other known enzymes of polynucleotide metabolism are discussed in some detail.

The uptake of ribonucleoside triphosphates by a supernatant fraction was investigated. The uptake of UTP required the presence of Mg^{++} ions and an added RNA primer. The incorporation of UTP could adequately be described as terminal addition of one to two ribonucleotides to the 2′(3′) ends of pre-existing RNA primers. The enzyme(s) appeared to require free 2′(3′) hydroxyl groups on the primer molecules. The significance of this activity is discussed.